

**ADVANCES IN
STRUCTURAL BIOLOGY**

Editor: SUDARSHAN K. MALHOTRA

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CONTENTS

LIST OF CONTRIBUTORS	vii
PREFACE <i>Sudarshan K. Malhotra</i>	ix
FLUORESCENT <i>IN SITU</i> HYBRIDIZATION (FISH) AS AN ADJUNCT TO CONVENTIONAL CYTOGENETICS: ANALYSIS OF METAPHASE AND INTERPHASE CELLS <i>Hon Fong L. Mark</i>	1
THE CONTRIBUTION OF DEFECTS IN INSULIN SIGNALING IN SKELETAL MUSCLE TO INSULIN RESISTANCE AND TYPE 2 DIABETES: CELLULAR AND MOLECULAR ASPECTS <i>Karen C. McCowen and E. Dale Abel</i>	41
CYTOPATHIC HYPOXIA IN SEPTIC SHOCK <i>David R. Schwartz, Atul Malhotra, Mitchell P. Fink</i>	65
BONE HEALING AND THE DIFFERENTIATION OF OSTEOPROGENITOR CELLS IN MAMMALS <i>Doreen E. Ashhurst</i>	83
POLYAMINE-TRAVELED PATHWAYS: SIGNIFICANCE IN HEALTH AND DISEASE <i>Ulka R. Tipnis</i>	117
REACTIVE ASTROCYTES, THEIR ROLES IN CNS INJURY, AND REPAIR MECHANISMS <i>Jean-Luc Ridet and Alain Privat</i>	147
PLASTICITY AND RIGIDITY IN THE NERVOUS SYSTEM: LESSONS FROM THE SPINAL CORD <i>Håkan Aldskogius</i>	187

**CERULOPLASMIN: STRUCTURE AND FUNCTION
OF A FERROXIDASE***Samuel David and Bharatkumar N. Patel*

211

**EXPRESSION OF A NOVEL NUCLEAR PROTEIN IS
CORRELATED WITH BRAIN DEVELOPMENT***Arumugham Raghunathan and Mohan C. Vemuri*

239

**STRUCTURE-FUNCTION RELATIONSHIPS OF
THE NUCLEAR ENVELOPE***Christopher Maske and David J. Vaux*

261

MEMBRANE PORES*C. Lindsay Bashford and Charles A. Pasternak*

299

INDEX

323

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PREFACE

In keeping with the broad objectives set for the serial publication of *Advances in Structural Biology*, Volume 6 contains exhaustive articles from experts in diverse areas of biomedical research. The common thread among the various articles is their relevance to the applications of cell biology to human health.

I am most appreciative of the interest and efforts of the contributors to this volume. I would also like to acknowledge others involved with the volume. Theodor K. Shnitka, M.D., has been most helpful in reading several of the articles included in this volume. Ms. Brenda Metherell has provided invaluable and efficient secretarial help in compilation of this volume. It is a pleasure to acknowledge here her contribution in the preparation of the volume. The cooperation of the production staff at JAI Press, particularly that of Mr. Christian Costeines, production editor, is gratefully acknowledged.

Sudarshan K. Malhotra
Editor

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FLUORESCENT *IN SITU* HYBRIDIZATION (FISH) AS AN ADJUNCT TO CONVENTIONAL CYTOGENETICS

ANALYSIS OF METAPHASE AND INTERPHASE CELLS

Hon Fong L. Mark

I.	Conventional Cytogenetics	2
A.	Conventional Cytogenetics Based on Banding	2
B.	Solid Staining	4
C.	G-Banding	4
D.	High-Resolution Banding	5
E.	Q-Banding	6
F.	C-Banding	7
G.	R-Banding	7
H.	Additional Comments on Conventional Cytogenetics	8
II.	<i>In Situ</i> Hybridization	9
III.	Fluorescent <i>In Situ</i> Hybridization (FISH)	10

Advances in Structural Biology, Volume 6, pages 1-39.

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IV.	Clinical Applications of FISH	12
A.	Autosomal Marker Chromosome Identification and Detection of Mosaicism	13
B.	Sex Chromosome Identification and Detection of Mosaicism	14
C.	Detection of Microdeletions and Microduplications	15
D.	Rapid Aneuploidy Detection and Other Applications in Prenatal Diagnosis	17
E.	STAT Chromosomes	18
F.	Detection of Chromosome Aneuploidy in Cancer using FISH	18
G.	Detection of Structural Rearrangements in Cancer	20
H.	Application of FISH in Bone Marrow Transplants	21
I.	Applications for Cancer Specimens with Low Mitotic Index, Suboptimal Preparations, Nondividing and Terminally Differentiated Cells	21
J.	FISH Analysis of Single-Cell Trisomies for Determination of Clonality	22
K.	Other Clinical Applications	23
V.	Research Applications of FISH	24
A.	Research using FISH on Specimens of Various Origins	24
B.	Interphase Cytogenetics Using Archival Specimens	24
C.	FISH for Detecting Chromosome Aneuploidy	27
D.	FISH for Detecting Oncogene Amplifications	28
E.	Other Miscellaneous Applications	28
VI.	Emerging FISH Technologies	29
A.	Comparative Genomic Hybridization	30
B.	Spectral Karyotyping	32
C.	Other Comments	32
VII.	Concluding Comments	34
	Acknowledgments	34
	References	35

I. CONVENTIONAL CYTOGENETICS

A. Conventional Cytogenetics Based on Banding

Cytogenetics is a subspecialty of medical genetics. Other medical genetics subspecialties include clinical genetics, biochemical genetics, and molecular genetics. Conventional cytogenetic analysis via banding is a powerful, established technique that can provide a picture of the human genome at a glance. What the clinical cytogeneticist refers to as "banding" is the staining method by which dark and light differential staining is induced along the lengths of the chromosomes. According to the Paris Conference (1972), a chromosome band is the part of a chromosome that can be distinguished from adjacent segments by appearing darker or lighter by one or more techniques. By this definition, chromosomes con-

sist of a continuous series of dark and light bands, with no interbands. Three levels of resolution have been standardized, namely, 400, 550, and 850 bands per haploid karyotype. Further descriptions of the universally adopted cytogenetic nomenclature can be found in An International System for Human Cytogenetic Nomenclature (Mitelman, 1995).

Prior to the advent of banding in the late 1960s and early 1970s, chromosomes were grouped and classified based on size, shape, the position of their centromeres, and the gross chromosome morphology. Unequivocal identification of each unbanded chromosome of the human genome was not possible. The development of Q-banding in 1970 (Caspersson et al., 1968, 1970; Paris Conference, 1972) ushered in an era where many protocols for banding flourished, leading to an unprecedented number of discoveries of numerical and structural chromosomal abnormalities. More descriptive terms were coined, such as the QFQ-technique for Q-banding and the GTG-technique for G-banding. These three-letter codes were described in the Paris Conference Supplement (1975). The first letter describes the kind of banding. The second letter stands for the technique or agent used to induce banding. The third letter signifies the kind of stain employed. For example, GTG stands for G-banding by trypsin using Giemsa as a stain (Harnden and Klinger, 1985).

Most laboratories in the United States use GTG-banding for routine karyotyping. In a routine cytogenetic analysis, a short-term 72-hour-culture of peripheral blood lymphocytes is established by adding the mitogen phytohemagglutinin (PHA), referred to as a stimulated culture. Briefer culture without such an agent is referred to as an unstimulated culture. The former is used most frequently to rule out constitutional abnormalities, whereas unstimulated cultures of peripheral blood or more typically, bone marrow aspirate, is used for the study of neoplastic cells. Long-term tissue cultures of almost any solid tissues are established for a variety of reasons, such as mosaicism detection.

Harvesting of chromosomes for conventional cytogenetics (Moorhead et al., 1960) is a rather lengthy and tedious process. Colcemid, a derivative of colchicine, is usually used to arrest the chromosomes in metaphase. This is followed by a hypotonic treatment to cause the cells to take up water and swell so that the chromosomes can spread well when dropped onto glass slides at a later step. After the hypotonic treatment step, the cell pellet is fixed in several changes of fixative consisting of three parts of methanol to one part of glacial acetic acid. After repeated rinsings, the cells are then dropped onto glass slides to facilitate chromosome spreading and air-dried. Slides are aged for a variable amount of time, then banded and stained by one of the banding protocols. G-banding using trypsin and Giemsa stain is the routine method in the United States (Seabright, 1971; Sumner et al., 1971) because of its simplicity. Prior to the advent of modern-day imaging systems, photographs were taken of the best metaphase spreads, enlarged and hand-cut to separate the images of the chromosomes for identification. Most cytogenetics laboratories now own computer-assisted karyotyping systems. Karyotyp-

ing consists of arranging the 46 chromosomes in the human genome according to shape, size and banding patterns. Thus, conventional cytogenetics is a labor-intensive process requiring highly trained personnel. In addition, conventional cytogenetics depends entirely on the availability of high-quality metaphase cells, thus excluding from analysis the vast majority of cells that are in interphase (Anastasi, 1991). Details of conventional cytogenetics have been described elsewhere (Mark et al., 1993, 1994b, 1995a, 1995b, 1995c; 1996a, 1996b, 1996c; 1997a).

B. Solid Staining

Solidly stained or unbanded chromosome preparations are largely obsolete since the introduction of banding methods in the late 1960s and early 1970s except for selected applications. The scoring of chromosome and chromatid breaks and gaps, for example, can be facilitated by solid staining (see, e.g., Mark et al., 1994c; 1995d, 1995e). Satellites, secondary constrictions, dicentrics, ring chromosomes, double minutes, and fragile sites can be better visualized by solid staining. Chromosome morphometry can also be better performed with nonbanded chromosomes (Priest, 1994). The stains employed are usually one of the Romanovsky-type dyes such as Giemsa, Leishman's, or Wright's stains.

C. G-Banding

Giemsa banding (G-banding) is the most widely used technique for the routine analysis of mammalian chromosomes in cytogenetics laboratories. With this method a series of dark and light stained regions along the length of the chromosome are produced and are grossly similar to the Q-bands discussed below. A number of protocols are used to induce G-bands. Most common are the treatment of slides with a protease such as trypsin or the incubation of the slides in hot saline-citrate (Seabright, 1971; Sumner et al., 1971) after appropriate slide aging. The chromosome banding patterns resulting from these treatments are distinct and quite consistent from experiment to experiment, leading some to postulate that G-bands reflect the inherent genetic composition and underlying structure of the chromosome. Dark G-bands have been found to correlate with pachytene chromomeres in meiosis. Generally, they replicate their DNA late in the S-phase, contain DNA rich in adenine and thymine (AT) base pairs, appear to contain relatively few active genes, and may differ from light bands in terms of protein composition. It has been hypothesized that the differential extraction of protein during fixation and banding pretreatments from different regions of the chromosome are important in the mechanism by which G-bands are obtained (see discussion by Rooney and Czepulkowski, 1992).

Although it has been approximately 30 years since chromosome banding was first discovered, the optimal banding conditions for each preparation are still often

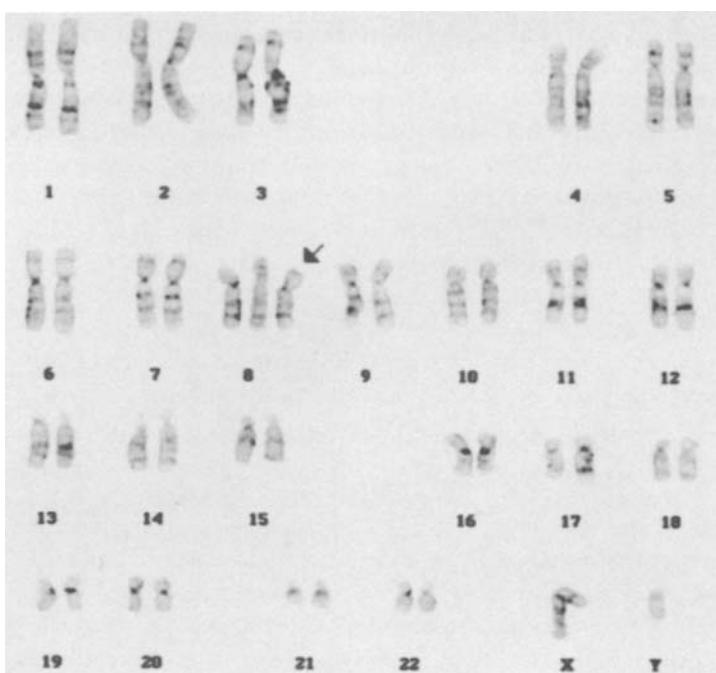


Figure 1. Computer-generated GTG-banded karyotype for illustrating chromosome 8 trisomy. Arrow points to an extra chromosome 8. Reprinted with permission of Medicine and Health/Rhode Island.

determined by trial and error. Cytogenetic technology is still more of an art than a science. An example of a GTG-banded karyotype is shown in Figure 1.

D. High-Resolution Banding

Although early G-banding was instrumental in delineating many structural chromosomal abnormalities, the level of resolution resulting from this technique is relatively low, with 400 to 450 bands per haploid genome, depending on the nature of the specimen. To obtain a higher level of resolution, at a band level of 550 to 850 or even greater, longer chromosomes at an earlier stage of mitosis, such as prophase or prometaphase, are needed. Protocols utilized to obtain such high-resolution G-banding were first described by Yunis and Sanchez (1975) and Yunis (1976). High-resolution banding protocols involve cell synchronization with agents such as methotrexate followed by a subsequent removal and the use of short exposure times to Colcemid or Velban. High-resolution banding, which is a mod-

ified G-banding technique, is most useful for the delineation of structural chromosomal rearrangements, such as microdeletions and microduplications. Examples of these include deletions and cryptic translocations involving 17p13.3 in Miller-Dieker syndrome, deletions of chromosome 15q11.2 in Prader-Willi, and Angelman syndromes, 22q11.2 deletions in DiGeorge syndrome and velocardiofacial syndrome, deletions of 17p11.2 in the Smith-Magenis syndrome, deletions of 7q11.23 in Williams syndrome, deletions of 4p16.3 in Wolf-Hirschhorn syndrome, deletions of 5p15.2 in Cri-du-Chat syndrome, deletions of Xp22.3 in Steroid Sulfatase Deficiency/Kallman syndrome, and microduplication at 17p11.2, which leads to Charcot-Marie-Tooth disease. However, even with high-resolution analysis, many of these deletions and duplications are not visible. For the detection of subtle structural rearrangements, FISH with molecular probes is a more sensitive technique than G-banding and high-resolution banding analysis (Mark, Jenkins, and Miller, 1997a).

E. Q-Banding

Quinacrine banding (Q-banding) using either quinacrine mustard or quinacrine dihydrochloride was the first banding method reported (Caspersson et al., 1968). Prior to Q-banding, the only chromosomes in the human genome that could be unequivocally identified by size and morphology alone were chromosomes 1, 2, 3, and 16 and the Y chromosome.

Quinacrine dihydrochloride (quinacrine, atebrin) is an acridine dye that binds to DNA by intercalation or by external ionic binding. Upon treatment, the chromosomes exhibit a series of bright and dull fluorescent bands. The distal long arm of the Y chromosome is particularly bright with Q-banding. The Q-banding pattern resembles that of G-banding, with Q-bright bands corresponding to G-dark bands. Notable exceptions are centromeric regions of chromosomes 1, 9, and 16 and the acrocentric satellite regions.

As in G-banding, it has been postulated that the pattern of banding reflects the underlying chromosome structure and genetic composition of the chromatin. It has been hypothesized that regions that fluoresce brightly are rich in AT nucleotides (Ellison and Barr, 1972), whereas the guanosine-cytidine (GC)-rich areas of the chromosomes tend to exhibit duller fluorescence (Comings et al., 1975; Comings, 1978).

One of the most useful features of this method of banding is the identification of the human Y chromosome. With Q-banding, the distal long arm (Yq12) of a normal Y chromosome appears very bright and is easily distinguishable. Thus, Q-banding is especially useful for identifying sex chromosome variants, such as XYY. Prior to FISH and the advent of interphase cytogenetics using FISH, the fluorescent Y body (or Y-chromatin) was often used to examine Y chromosome copy number in interphase cells (Pearson et al., 1970).

Although simple, quick, and reliable, Q-banding has two serious drawbacks. One is the requirements of special optics, filters, and light source for fluorescent microscopy, which are expensive. The other is the rapid quenching of the fluorescence, making Q-banding less than ideal for microscopic analysis, especially compared with methods such as G-banding which produce permanent preparations. For these reasons, most cytogeneticists today no longer perform Q-banding on a routine basis.

F. C-Banding

Constitutive heterochromatin, first described by Heitz (1928), constitutes approximately 20% of the human genome. It is the structural chromosomal material seen as dark staining material in interphase as well as during mitosis (Rooney and Czepulkowski, 1992). Constitutive heterochromatin is distinguished from facultative heterochromatin which is represented by the inactivated X chromosome in mammalian females (Lyon, 1962) and which manifests itself as the Barr body or sex chromatin (Barr and Bertram, 1949). Whereas constitutive heterochromatin can be visualized with the C-banding technique, facultative heterochromatin cannot.

Constitutive heterochromatin banding (C-banding) stains constitutive heterochromatin located in pericentromeric regions of all human chromosomes, but is most prominent in the secondary constrictions of chromosomes 1, 9, and 16 and the distal long arm of the Y chromosome. C-banding therefore can be used sometimes to characterize karyotypic abnormalities involving these chromosomes. C-dark bands are postulated to contain satellite DNA and are ideal for studying heteromorphic variants in the human genome, such as variations in the lengths and positions of the secondary constriction regions of chromosomes 1, 9, and 16 and distal Yq. C-banding protocols involve treatment with acid, alkali, and hot saline and subsequent staining with Giemsa. A popular protocol uses a saturated solution of barium hydroxide (Sumner, 1972), whereas the original method (Arrighi and Hsu, 1971) used sodium hydroxide as the denaturing agent. The formation of C-bands is postulated to be due to preferential destruction of DNA in non-C-banded regions of the chromosomes (Comings et al., 1973; Pathak and Arrighi, 1973), but the mechanism of C-banding is not at all clear.

G. R-Banding

Reverse or R-banding (Dutrillaux and Lejeune, 1971) is so called because bands that are pale by G-banding often appear dark by R-banding, and vice versa. In general, R-banding produces a reverse pattern to that obtained with either Q- or G-banding. R-bands replicate their DNA early and are said to contain active genes.

R-banding can be induced by incubation in a saline solution at a high temperature followed by Giemsa staining, in which case metaphases are analyzed by conventional brightfield microscopy. This is the so-called RHG technique, or R-banding by heat using Giemsa. It can also be obtained by staining with acridine orange after hot phosphate buffer treatment that results in bands being green and red in color, in which case fluorescent microscopy is required. The latter is called the RFA technique (Bobrow and Madan, 1973), or R-banding by fluorescence using acridine orange. Other variations of the protocols used to induce R-banding are also possible.

Although the exact mechanisms of chromosome banding remain unclear, the fact that R-bands fluoresce yellow-green and G- and Q-bands fluoresce orange-red is consistent with the hypothesis that R-bands are GC-rich and are more resistant to denaturation than AT-rich regions, as acridine orange fluoresce yellow-green when bound to double-stranded DNA and orange-red when bound to single-stranded DNA. The main advantage of using R-banding over G-banding is that the telomeric regions of certain chromosomes that stain faintly using Q- and G-banding can be better visualized using R-banding (Wyandt et al., 1973, 1974). This method of banding is useful in cases where a structural chromosomal rearrangement near the terminal region of a chromosome is suspected.

H. Additional Comments on Conventional Cytogenetics

Other less commonly used staining and banding techniques include nucleolar organizer regions (NOR) staining, in which the genes that are thought to be actively transcribed can be selectively stained using silver nitrate (Goodpasture and Bloom, 1975; Howell et al., 1975; Bloom and Goodpasture, 1976). With silver staining, NOR bands appear as one or more dotlike structures of varying size located on the stalks (not the satellites) of the acrocentric chromosomes. Studies have also shown that only the active NORs are impregnated by silver. Another technique that is only used rarely is differential replication staining. Other conventional cytogenetic techniques include 4',6-diamidino-2-phenylindole (DAPI)/distamycin A (DA) staining (Schweizer et al., 1978), telomere staining (Bobrow et al. 1972), CT banding (Chamla and Ruffie, 1976; Scheres, 1976) and kinetochore staining, all of which are nonroutine techniques.

A multitude of other banding and staining techniques induced by a variety of agents, including antibiotics, antibodies and restriction enzymes, have been described by Verma and Babu (1995). For example, in restriction endonuclease banding, the treatment of slides with restriction enzymes prior to staining with Giemsa generally produces a modified C-banding pattern depending on the choice of enzymes. A banding pattern similar to G-banding can be obtained with *Hae*III (Rooney and Czepulkowski, 1992; Verma and Babu, 1995). This particu-

lar technique is useful in occasional instances where one of the more common techniques is not informative and newer molecular technologies such as FISH are not available.

Most of the above staining and banding techniques depend on brightfield microscopy, discussed thoroughly in many other texts (e.g., Rooney and Czepulkowski, 1992; Kaplan and Dale, 1994; Barch et al. 1997), the details of which are beyond the scope of this chapter. Q-banding and fluorescent R-banding, on the other hand, need instrumentation for fluorescent microscopy, also discussed extensively elsewhere (see above).

Summarizing, G-banding is the method of choice for many cytogenetics laboratories involved in diagnostic testing. C-banding is used occasionally to study centromere position, dicentric chromosomes, and Y chromosome variants. Q-banding is rarely used nowadays, although in the past it had been used more often for routine analysis as well as for the study of heteromorphisms of the centromeres, shortarms of the acrocentric chromosomes, and the distal long arm of the Y chromosome. The application of solid staining has been restricted as described above. NOR (silver) staining was used to analyze short arm variants of the acrocentric chromosomes and for the identification of small markers. With the advent of FISH and other FISH-based techniques to be discussed in the remaining sections of this chapter, the importance of some of the older staining and banding techniques has been somewhat diminished. However, conventional cytogenetics using G-banding remains a highly informative test and will most likely continue to serve as the preferred standard of reference.

II. *IN SITU* HYBRIDIZATION

In situ hybridization with radionuclide-labeled probes has been reported since the late 1960s (Pardue and Gall, 1969). Autoradiography, however, requires long periods of exposure that are not practical for most applications. Biotinylated DNA probes and probes modified with other reporter molecules were introduced in the 1980s (Burns et al., 1985; Manuelidis, 1985a, 1985b; Nederlof et al., 1989; Guttenbach and Schmid, 1990; Lichter and Ward, 1990; Guttenbach et al., 1995). These early colorimetric *in situ* hybridization assays were performed with immunocytochemical stains, such as horseradish peroxidase, and were detected with a light microscope. Some laboratories today still adhere to these systems because of preference, while others have little choice because of financial constraints. Methods of nonisotopic *in situ* hybridization possess several advantages over the older radioactive methods, such as high sensitivity and specificity, rapid results, probe stability, and direct mapping without fastidious statistical analysis (Mark et al., 1997a; Viegas-Pequignot, 1992).

III. FLUORESCENT *IN SITU* HYBRIDIZATION (FISH)

With the advent of fluorescence-based methods. (FISH) (Pinkel et al., 1986), many laboratories began to use this protocol for both clinical and research applications. Regardless of the methodology for detection, *in situ* hybridization possesses the advantage of being able to analyze nondividing cells. This ability for cytogenetic analysis to be performed on interphase cells, called interphase cytogenetics, has indeed revolutionized the field of cytogenetics.

Interphase cytogenetic analysis can be performed using a variety of DNA probes. These include α -satellite probes or centromeric probes, whole chromosome painting probes and unique sequence probes.

The most popular probes used are α -satellite DNA probes (Willard, 1989). Alpha-satellite DNA sequences are organized as tandem repeats of unique 171 base pair sequences that are present in as many as 5000 copies. For interphase studies, a DNA probe to the pericentromeric α -satellite DNA of a specific chromosome is biochemically modified by nonisotopic methods, such as nick translation with biotinylated deoxyuridine triphosphate (dUTP), and then hybridized to cells using routine hybridization techniques. Because of the high copy number of the target DNA, cumbersome, albeit sensitive, radioactive detection methods are not required. Instead, rapid, simple-to-use, nonisotopic detection methods can be employed. Commonly used for the detection of biotinylated probes are fluoresceinated avidin and avidin conjugated with a detector enzyme such as alkaline phosphatase. Both produce punctate signals that can be recognized microscopically. The advent of digital imaging microscopy further provides improvements in signal detection and signal-to-noise ratios.

For detection of structural chromosomal abnormalities, chromosome painting or whole chromosome probes can be used. These probes are comprised of numerous unique and repetitive chromosome-specific sequences covering the entire chromosome, and are usually derived from the isolation of somatic or radiation hybrids, flow sorting of the specific chromosomes, or microdissection of specific chromosomes with polymerase chain reaction (PCR) amplification of the DNA. Painting probes are designed for use on metaphase chromosome preparations, not interphase cells, where their appearance is too diffuse for clear interpretation.

Unique sequence probes are especially useful for purposes such as microdeletion detection. They are also useful for other purposes such as oncogene amplification and studies of specific chromosome loci.

An overview of FISH can be found in Blancato and Haddad (in press). Various applications of FISH are discussed in Mark (1994a). In a typical FISH experiment, the probes and the slides are denatured using heat and formamide so that the single-stranded probe can hybridize to its homologous sequences on the denatured chromosome. The probe is usually prelabeled with a hapten such as biotin or digoxigenin or it may be directly labeled. Hybridization of α -satellite sequences are usually carried out at 37° C and postwashed at 72° C. Classical satellite chro-

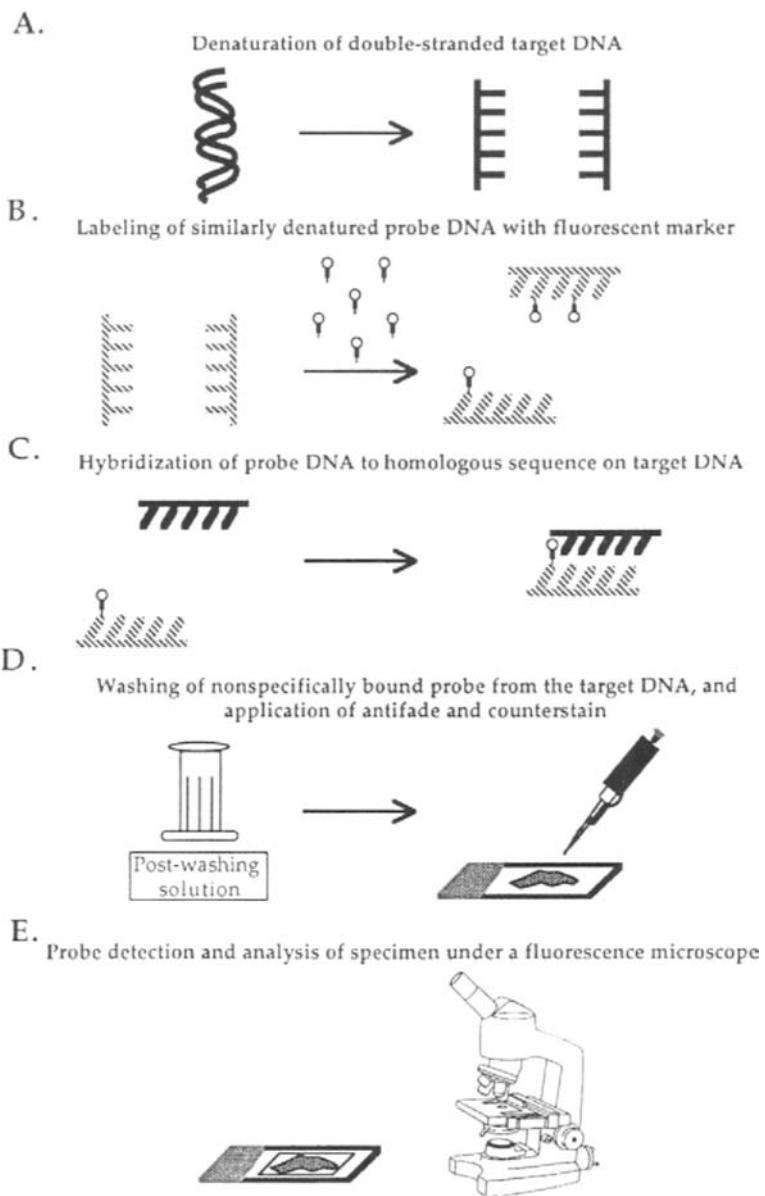


Figure 2. Fluorescent *in situ* hybridization (FISH). Reprinted with permission of Cytobios.

mosome-specific probes hybridize to the short repeats located in the pericentric heterochromatin of chromosomes 1, 9, 15, and 16 and to the long arm of the Y chromosome. Classical satellite probes labeled with digoxigenin are hybridized at 37° C and post-washed at 72° C using 0.25X standard sodium citrate (SSC). Classical satellite probes labeled with biotin are hybridized at 37° C and post-washed at 72° C using .05X SSC.

The FISH procedure involves the pretreatment of slides in 2X SSC, dehydration in ethanol, the denaturation of slides in a 70% formamide solution at 72° C, dehydration in cold ethanol, the application of heat-denatured probes, and hybridization in a moist chamber at 37° C for 1.5 to 2.0 hours. After coverslips are removed, slides are postwashed in 0.25X or 0.5 X SSC at 72° C for 5 min. After the slides are washed in a phosphate-buffered detergent, probe detection is carried out using an appropriate detection reagent, which is applied in subdued light. The final steps include the application of a counterstain, antifade, and coverslips.

Detection of a biotin-labeled probe is accomplished through the use of fluorescence-tagged avidin or streptavidin, which has a high affinity for biotin. Detection of a digoxigenin-labeled probe is accomplished through the use of an antibody tagged with a fluorescent dye. Visualization of the nuclei is accomplished through the use of counterstains, the most common of which are propidium iodide (PI) and 4,6-diamidino-2-phenylindole (DAPI). When the probe is labeled with fluorescein isothiocyanate (FITC), the counterstain propidium iodide is often used. When dual color fluorescence is performed, where one signal appears orange and the other signal green, DAPI counterstain is preferred. The DAPI counterstain provides the additional advantage of a reverse banding pattern for chromosome identification. Visualization of the hybridization probes and the counterstained nuclei is achieved through fluorescent microscopy. Slides are usually viewed under a 100X objective depending on the specimen being examined. Mounting media containing an antifading agent such as diphenylene diamine are added to prevent rapid quenching of the fluorescence. Metaphases or interphases are then photographed with an epifluorescence photomicroscope equipped with an FITC exciter filter set and Ektachrome ASA 400 color film, or captured for computer imaging. The steps of FISH are simplified and illustrated in Figure 2.

IV. CLINICAL APPLICATIONS OF FISH

Chromosomal abnormalities can be either numerical or structural. Numerical abnormalities can be either gain or loss of one or more chromosomes. Structural abnormalities include reciprocal and Robertsonian translocations, paracentric and pericentric inversions, deletions, tandem or inverted duplications, insertions, amplifications, ring chromosomes, isochromosomes, dicentric chromosomes, acentric fragments, and marker chromosomes.

An accurate identification of chromosomal abnormalities and mosaicism detection is important for genetic counseling relating to prenatal diagnosis, and as part of the workup for patients with cancer, congenital malformations, developmental delay, or mental retardation. FISH has been used with an increasing frequency to augment the power of conventional cytogenetics. Numerical chromosomal abnormalities can be detected readily using chromosome enumeration probes. Chromosome painting, on the other hand, can be used to identify and clarify structural rearrangements, such as cryptic and semicryptic translocations.

A. Autosomal Marker Chromosome Identification and Detection of Mosaicism

An International System for Human Cytogenetic Nomenclature (Mitelman, 1995) defines a marker chromosome as a structurally abnormal chromosome in which no part can be identified. They are sometimes called extra structurally abnormal chromosomes (ESACs) or supernumerary chromosomes.

Marker chromosomes can be classified into three groups for convenience, although each of the three groups contains markers of quite diverse origins. Rooney and Czepulkowski (1992) have provided an excellent overall description of marker chromosomes, which is summarized below.

Bisatellited markers, as the name implies, have satellites on both arms of the chromosome. They are assumed to be derived from the acrocentric chromosomes, which are chromosomes 13, 14, 15, 21, and 22. Chromosome 15, in particular, is often implicated in the origins of these markers.

Bisatellited markers may contain a single centromere or two centromeres. The two centromeres may be separated by euchromatin. One of the centromeres may also be inactive, in which case the marker is a pseudodicentric. The region between the two centromeres may or may not contain G- or R-bands. The two centromeres may be adjacent to the breakpoints very close to the centromeric region; they contain little or no euchromatin. Many variants are possible.

Markers can have satellites on one end only. This is the second class of markers. An individual marker may show a terminal deletion, an interstitial deletion or it may be a translocation product, for example, from a 3:1 segregation of a balanced rearrangement such as $t(11;22)(q23;q11)$. It may also be *de novo*.

The third class of markers are nonsatellited markers, which include everything other than the above two classes of markers. Specific groups that have been identified include: (1) very small rings (usually heterochromatic) derived from the centromeric regions of chromosomes 1, 9, or 16; (2) isochromosomes derived from the short arms of chromosomes 12, 18, and the Y chromosome; and (3) deletions of the long arm of the Y chromosome. Small markers or rings involving many human chromosomes have been reported in the literature.

Miscellaneous structurally rearranged chromosomes also exist. The derivative of chromosome 22 from $t(11;22)(q23;q11)$, the isochromosome of the short arm

of chromosome 18, the pseudoisodicentric of chromosome 15 and the isochromosome of the short arm of chromosome 12 (mosaic in skin) are markers known to be associated with distinct phenotypes. One of the best-known example of a marker is an additional isodicentric of chromosome 22 found in the cat eye syndrome.

In a case report by Abuelo et al. (1995), the authors described how FISH was utilized to identify the origin of a marker in a child who presented with developmental delay. The marker was demonstrated to be an inverted duplication of chromosome 15, more properly called pseudodicentric 15 in this case.

Marker chromosomes occur in approximately 0.05% of live-born infants and in approximately 0.06% of amniocenteses. About one-half of the cases are familial and carry a low risk for an abnormal phenotype. However, nonfamilial cases may have cognitive, behavioral, and physical abnormalities. In many previously reported cases, conventional chromosome staining techniques could not determine the origin of the extra chromosomes. Only with recent advances in molecular cytogenetic technology could many supernumerary chromosomes be identified (Abuelo et al., 1995).

In another case, we detected mosaicism for an extra chromosome 8 (trisomy 8) using an α -satellite probe for chromosome 8 (Mark and Bier, 1997b). The identification of structurally rearranged chromosomes can be achieved by using either "painting" probes or α -satellite probes. For example, using the latter we have identified an individual with an isochromosome of the short arms of chromosome 18, a well-defined syndrome described by Callen et al. (1990), Callen (1991), and Phelan et al. (1991). According to Callen (1991), patients possessing the i(18p) marker demonstrate low birth weight, typical faces, neonatal hypotonia with subsequent limb spasticity, short stature, microcephaly, mental retardation, and seizure disorders.

Although small marker chromosomes themselves cannot always be readily characterized by conventional methodology, it is possible to trace their chromosomal origins through microdissection of the marker, followed by FISH, using the microdissected material as a probe.

For larger-sized markers, identification of at least part of the marker is usually possible, in which case the marker is instead called a "derivative" chromosome. FISH, using chromosome painting probes as well as chromosome enumeration probes, is useful for the identification and confirmation of chromosomal origins of the markers in these cases.

B. Sex Chromosome Identification and Detection of Mosaicism

An immediate consequence of the ability to assess chromosome copy number in interphase as well as metaphase cells is the creation of a potentially large sample of cells for mosaicism study. The utility of FISH using an X chromosome and a Y chromosome probe was illustrated by a case report by Mark et al. (1995f). The patient was an 18-year-old woman referred for delayed puberty, short stat-

ure, hypergonadotropic hypogonadism, primary amenorrhea, and growth retardation. Her past medical history was unremarkable with the exception of a learning disability. Cytogenetic analysis was ordered to rule out a chromosomal basis for her clinical findings. GTG-banding analysis of peripheral blood lymphocytes revealed the presence of predominantly 46,XY cells. Using dual-color X chromosome-specific and Y chromosome-specific probes, a FISH analysis undertaken to further assess the contribution of a minor cell line yielded frequencies of 87% cells with the 46,XY constitution and 9% cells with the 45,X constitution. To establish unequivocally the presence of mosaicism, a skin biopsy was obtained for fibroblast culture, which further corroborated the results of the peripheral blood study. The FISH analysis revealed that 74% of the cells were 46,XY and 12% were 45,X. The unequivocal presence of XY cells puts the patient at risk for neoplastic transformation of the gonads. Laparoscopy and surgical removal of the patient's presumptive streak gonads were therefore undertaken. Cytogenetic results derived from the gonadal tissues further confirmed findings of the previous cytogenetic analyses.

In another study (Mark et al., 1996d), a combined cytogenetic and molecular approach was employed to detect low-percentage mosaicism for a minor cell line in a 15½-year-old patient with delayed puberty and short stature. The use of DNA analysis further helped delineate the nature of a marker present in a low percentage of cells initially detected using cytogenetics. Adoption of a combined cytogenetic and molecular approach can be useful for the diagnosis and management of patients with specific sex chromosome aneuploidy. It has been estimated that monosomy X with a normal male cell line (45,X/46,XY) accounts for only 4% of cases of Turner syndrome. However, the occurrence of neoplasia in dysgenetic gonads associated with karyotypes containing the Y chromosome is well-known. Gonadoblastoma is the most common tumor arising in dysgenetic gonads, although dysgerminoma and other histologic types may also occur. Because the risk of a gonadoblastoma may be as high as 30% in Turner subjects with Y-chromosomal material, removal of the streak gonads is recommended in these cases. Thus, determining with certainty the presence or absence of Y-chromosomal material is of critical importance, because a recommendation for a surgical procedure, gonadectomy, is based on this information. The X chromosome probe is shown in Figure 3, and the Y chromosome probe is shown in Figure 4. Dual X/Y probes are available but are not shown here because of the difficulty of discerning two color signals with black-and-white photography.

C. Detection of Microdeletions and Microduplications

A microdeletion is a small deletion of chromosomal material often undetectable by chromosome banding. One of the most powerful methods for detecting microdeletions and microduplications is FISH. Indeed, Ledbetter (1995) attrib-



Figure 3. Fluorescent *in situ* hybridization using an X chromosome-specific α -satellite probe. Reproduced with permission from a color photograph from the covers of issues of *Pathobiology* for the year 1997.

uted the “death of high resolution cytogenetics” to this particular application of FISH. Some of the specific applications of FISH include the detection of deletions and cryptic translocations involving 17p13.3 in Miller-Dieker syndrome (Kuwano et al., 1991), the detection of deletions of chromosome 15q11.2 in Prader-Willi and Angelman syndromes (Kuwano et al., 1992), and the detection of 22q11.2 deletions in DiGeorge and velocardiofacial syndromes (Desmaze et al., 1993; Driscoll et al., 1993). For a growing number of microdeletion syndromes, a combination of routine cytogenetics and FISH analysis may be the optimal protocol. Included in this list of syndromes are the following: deletion of 17p11.2 in Smith-Magenis syndrome, deletion of 7q11.23 in Williams syndrome, deletion of 4p16.3 in Wolf-Hirschhorn syndrome, deletion of 5p15.2 in the Cri-du-Chat syndrome, and deletion of Xp22.3 in steroid sulfatase deficiency/Kallman syndrome.

Molecular cytogenetics can also be applied for the detection of microduplications, such as the microduplication in 17p11.2 in Charcot-Marie-Tooth disease (Type 1A).



Figure 4. Fluorescent *in situ* hybridization using a Y chromosome-specific probe cocktail.

D. Rapid Aneuploidy Detection and Other Applications in Prenatal Diagnosis

Rapid aneuploidy detection in prenatal diagnosis using FISH technology usually involves the analysis of interphase nuclei rather than metaphase chromosome spreads. The test is often used to detect the most common aneuploidies, trisomy 13 (Patau syndrome), trisomy 18 (Edward syndrome), trisomy 21 (Down syndrome), and the sex chromosome aneuploidies in Klinefelter syndrome (47,XXY, 48,XXYY) and Turner syndrome (45,X). The major advantage of this technique is that aneuploidy in the fetus is detected within 48 hours rather than in 7 to 10 days required for standard chromosome analysis from *in vitro* culture. Because concerns have been expressed about the sensitivity and specificity of this technique, conventional cytogenetic studies are recommended for confirmation of FISH findings.

According to Mark et al. (1997a), the main utility of FISH for prenatal applications is for select situations such as very late gestational age pregnancies (i.e., greater than 22.5 weeks) with ultrasound-identified abnormalities that are relatively specific for FISH-diagnosable aneuploidies. These cases include cystic

hygroma (monosomy X, trisomy 18, and trisomy 21), holoprosencephaly with cleft lip (trisomy 13), clenched fists with overlapping fingers and a 2-vessel cord (e.g., trisomy 18), and in biochemical screen-positive populations. For those cases, FISH testing may be clinically applicable and may prove to be cost-effective.

FISH also holds promise for use in preimplantation genetics. It may also have potential applications in the detection of fetal cells in maternal blood, a new and exciting area of investigation that may be feasible as a routine test in the future, as amniocentesis and chorionic villus sampling (CVS) are invasive techniques that pose small but significant risks. FISH may be useful for the detection of certain chromosomal abnormalities after fetal cell sorting. Male fetal cells can be detected using classical and α -satellite probes derived from the Y chromosome. The application of FISH for confined placental mosaicism is yet another application. Confined placental mosaicism (CPM) is a tissue-specific mosaicism affecting only the placenta (Lomax et al., 1994). This is usually detected in cases where CVS karyotypes show mosaicism, but follow-up amniocentesis or fetal blood samplings show normal results. It has been postulated that these localized abnormalities in the placenta are responsible for their compromised developments and growth retardation in the fetuses (Lennow et al., 1993). Finally, metaphase FISH is a powerful adjunct to conventional cytogenetics in identifying numerical and structural chromosomal abnormalities such as the chromosomal origin of supernumerary marker chromosomes, the origin of the derivative chromosomes found in unbalanced rearrangements, and the breakpoints in balanced rearrangements in prenatal diagnostic samples. Issues relating to prenatal diagnosis are further discussed in Mark, et al. (1997a), where the authors stressed that caution should be exercised when interpreting FISH results, consistent with position statements of the New England Regional Genetics Group (1992) and the recommendations of the American College of Medical Genetics (1994).

E. STAT Chromosomes

STAT chromosomes can be performed using slides of fixed cells from the bone marrow or peripheral blood. This technique has been utilized only in cases where an answer must be obtained rather quickly, such as in cases where there is a question of a trisomy in a newborn, such as trisomy 13 or trisomy 18. FISH can be used as an adjunct to conventional cytogenetics for STAT chromosomes with the appropriate probes by using slides prepared from fixed cells from bone marrow or even bone marrow smears.

F. Detection of Chromosome Aneuploidy in Cancer Using FISH

One of the most important uses of FISH is for chromosome enumeration in the detection of aneuploidies such as trisomy 8, monosomy 7, trisomy 13 and trisomy 9 (see, e.g., Wyandt et al., 1998). Mark et al. (1995g) used FISH to assess the pro-

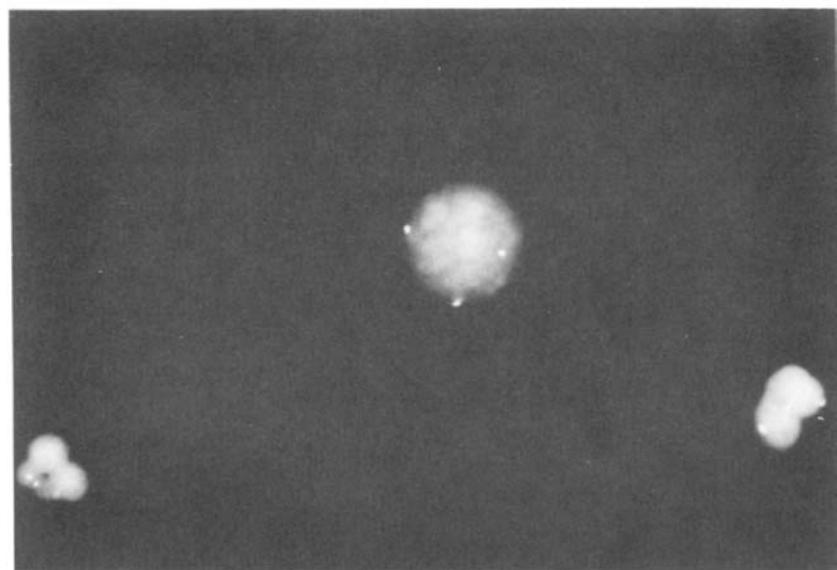
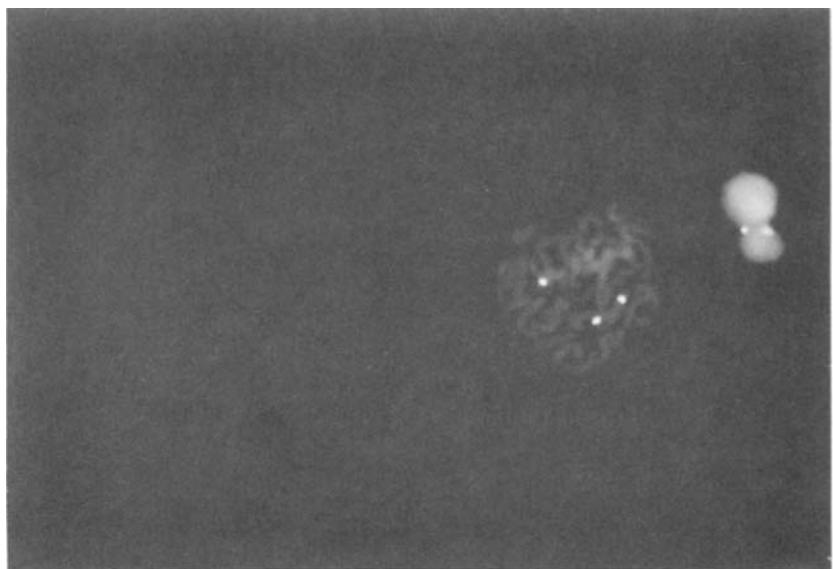


Figure 5. Fluorescent *in situ* hybridization using a chromosome 8-specific α -satellite probe, demonstrating three positive hybridization signals for chromosome 8.

portion of cells with trisomy 4 in a patient with acute nonlymphoblastic leukemia, and recognized FISH as a potentially useful tool for monitoring the minimal residual disease in this patient. Another study described the concurrence of congenital trisomy 8 mosaicism and gestational trophoblastic disease in a 42-year-old woman (Mark et al., 1995a). Subsequently, archival materials from this patient were retrieved, and FISH was used to assess the chromosome 8 copy number in the cells of various tissues (Mark et al., 1995h). Figure 5 illustrates the detection of trisomy 8 using FISH. A GTG-banded karyotype of a trisomic 8 cell is shown in Figure 1.

G. Detection of Structural Rearrangements in Cancer

The Philadelphia (Ph) chromosome was the first consistent chromosome abnormality associated with a single cancer type, chronic myelogenous leukemia (CML). Using a "new" banding technique in the 1970s, Rowley (1973) identified the Ph chromosome as a reciprocal translocation between chromosomes 9 and 22. The Ph translocation can be detected even in suboptimal metaphase cells and in interphase nuclei through the use of translocation FISH probes. Young et al. (1996) used FISH with paired painting probes in various combinations and the Oncor *bcr/abl* probe to identify a unique complex translocation involving chromosomes 9, 13, 15, 17, and 22 associated with Ph chromosome-positive CML. With the increasing availability of newer clinical cytogenetic technologies, many steps in the cumbersome process as described will become unnecessary.

The *bcr/abl* translocation DNA probe described by Young et al. (1996) was a commercial probe purchased from Oncor (Gaithersburg, MD) for identifying the reciprocal translocation, t(9;22)(q34;q11.2), characteristics of CML. The locus for *bcr* is on 22q11.2 whereas *abl* is on 9q34. In CML, the *bcr* gene is fused to the *abl* oncogene. The Philadelphia (Ph) chromosome is found by conventional cytogenetics in approximately 90% of CML cases.

Besides *bcr/abl*, there are other important cancer probes. The t(15;17) translocation DNA probe is also by Oncor. The *PML* gene is located on 15q22 whereas the retinoic acid receptor alpha (*RARA*) gene is on 17q12. Acute promyelocytic leukemia (APL) is characterized by the fusion of the *PML* gene and the *RARA* gene. A reciprocal translocation, t(15;17)(q22;q12), affects that fusion, and is found by conventional cytogenetics in >70% of APL cases. The Oncor t(15;17) Translocation DNA Probe identifies this reciprocal translocation in interphase nuclei and metaphase cells by two-color FISH.

The Oncor inv(16) DNA Probe is intended for use on research samples for the identification of the 16p13 breakpoint associated with inv(16)(p13q22) in metaphase spreads by *in situ* hybridization. The inv(16)(p13q22) is seen in acute myelomonocytic leukemia (AMML) cells with abnormal bone marrow eosinophilia (M4Eo). An observed disappearance of the inversion chromosome on remission in treated patients has been reported. The Oncor inv(16) DNA Probe spans the 16p13 region. If a break in 16p13 occurs, the 16p13 signal will split.

H. Application of FISH in Bone Marrow Transplants

One of the most important applications of FISH in cancer is the monitoring of engraftment in sex-mismatched bone-marrow transplants (BMT). Conventional cytogenetics has the capability of distinguishing female (XX) cells and male (XY) cells in metaphase spreads. FISH can distinguish between host and donor cells in both interphase and metaphase cells. By detecting the ratio of XX to XY cells using FISH with the X chromosome and Y chromosome probes in a post-BMT sample, the degree of success for engraftment can be assessed and disease recurrence can be better predicted.

I. Applications for Cancer Specimens with Low Mitotic Index, Suboptimal Preparations, Nondividing and Terminally Differentiated Cells

Conventional cytogenetics of cancer has been hampered by the requirement that there be a sufficient number of dividing cells of adequate quality for karyotypic analysis. Mark (1994a) noted that FISH is a useful adjunct technique for the analysis of tumor cells in cases in which there are insufficient metaphases for karyotypic analysis, where the quality of the preparation is suboptimal, where the structural chromosomal rearrangements are complex, or where there is extensive aneuploidy. The utility of FISH in the cytogenetic characterization of cervical cancer cells with multiple numerical and structural chromosomal abnormalities has been described by Mark et al. (1995c).

Equally inaccessible for karyotyping are terminally differentiated cells. FISH is a useful adjunct to conventional cytogenetics in cases where dividing cells are either inadequate or lacking in number (low mitotic index) or the quality of the metaphase preparation is suboptimal (Mark et al., 1998c). FISH is clearly superior to conventional cytogenetics in its ability to screen a large number of cells in a relatively short period of time. Furthermore, FISH does not require a new specimen. It can be performed on the original specimen submitted for routine cytogenetic analysis or even hematologic evaluation. Both air-dried slides and flame-dried slides prepared from fixed cells are suitable for FISH. FISH can be performed on either unstained or previously stained cells. FISH has been performed on archival material nearly 20 years old (Mark et al., 1995h; Mark et al., 1997c) Thus, FISH greatly expands the capabilities of the clinical cytogenetics laboratory in the study of cancer cells.

FISH can also be used to detect very low levels of disease (i.e., minimal residual disease). The ability to detect minimal residual disease by PCR has assisted in designing the appropriate therapy, in predicting the outcome of treatment and in creating novel molecular therapies. However, as Cotter (1997) noted, molecular analysis has its limitations. The analysis is primarily performed on a "soup" of DNA or RNA from both normal and diseased cells. Thus, using PCR, it is impos-

sible to identify the individual cell bearing the abnormality or to determine its phenotypic characteristics. In addition, only a small focused area of genetic change can be assessed. Other relevant genetic alterations that are outside the region of interest or in other parts of the genome may not be detected. As Cotter noted, "cytogenetics took a quantum leap in detection methodology with FISH and was able to fill some of the shortcomings of PCR-based technology."

Using FISH, useful information on remission and relapse can be obtained and applied prognostically to determine treatment options and to monitor the response to therapy. The presence of even a very low percentage of leukemic cells can be detected by FISH.

J. FISH Analysis of Single-Cell Trisomies for Determination of Clonality

In addition to its use for the identification of clonal trisomies associated with specific hematopoietic disorders, FISH can be used to study cases of single-cell trisomies identified in standard 20-cell GTG-banded analyses.

An International System for Human Cytogenetic Nomenclature (Mitelman, 1995) defines a clone as a cell population derived from a single progenitor cell. It is common practice to infer a clonal origin when a number of cells have the same or closely related abnormal chromosome complements. It is generally accepted that numerical abnormalities are reported only if two or more cells with the same abnormalities are found in the sample (see, e.g., Mark, 1994d). One-cell numerical abnormalities in standard 20-cell cytogenetic analyses are, by convention, considered random and nonclonal.

Nonclonal chromosomal abnormalities were the subject of study in two prior reports. Chen et al. (1993) used interphase FISH to analyze hematologic (bone marrow or peripheral blood) samples and demonstrated clonality of single-trisomic cells in six cases of myelodysplastic syndromes (MDS), two cases of acute myeloid leukemia (AML), and one case each of a myeloproliferative disorder and chronic lymphocytic leukemia. Kasprzyk et al. (1995) asked whether single-cell trisomies in bone marrow samples were truly random events or whether they represent cells from a mitotically inactive or minor clone. After using FISH to study a total of seven patients with single abnormal cells at diagnosis, these investigators concluded that single-cell abnormalities may not be clonal and "do not represent the tip of an iceberg." They thus seemingly contradicted the conclusions of Chen et al. (1993). In view of these conflicting data and the important implications for subsequent patient follow-up, Mark et al. (1997d) decided to conduct a retrospective study of single-cell trisomies in patients previously studied by routine GTG-banding at the cytogenetics laboratory at Rhode Island Hospital from July 1, 1990, to November 30, 1996. FISH was performed using chromosome enumeration probes on previously fixed bone marrow and, in some cases, peripheral blood slides from patients identified to have single-cell trisomies of selected chromo-

somes in routine 20-cell GTG-banded analyses. The results of the study of a cohort of 16 patients with available and analyzable slides indicated that a single unifying answer to the above question does not seem to exist. Although some cases are apparently random events as predicted by chance, other cases appear to represent "the tip of an iceberg." It is therefore important for cancer cytogeneticists to interpret the results of each patient on a case-by-case basis and to formulate the most optimal strategy for follow-up in the particular case under study. FISH is an invaluable tool in studying cases such as these.

K. Other Clinical Applications

Other cancer applications include detection of gene deletions (such as p53) and amplifications (such as *HER-2/neu* and *N-myc*), analysis of archival tumor materials including those of breast cancer, prostate cancer, bladder cancer, ovarian cancer, neuroectodermal tumor, carcinoma of testes, endometrial carcinoma, stomach cancer, verification of clonality, sex-mismatched bone marrow transplant, monitor of remission and relapse, and detection of minimal residual disease.

Tissues that are amenable to interphase cytogenetics include, but are not limited to, body fluids such as cerebrospinal fluids, frozen and fixed suspensions of blood, bone marrow and tumor, hair follicles, urine sediments in bladder and cervical cancer, other exfoliate cells, and other cytologic specimens. Smears that can be used for FISH include bone marrow smears, cervical (Pap) smears, and buccal and semen smears. FISH can be performed on cytopsins and touch preparations as well as formalin-fixed, paraffin-embedded archival materials. Besides confirmation of conventional cytogenetic results, the main advantages of FISH are in the following areas: (1) in cases where there are insufficient metaphase cells for analysis or the cells are of suboptimal quality; (2) in cases where there is a need for an increased sample size because interphase cells can be analyzed as well as metaphase cells; and (3) in cases where markers are found and unequivocal identification is not possible using GTG-banding alone.

A recent case history that comes to mind is that by Mark, Feldman and Sigman (1999). The patient was referred to our laboratory for chromosome analysis because of infertility. The phenotype was near normal except for small testes and azoospermia. Conventional cytogenetics detected an extra D-sized marker chromosome. The marker was unequivocally identified by FISH as an X chromosome with most of the short arm deleted, giving rise to a variant form of Klinefelter syndrome.

Besides endocrinology and infertility, FISH as an adjunct to conventional cytogenetics can impact many other fields, such as genetic counseling. Applications of FISH in cancer and prenatal diagnosis as discussed here is but a tiny sampling of the immense potential of FISH in clinical practice.

V. RESEARCH APPLICATIONS OF FISH

A. Research Using FISH on Specimens of Various Origins

Sex chromatin (Barr body) analysis of buccal mucosal cells has been recognized for many years as an inexpensive, noninvasive, and rapid means of sex determination. Conventional Barr body analysis using Papanicolaou stain was discontinued as a routine test because of its unreliability and failure to detect mosaicism and other chromosomal abnormalities. With the advent of recombinant DNA technology and the availability of molecular probes, however, the value of this simple, albeit obsolete, test was reevaluated. We reported results of our laboratory's experience in optimizing a FISH assay on buccal mucosal cells using dual color X/Y chromosome probes (Mark et al., 1996e). Use of FISH on this tissue may be important under certain circumstances when other sources for conventional cytogenetic analysis, such as peripheral blood or bone marrow, are not available for a variety of reasons, as discussed in Mark et al. (1996e). Buccal smears can be utilized in the absence of a more suitable specimen. Obtaining a buccal smear specimen is a relatively simple noninvasive procedure that requires only minimally trained personnel. Part of the specimen can also be sent for DNA analysis.

Even in cases in which other tissues are available, buccal smear results can be used to corroborate data derived from these tissues or to assess the proportions of different cell lines in cases of mosaicism. Because the FISH procedure is rapid, it may be useful as an adjunct to routine 3-day stimulated peripheral blood cytogenetic studies for diagnosing newborns with ambiguous genitalia.

Similarly, FISH can be performed on cervical smears. The Papanicolaou smear has been established as a useful cytological screening tool that has greatly contributed to the reduction of cervical cancer-related mortalities. This test, however, cannot reveal underlying genetic damage (e.g., numerical abnormalities) that predispose the individual to a potentially life-threatening neoplasm. The interphase FISH assay is feasible as a potential future screening tool for cervical cancer (Mark et al., 1997e).

Other body tissues that are amenable to interphase cytogenetic analysis using α -satellite probes include, but are not limited to, cerebrospinal fluid, frozen and fixed suspensions of blood, bone marrow and tumor, hair follicles, urine sediments in bladder, and cervical cancer and other exfoliated cells. The multiple applications of interphase cytogenetics have been discussed extensively elsewhere (Lichter and Ward, 1990; Mark, 1994a; Mark, 1998a).

B. Interphase Cytogenetics Using Archival Specimens

The conventional cytogenetics of cancer tissues, especially solid tumors, has been hampered by a number of factors (Nederlof et al., 1989; Sandberg, 1990;

Mark, 1994a; Heim and Mitelman, 1995; Afify et al. 1996). One is the difficulty in establishing cell culture. Another is the susceptibility to culture contamination despite adherence to sterile techniques. The tumor tissue may grow at a very slow rate. The tumor cells may also be overgrown by normal stromal cells. The culture often has a low mitotic index, leading to insufficient metaphase cells for karyotyping. The banding of the metaphases is at times suboptimal, with fuzzy chromosomes. Even if all these factors could be overcome, the resultant metaphases could all be normal, leading one to wonder whether tumor cells or normal cells were being analyzed. The resultant karyotype, on the other hand, could be extremely complex with multiple numerical and structural abnormalities, leading to difficulties in interpretation. The possibility that the abnormal karyotype could be an artifact of culture also cannot be completely ruled out. Due to these various factors, the conventional cytogenetics of solid tumors has not kept pace with that of the hematopoietic disorders and few tumors are well characterized from a conventional cytogenetic standpoint.

Although fresh tumor tissues have become scarce due to improved cancer screening, other materials for study are still relatively abundant. Surgical specimens in the form of formalin-fixed, paraffin-embedded blocks in a typical department of pathology have become a valuable resource. Two basic methods of processing can be utilized. One involves cutting a thick section of approximately 50 µm from the paraffin block and preparing slides from disaggregated cells. The other involves using 3 to 7 µm thin sections. Both kinds of slides are amenable for FISH analysis, as are slides from touch preparations and slides prepared from fresh tissues.

FISH is especially suited for retrospective studies in which a cytogenetic study has not been part of the patient's initial work-up. FISH analysis allows for the current cytogenetic results to be coordinated with previous studies.

Probes such as *bcr/abl*, *p53*, *HER-2/neu*, *c-myc*, and *N-myc* can be used to answer specific research questions. FISH analysis can be performed on archival materials of a wide variety of tumors, including bladder tumors (Hopman et al., 1988, 1989, 1991), gestational trophoblastic disease (Mark et al., 1997c), breast cancer (Devilee et al., 1988; Afify, et al. 1996; Afify and Mark, 1997; Mark et al., 1997f), neuroectodermal tumors (Cremer et al., 1988), carcinoma of the testes (Giwercman et al., 1990), prostate cancer (Zitzelsberger et al., 1994; Ross et al., 1997a, 1997b) and rhabdomyosarcoma (Afify and Mark, 1999; Mark et al., 1998b). We have even analyzed 20-year-old archival materials of gestational trophoblastic disease using FISH (Mark et al., 1995h). Here, we briefly summary our experience with breast cancer using FISH.

Breast cancer affects approximately 1 in 8 women in the United States. The current criteria for breast cancer staging include pathologic parameters such as tumor size, histological classification, and nodal involvement; cytologic parameters such as nuclear grade; and biologic parameters such as patient age, hormone receptor status, and oncogene amplification. Although there is evidence that some of these

factors may be of prognostic significance, information currently available is still fragmented and incomplete. Clearly, more biomarkers are needed. We began our FISH studies in an attempt to address this need, as molecular cytogenetics has until recently been an underdeveloped area of breast cancer research.

The tumors were examined morphologically by hematoxylin and eosin staining. Tumors were then analyzed cytogenetically using FISH and a chromosome 8-specific, α -satellite probe. Various approaches for specimen preparations were tested to compare their adequacies according to defined criteria. The approaches tested included modifications in specimen preparation such as cell disaggregation from formalin-fixed, paraffin-embedded tissue sections prior to FISH, FISH on thin sections from paraffin blocks, FISH on touch preparations, and FISH from suspensions of cells from fresh tumors. A total of 34 specimens were analyzed in the first study (Afify et al., 1996). The second and third studies (Afify and Mark, 1997; Mark et al., 1997f) focused on stage I and stage II disease and employed sample sizes of 34 and 36 cases, respectively. Our data thus far indicate that chromosome 8 trisomy in infiltrating ductal carcinoma is associated with an aggressive biological behavior and a poor prognosis. Although these results need to be further confirmed and extended in other centers, the data that currently exist suggest that chromosome 8 copy number may be utilized as a possible biomarker to predict patient prognosis. FISH showing 3 and 4 copies of chromosome 8 in breast cancer cells is shown in Figure 6.

As mentioned above, the advantages of interphase cytogenetics include the fact that it is not restricted to cells arrested in metaphase, thus allowing for an analysis of a large numbers of cells. The ability to analyze large numbers of cells is significant in that it permits the detection of low-frequency abnormalities that are otherwise difficult to detect (Anastasi, 1991). Thus, specimens that would not be

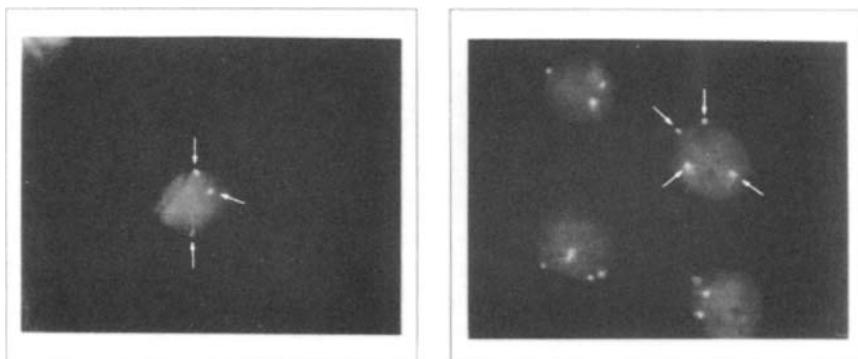


Figure 6. Trisomy and tetrasomy for chromosome 8 in breast cancer cells. Reprinted with permission, Medicine and Health/Rhode Island.

otherwise suitable for analysis by conventional techniques could still yield useful cytogenetic information. Interphase analysis could thus be used to study cells from tumors with low proliferative activity or from tumors that are difficult to maintain in short-term cultures. Additionally, interphase cytogenetics is rapid because it does not require special training for interpretation. Interphase cytogenetics allows some correlation of cytogenetic findings with morphology, which is not possible with routine cytogenetic study because all nuclear details are lost in a metaphase cell. When used as an adjunct, interphase cytogenetics offers benefits in both the study of malignant cells and in the management of patients with malignant disorders. It is ideal for archival tumor tissues that are amenable to immunocytohistochemical studies, but are not amenable to conventional cytogenetic analysis.

C. FISH for Detecting Chromosome Aneuploidy

Aneuploidy has been explored as a biomarker for stratifying many cancers. Traditionally, flow cytometry has been the method of choice for ploidy analysis. Many studies suggest that DNA ploidy analysis using flow cytometry and static image cytometry can provide independent prognostic information in addition to stage and histological grade (Adolfsson, 1994; Lieber et al., 1995). However, flow cytometry for detecting aneuploidy has its own limitations for sensitivity (Hopman et al., 1990; Takahashi et al., 1994; Visakorpi et al., 1994). For example, Visakorpi et al. (1994) found that FISH using three selected chromosome-specific probes is two to three times more sensitive than cytometric DNA-content analysis in aneuploidy detection. DNA flow cytometry, especially when performed from paraffin-embedded tumors, cannot distinguish aneuploid cell clones that have only a few numerical chromosomal changes from diploid clones. Early stage tumors that are often near diploid or that show balanced chromosomal abnormalities are not detected as DNA abnormal. Mesker et al. (1996), for example, noted that the diagnostic value of DNA ploidy measurements by flow or with image cytometry with respect to the detection of DNA aneuploidy is limited, as only relatively large changes in total DNA content can be detected (i.e., at best 2%, depending on the accuracy of the measurements). Generally, the equivalent of a gain or loss of one large or several small chromosomes (4% change in total DNA content) is required for adequate detection by flow cytometry (Koss et al., 1989; Persons et al., 1994; Mark et al., 1997g). Thus, although the ability to readily evaluate large numbers of nuclei is a strength of flow cytometry, a small population of abnormal cells may be difficult to detect within a much larger population of normal cells.

Interphase cytogenetics is ideal for studying aneuploidy and has the potential of revolutionizing the field of surgical pathology because of its increased sensitivity over flow cytometry and static image analysis in aneuploidy detection (Persons et al., 1993; Brown et al., 1994; Takahashi et al., 1994). Previous studies have found that PCR techniques also may not be as sensitive as FISH due to tumor heterogeneity (Deubler et al., 1997). In short, interphase cytogenetics using FISH with

chromosome enumeration probes is ideally suited for the study of cancer using archival materials.

D. FISH for Detecting Oncogene Amplifications

Cytogenetic manifestations of gene amplification are homogenously staining regions (HSRs) and double minutes (DMs). The former are amplified sequences on chromosomes, whereas the latter are extrachromosomal amplicons. Gene amplification is a characteristic of cancer cells whereby specific proteins important for carcinogenesis are produced. Oncogene overexpression has traditionally been detected using immunohistochemistry (IHC). Detection of oncogene amplification using FISH is a more sensitive technique that may overcome some of the difficulties of IHC and also Southern blot analysis. The most frequently studied oncogenes include *HER-2/neu* in breast cancer, *N-myc* in neuroblastoma, and *c-myc* in breast and ovarian cancers. *HER-2/neu* is associated with poor prognosis in node-negative breast cancer and is a better predictor of poor clinical outcomes than tumor size (Press et al., 1997). Studies of gene amplification is one of the most active areas of cancer research. Our laboratory, for example, has successfully detected *HER-2/neu* oncogene amplification in rhabdomyosarcoma, and breast, ovarian and cervical cancer (Mark et al., 1998b).

E. Other Miscellaneous Applications

Gene Mapping

Somatic cell hybrids containing one or more human chromosomes in a rodent background have been used extensively in gene mapping. For mapping of sub-chromosomal fragments, radiation hybrids are employed. The utility of the irradiation-fusion approach, however, depends on the purity of the hybrid cell lines. Initial characterization and periodic verification are therefore of paramount importance. Both conventional techniques (e.g., GTG-banding and G-11 staining) and molecular cytogenetic techniques are useful; but FISH is more sensitive in detecting human chromosomes or chromosomal fragments (Mark et al., 1992).

One of the first uses of FISH was for localizing DNA sequences onto metaphase chromosomes as part of the efforts of the Human Genome Project. The same FISH technology can be extended to map viral integration sites. Using FISH and chromosome morphometry, Mark and Chow (1995b), for example, localized the gene encoding the Secretin Receptor, SCTR, on human chromosome 2q14.1. Similarly, the site of integration of human papillomavirus sequences in a cervical tumor cell line was mapped by the technique of FISH (Mark et al., 1996c).

Genotoxicity Studies

Dicentric chromosomes have been traditionally utilized as an index of chromosome damage. However, chromosomal translocations are more stable with time. FISH using chromosome painting probes is a powerful tool for detecting translocations and in studying the genotoxic responses to environmental genotoxins. FISH with chromosome-specific α -satellite probes, on the other hand, can detect aneuploidies resulting from chromosome nondisjunction. This type of study can yield important information on the frequency of spontaneous and induced genetic damage and on the effects of various mutagens (aneugens) and carcinogens. Eastmond and Pinkel (1989), for example, studied aneuploidy-inducing agents in human lymphocytes using FISH.

Sequential Flow Cytometry and FISH

Both flow cytometry and FISH are useful techniques for the analysis of cancer tissues. When the two methods are used to study the same specimens, they are usually performed separately, in parallel. This protocol is problematic in cases where there is a scarcity of material, making completion of both studies impossible. FISH procedures that utilize excess material discarded from flow cytometry would be advantageous. Thus, Mark et al. (1997g) described a protocol for performing sequential flow cytometry and FISH using formalin-fixed, paraffin-embedded archival material of breast cancer tissues. Analogously, sequential hematopathology and FISH can also be performed.

FISH-Based Assay for Aneuploidy Detection in Sperm

It is estimated that in humans a minimum of 10% of all recognized conceptions are chromosomally abnormal, and that 1 to 2% are the result of fertilization by spermatozoa with chromosomal abnormalities. FISH is a suitable technique for the analysis of sperm in infertility as well as in studies of genotoxicology (Mark et al., 1995i; Sigman and Mark, in press).

VI. EMERGING FISH TECHNOLOGIES

Recent advances in molecular technology have led to the development of newer techniques that combine the sensitivity and specificity of FISH with the global screening ability of conventional cytogenetics. We will provide a brief overview of two such technologies, comparative genomic hybridization (CGH) and spectral karyotyping (SKY).

A. Comparative Genomic Hybridization

The principle of CGH is based on what Kallioniemi et al. (1992) called a copy number karyotype, in which tumor genomic DNA and normal genomic DNA are hybridized, in fixed proportions, to metaphase spreads with normal chromosome complements. With the advent of CGH, a map of DNA sequence copy number changes can be produced with respect to size, frequency, number, location and effect on the entire genome (Kallioniemi et al., 1993, 1994).

The hybridization of both types of genomic DNA is conducted in concert in order to induce competition between the tumor genomic DNA and the normal genomic DNA for specific DNA sequences on the normal metaphase spreads. Normally the test DNA is detected with fluorescein isothiocyanate, which produces a green fluorescence, and the reference DNA with rhodamine, which produces a red fluorescence. How much DNA is bound from either the tumor or normal genomic DNA is characterized by the intensity with which they fluoresce.

In order to determine whether a chromosomal loss or gain has occurred, the fluorescence ratio of the green-red intensities is gauged and then calculated. From the number obtained through a ratio of test DNA to reference DNA, the approximate copy number of the homologous sequences on each chromosome between the two genomes can be quantified. Disomy should yield a ratio of 1.0; trisomy would be expected to increase the ratio to 1.5; tetrasomy increases it further to 2.0; and monosomy has a ratio of 0.5. Interspersed repetitive sequences that can lead to cross-hybridization and interfere with the fine gradations in the signal intensities are suppressed by saturation with human Cot 1 DNA. A schematic representation of the CGH protocol is given in Figure 7.

Whereas conventional cytogenetics require dividing cells from the test subject, CGH only requires the availability of genomic DNA so that even archival materials can be studied. CGH requires no prior knowledge of the genomic region to be studied.

However, CGH is not without limitations. Indeed, the following shortcomings of CGH have prevented its routine application in the average clinical cytogenetics laboratory. For example, CGH can spot sequence copy number changes only if greater than 50% of the cells analyzed contain a chromosome gain or loss. This means that the sequence copy changes in tumors which have many heterogeneous populations of normal and stromal cells may very likely evade detection. In addition, CGH can recognize gains and losses of DNA regions only with respect to the average copy number of the complete tumor specimen. CGH cannot identify balanced chromosome abnormalities for which there are no copy number changes, such as those found in balanced translocations, inversions, and intrachromosomal rearrangements, which represent a significant proportion of chromosomal abnormalities. Finally, its dependence on expensive imaging equipment for analyzing results financially taxes the budget of cytogenetics laboratories.

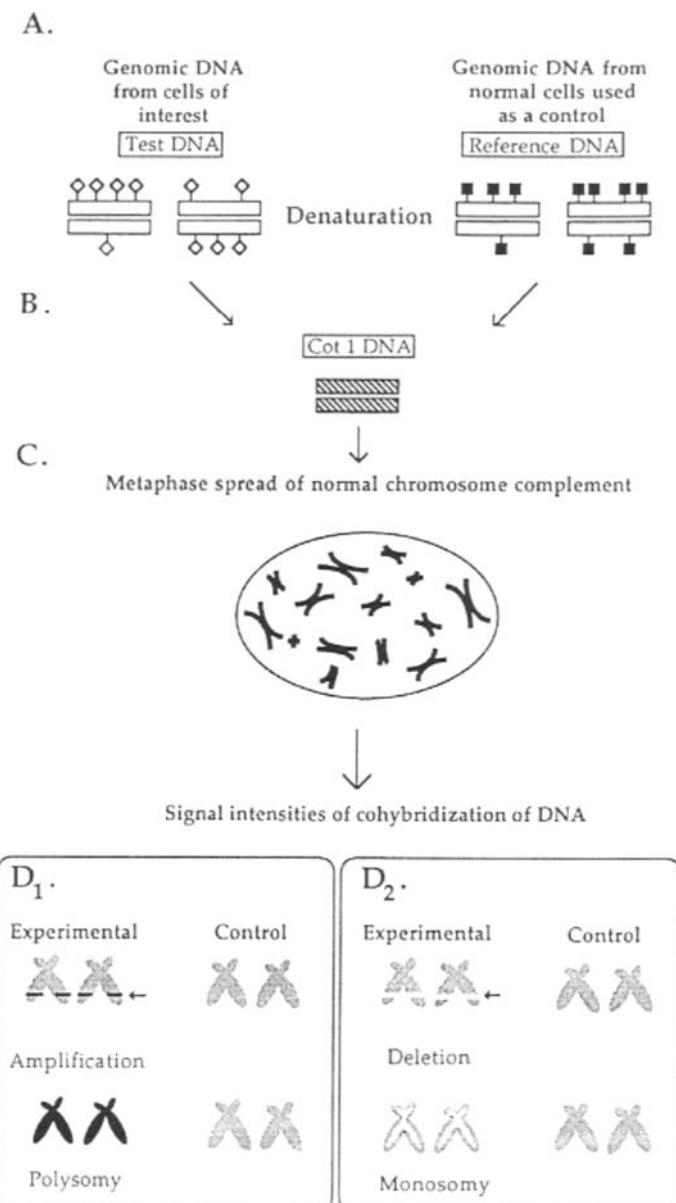


Figure 7. Comparative genomic hybridization (CGH). Reprinted with permission of Cytobios.

B. Spectral Karyotyping

SKY is an evolving molecular cytogenetic technique that permits an examination of the entire genome in a single hybridization. Based on the ability of a digital image analysis system to designate a specific color to a particular mixture of fluorochromes, SKY has the capability to create a different paint color for each chromosome to cover the whole genome. Painting the chromosomes this way, SKY can identify balanced rearrangements, cryptic translocations and marker chromosomes in all tissues, including solid tumors (Schrock et al., 1996).

The methodology of SKY essentially involves 3 parts: preparation of metaphase spreads, hybridization of the chromosomal preparation to the probe cocktail; and spectral imaging of the results. With SKY, each chromosome is labeled with its own unique concoction of fluorochromes. Once every chromosome has been labeled, a probe set that comprises all 24 chromosomes is hybridized to normal metaphase spreads in a process called suppression hybridization. This step blocks the cross-hybridization of repetitive sequences in the genomic libraries that would significantly alter spectral measurements for image acquisition. Finally, an image displaying a spectral karyotype of all chromosomes can be visualized through spectral imaging, in which the overlapping fluorochromes are separated and classified according to the emission spectra produced from each pixel, using epifluorescence microscopy, charge-coupled device imaging, and Fourier spectroscopy. A simplistic summary of SKY is depicted in Figure 8.

The power of SKY is that it can detect small translocations and insertions beyond the resolution of conventional cytogenetics. It is especially useful for analyzing solid tumors. SKY, however, lacks the capacity to detect certain chromosomal aberrations. SKY can neither identify microdeletions nor microduplications. It cannot detect pericentric or paracentric inversions. It also entails the use of highly specialized and expensive equipment as well as costly painting probes for labeling each chromosome.

C. Other Comments

Because techniques such as CGH (Kallioniemi et al., 1992, 1993, 1994) and SKY require specialized instrumentation and trained personnel, the exact roles that these new emerging technologies will play in the average clinical cytogenetic laboratory in the current climate of managed care, and cost containment (Mark et al., 1996f) is yet to be determined. Meanwhile, interphase cytogenetics using commercially available chromosome enumeration probes is increasingly being utilized in the average clinical cytogenetics laboratory for a multitude of applications.

One of the latest developments in the field is in the area of research on chromosome-specific subtelomeric probes (being conducted in the laboratory of Dr. David Ledbetter in Chicago, IL). Telomeres are repetitive structures at the ends of

A.

Preparation of metaphase spreads

**B.**

Labeling of probes and hybridization to the chromosomes

**C.**

Spectral imaging and production of a rainbow-colored karyotype whereby each chromosome can be distinguished by a different color

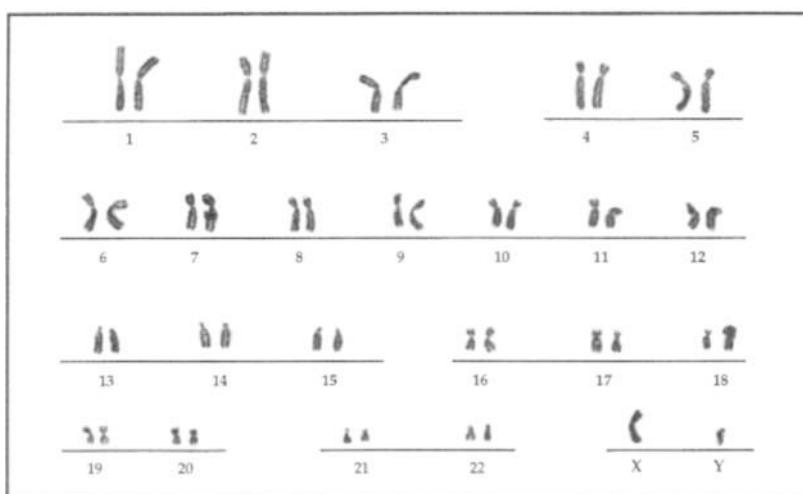


Figure 8. Spectral karyotyping (SKY). Reprinted with permission of Cytobios.

chromosomes consisting of a simple six-nucleotide repeat, TTAGGG. The telomere band, however, also includes "subtelomeric repeats" or "telomere-associated repeats." Subtelomeric regions of chromosomes are believed to be gene-rich (Saccone et al., 1992; Wong et al., 1997); thus, rearrangements involving these regions may have grave phenotypic consequences.

VII. CONCLUDING COMMENTS

The advent of FISH was made possible by the explosion in recombinant DNA technology and the Human Genome Project. It offers an unprecedented opportunity for the analysis of a large number of non-dividing cells in the interphase stage. Thus, FISH is sometimes called interphase cytogenetics.

FISH is the most direct method for visualizing a specific DNA sequence on the metaphase chromosome or an interphase cell. It allows for both enumeration and localization of a DNA sequence underlying a cytogenetic abnormality. In addition, FISH requires no additional acquisitions of new samples. It can be performed on unstained or previously Giemsa-stained slides and also unstained as well as previously Wright-stained smears of both peripheral blood and bone marrow. It can also be performed on many other tissues, as well as archival materials previously not amenable to cytogenetic study.

The 1990s has seen the establishment of conventional cytogenetics as part of the routine management of patients with many disease types. Since our last survey of the current status of the field (Mark, 1994a, 1994e) the increase in the number of reports where FISH is mentioned has been astronomical. By July of 1997, several cancer probes had already gained U.S. Food and Drug Administration approval. Vysis (Downers Grove, IL) had received 510(K) clearance for the CEP X-Spectrum OrangeTM/Y Spectrum GreenTM direct label chromosome enumeration DNA probe kit, CEP 8 Spectrum OrangeTM direct label chromosome enumeration DNA probe kit, and CEP 12 Spectrum OrangeTM direct label chromosome enumeration DNA probe kit (Chang and Mark, 1997). In the research arena, Oncor, Inc. (Gaithersburg, MD) has received FDA approval for a DNA probe to detect amplification of the *HER-2/neu* oncogene.

As Chang and Mark (1997) pointed out, since today's research may be tomorrow's clinical procedures, it behooves all physicians, scientists, and others interested in laboratory genetics to closely monitor developments in this rapidly evolving field.

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ter Cytogenetics Laboratory is also acknowledged. Dr. Mark is a clinical cytogeneticist board-certified by the American Board of Medical Genetics. She has been performing *in situ* hybridization as an adjunct to conventional cytogenetics since 1988 and has authored approximately 135 scientific publications and more than 300 abstracts/posters/presentations.

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THE CONTRIBUTION OF DEFECTS IN INSULIN SIGNALING IN SKELETAL MUSCLE TO INSULIN RESISTANCE AND TYPE 2 DIABETES

CELLULAR AND MOLECULAR ASPECTS

Karen C. McCowen and E. Dale Abel

I.	Background	42
II.	Insulin Signaling and Glucose Metabolism	42
III.	The Insulin Signaling Cascade	48
	A. Insulin Receptor	48
	B. IRS Proteins	48
	C. Phosphatidylinositol 3-Kinase	50
	D. Akt	50
	E. Glut4	51
IV.	Insulin Signaling in Insulin-Resistant States	52
	A. Gene Knockout Models	52
	B. Animal Models of Diabetes and Insulin Resistance	54
	C. Human Obesity and Type 2 Diabetes	55

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V.	Mechanisms of Insulin Signaling Abnormalities	56
A.	Tumor Necrosis Factor- α	56
B.	Glucosamine	57
C.	Phosphatases	58
D.	PC1	59
VI.	Summary and Conclusions	59
	References	60

I. BACKGROUND

Glucose homeostasis is maintained *in vivo* through a balance between glucose disposal in multiple tissues, glucose production by hepatocytes, and absorption via the small intestine. In mammalian cells, glucose crosses cell membranes by facilitated diffusion mediated by a family of closely related glucose transport proteins (Gluts). Glucose transport into cells is essential for survival. Insulin enhances disposal of glucose into certain tissues (i.e., skeletal muscle, adipose, and cardiac) by binding its receptor on the cell surface and activating a series of reactions that leads ultimately to glucose uptake by a specific transporter, Glut4. Enzymes that increase glycolysis and glycogen synthesis are stimulated by insulin so that intracellular glucose concentrations do not rise. Simultaneously, in liver, insulin inactivates the enzymes of gluconeogenesis and glycogenolysis, thus reducing endogenous glucose production.

Diabetes mellitus is a metabolic disorder characterized by elevated blood glucose and is classified most simply into two main types (McCown and Smith, 1998). The pathogenesis of Type 1 diabetes is on the basis of insulin deficiency, whereas the more common Type 2 is caused by a combination of insulin resistance and relative insulin insufficiency, the latter likely resulting from "postreceptor" signaling defects. This insulin resistance, when coupled with diminished insulin production, leads to decreased glucose disposal rates following a meal and elevated blood glucose levels (DeFronzo, 1992). Persons with severe insulin resistance but adequate beta cell reserve do not develop diabetes. Type 2 diabetes has a strong genetic predisposition; however, acquired conditions such as obesity can precipitate diabetes by exaggerating the inherited insulin resistance.

II. INSULIN SIGNALING AND GLUCOSE METABOLISM

The early steps of the insulin signaling pathway in skeletal muscle have been well characterized (Cheatham and Kahn, 1995). The hormone binds to the extracellular portion of the transmembrane receptor (Figure 1), which results in autophosphorylation of certain tyrosine residues of the intracellular portion. This latter event triggers a cascade of downstream phosphorylation events,

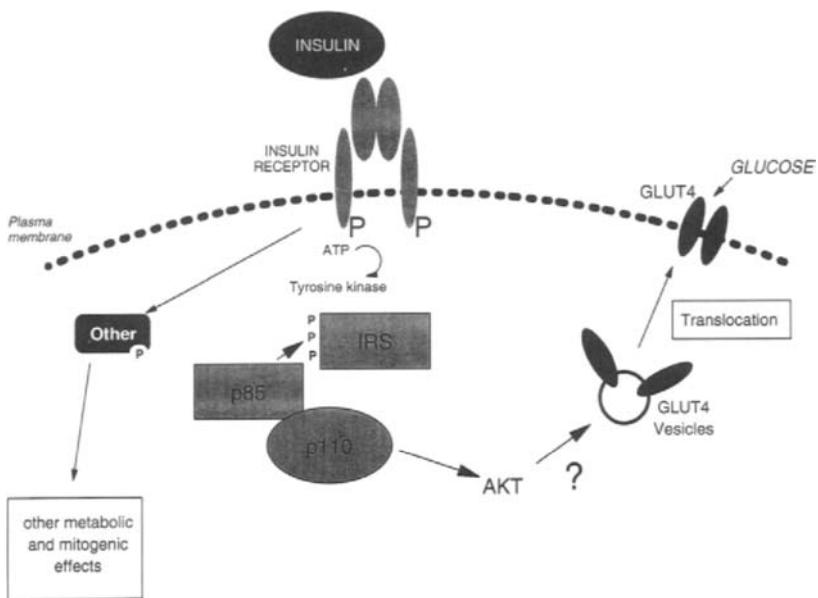


Figure 1. The insulin signaling pathway: from receptor to Glut4 and glucose uptake. P = phosphorylated tyrosine residues.

magnifying the initial signal. A family of docking proteins known as the insulin receptor substrates (IRSs) mediates a critical part of the signal process. Interaction with activated receptor leads in turn to phosphorylation on specific tyrosine residues of IRS proteins. Tyrosine phosphorylated IRS proteins are targeted by a number of different intracellular proteins that have in common a specific domain homologous to Src: the Src homology-2 (SH2) domain (Wandless, 1996). In this manner, IRS proteins can activate diverse intracellular processes. In skeletal muscle, this results in stimulation of 2 major events: (1) glucose metabolism and (2) mitogenesis. Signal is propagated when the regulatory subunit (p85) of a critical enzyme phosphatidylinositol 3-kinase (PI 3-K) binds to the phosphorylated IRS, allowing activation of the catalytic subunit of PI 3-K. The serine/threonine kinase Akt is then phosphorylated and may be one of the mediators of Glut4 translocation. Insulin ultimately causes Glut4 to shift from an intracellular pool (inactive) in the low-density microsomal membranes (LDM) to the plasma membrane and increases its intrinsic activity, both of which lead to a dramatic enhancement of glucose transport.

Activation of IRS proteins and PI 3-K by the insulin receptor is also critical for the intracellular disposal of glucose in skeletal muscle (Figure 2). Oxidative

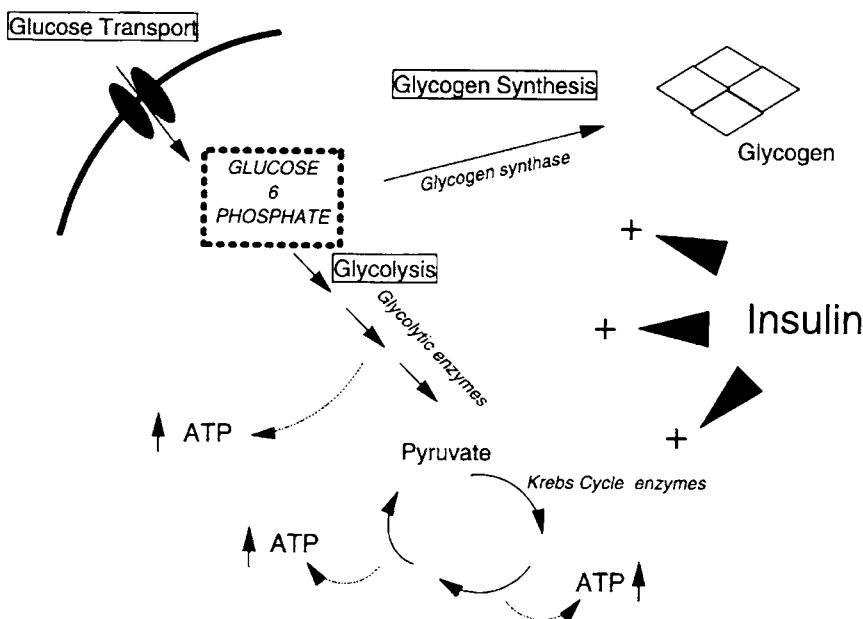


Figure 2. Intracellular disposal of glucose through glycolysis or for glycogen formation.

degradation of glucose to 3-carbon fragments and adenosine triphosphate (ATP) occurs in glycolysis and the Krebs cycle. These enzymatic processes are stimulated by the presence of glucose as well as through activation by insulin of certain key enzymes. Nonoxidative disposal, accounting for a greater proportion of any glucose load, occurs through storage of glucose as glycogen. Insulin, via its activation of PI 3-K and probably Akt, causes stimulation of glycogen synthase and simultaneous inhibition of glycogen breakdown, avoiding a futile cycle. The net result of the “metabolic” action of insulin in muscle is to raise both available and stored energy.

In hepatocytes and adipocytes the insulin signaling machinery is identical, although the terminal effects of insulin differ somewhat. In adipocytes, insulin regulates glucose uptake by Glut4 but also has a major role to promote fat storage and prevent lipolysis. Hepatocytes differ from muscle and fat in that the main glucose transport protein is Glut2, which is not insulin-responsive. Insulin regulates the function of a number of genes in liver, controls the rates of glycogen synthesis and breakdown as in muscle, and regulates lipogenesis.

Insulin has diverse effects, both immediate actions on various enzyme systems as well as longer-term effects involving DNA, lipid and protein synthesis, cell proliferation, and cell differentiation (White and Kahn, 1994) (Figure 3). The

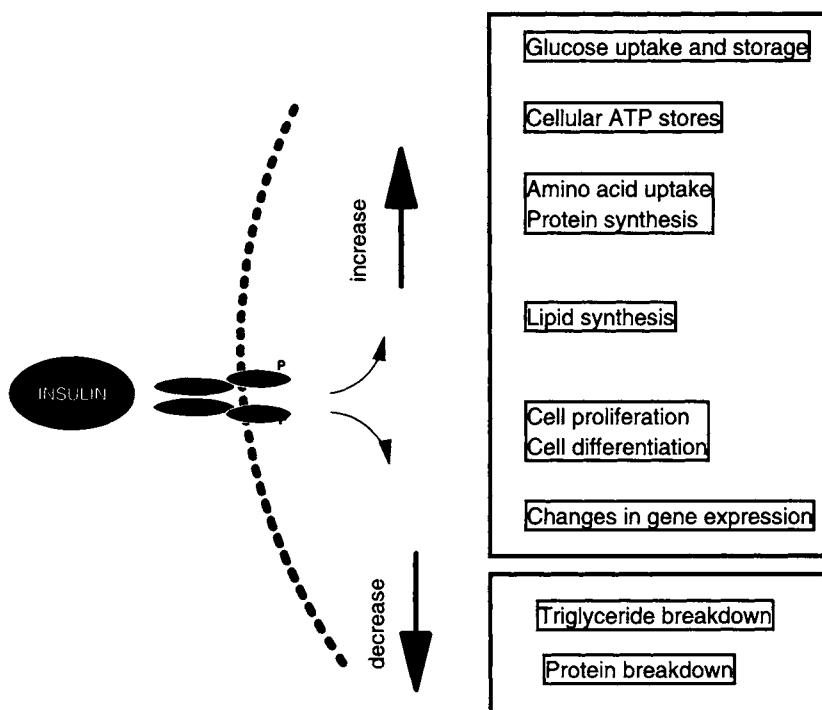


Figure 3. Multiplicity of effects of insulin.

proto-oncogenes *c-fos* and *c-jun* have been identified as intermediates in pathways that ultimately lead to insulin-stimulated tissue growth responses (Kim and Kahn, 1994). These actions occur through pathways that are parallel to and overlap the glucoregulatory cascade (Figure 4). Although IRS proteins and PI 3-K are probably involved in this function, several other proteins are candidate mediators of mitogenesis (Mendez et al., 1996; Rose et al., 1994). Grb2 is a cytoplasmic protein containing an SH2 domain that associates with a guanine nucleotide exchange factor, Sos (Chardin et al., 1995).

Insulin activates the Grb2-Sos interaction, which results in the stimulation of the guanosine triphosphate (GTP)-binding protein Ras by increasing its GTP:GDP (i.e., guanosine diphosphate) ratio (Myers, Jr. et al., 1994). Ras, a membrane-associated GTPase that has been demonstrated to play a major role in regulation of cell growth and tumorigenesis, is linked through a series of kinases with members of the family mitogen-activated protein kinases (MAPK). Within minutes of insulin

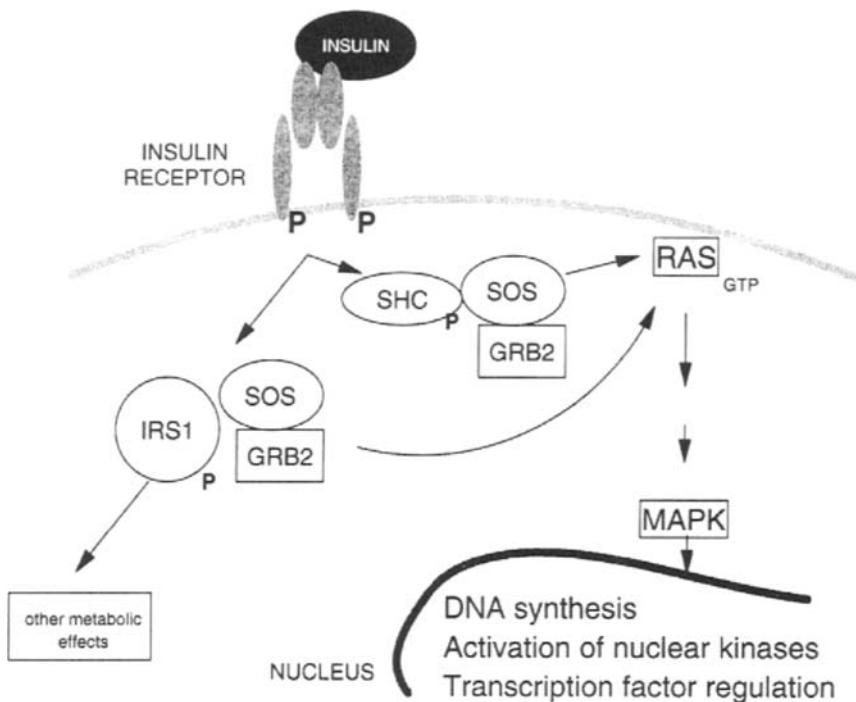


Figure 4. Activation by insulin of mitogenesis through the MAP kinase pathway. P = phosphorylated tyrosine residues.

receptor activation, dual phosphorylation on serine and threonine residues of 2 members of the family, Erk1 and Erk2, is evident (Denton and Tavare, 1995). In cultured cells, overexpression of activated Ras results in MAPK activation and leads to cellular transformation. Insulin stimulation can lead to activation of MAPK either through IRS protein association with Grb2, or by phosphorylation of a different adapter protein, Shc, directly by the tyrosine phosphorylated receptor (Skolnik et al., 1993). Shc too can directly interact with Grb2 and thus activate Ras. To sum up the recent available evidence, MAPK plays a role in many, if not all of the mitogenic effects of insulin (Heesom et al., 1997). Thus, through parallel but somewhat overlapping pathways, insulin signaling results in both mitogenic and metabolic downstream events.

In addition, insulin causes activation of various phosphatases (PTPases), that may reduce the degree of tyrosine phosphorylation of the signaling cascade and thus may deactivate or downregulate the system (Goldstein et al., 1998). In cellular and molecular studies, both transmembrane and soluble PTPases have been shown to have a direct impact on insulin action in cultured cells. In skele-

tal muscle, the 3 most abundant phosphatases thus far described are SHPTP2, LAR, and PTP-1B (Ahmad and Goldstein, 1995). All 3 have been shown to interact with various members of the insulin signaling cascade. SHPTP2 is a soluble phosphatase that contains SH2 domains, has been shown to interact with IRS1, and has potential to modulate signaling through this pathway (Lima et al., 1998). LAR is a transmembrane phosphatase that contains catalytic specificity in vitro for the insulin receptor kinase domain (Zhang et al., 1996). PTP-1B is abundantly expressed in tissues and is located predominantly in the endoplasmic reticulum (Bandyopadhyay et al., 1997; Seely et al., 1996). The relative

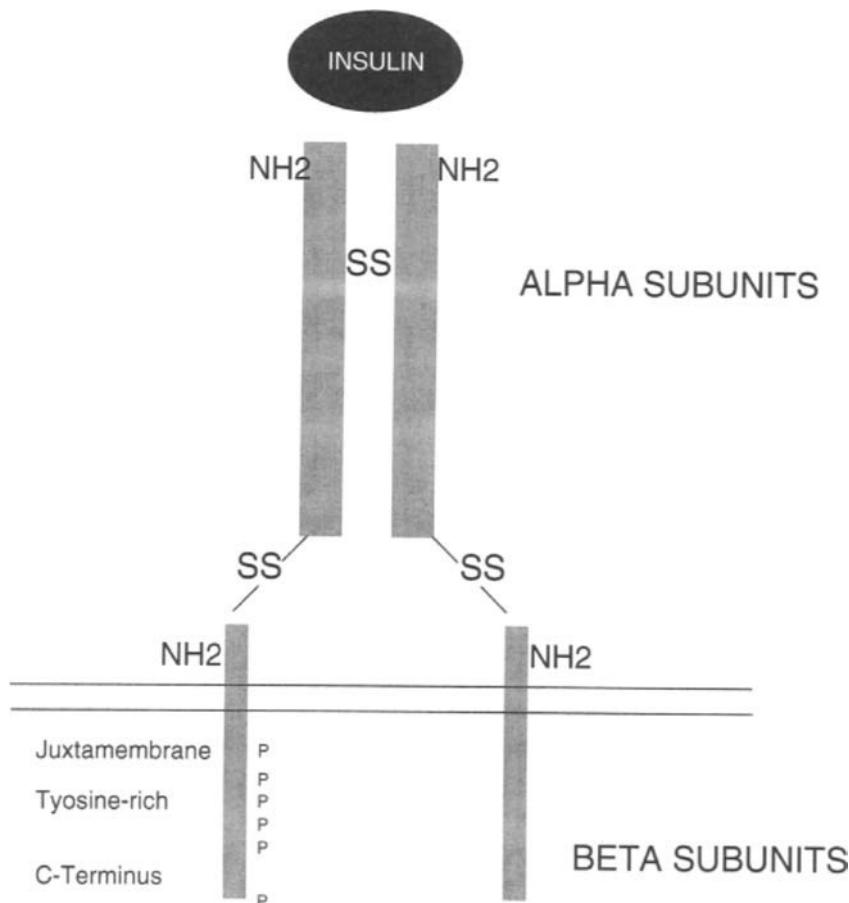


Figure 5. Structure of the insulin receptor. SS = disulphide bond. P = phosphorylated tyrosine residues. NH2 = amino terminus.

importance of the various PTPases for insulin signaling to glucose transport is currently incompletely understood.

III. THE INSULIN SIGNALING CASCADE

A. The Insulin Receptor

The receptor is a membrane-spanning glycoprotein tetramer, composed of 2 each α and β subunits (Figure 5) (Lee and Pilch, 1994). The α subunits form the hormone binding domain (De Meyts et al., 1990); insulin binding results in transmission of a poorly understood signal to the internal β subunits, resulting in receptor autophosphorylation, allowing the receptor to function as a protein kinase for intracellular substrates (Kasuga et al., 1983; Kasuga et al., 1982).

It has become clear that mutations in the gene for the insulin receptor are extremely rare causes of insulin resistance (Moller et al., 1994), and that even acquired defects are not responsible for typical diabetes. Alpha subunit mutations are associated with reduced insulin binding at the cell surface, as well as impairment in posttranslational processing and transport of the receptor to the cell membrane for insertion (Takahashi et al., 1998). Mutations in the juxtamembrane region of the β subunit distort the insulin signal, and are associated with impaired autophosphorylation. Similarly, mutations in the ATP binding site or in any of the three tyrosine residues in the kinase domain that are normally phosphorylated will also reduce the kinase activity of the receptor (Moller and Flier, 1988). It is in the tyrosine kinase domain that the greatest number of naturally occurring mutations occur. Insulin resistance associated with these mutations demonstrates the vital importance of the tyrosine kinase function of the insulin receptor in modulation of downstream actions of the hormone. When these mutant receptors were expressed in Chinese hamster ovary (CHO) cells, autophosphorylation did not occur nor were downstream substrates phosphorylated (Moller et al., 1990).

Clinically, patients with insulin receptor mutations have been described to have a wide variation in phenotype, ranging from profound insulin resistance to milder forms with hirsutism and acanthosis nigricans (Moller and Flier, 1991). However, direct examination of the genotype in patients with typical Type 2 diabetes for polymorphisms of the insulin receptor has not shown any specific associations (Kusari et al., 1991; Moller et al., 1989).

B. IRS Proteins

IRS-1 was the originally described member of what has become a large family of proteins, all of which are rapidly tyrosine phosphorylated by insulin with a time course and dose-response curve that mirror that of autophosphorylation of the insulin receptor (White et al., 1985). IRS-1 contains 22 potential sites for

phosphorylation on tyrosine residues—mainly in the carboxy terminus—and serves, when activated, as a docking site for many other downstream molecules containing SH2 sites (Myers, Jr. et al., 1994). IRS-1 is highly conserved between species, with the most highly conserved region being that with homology to plexin (plexin homology [PH] domain). Plexin is a platelet protein containing internal repeats and is found in a variety of signaling proteins, suggesting a role for IRS-1 in protein–protein interaction. Mutant IRS-1 molecules with PH domain deletions are associated with impaired responsiveness to insulin (Myers, Jr. et al., 1995). Another highly conserved region of IRS-1 is a stretch of approximately 115 amino acids that bind the phosphorylated insulin receptor, the phosphotyrosine binding (PTB) domain (van der Geer and Pawson, 1995).

One of the best-described actions of tyrosine-phosphorylated IRS proteins is to bind p85. This results in phosphorylation by the catalytic subunit of PI 3-K of the myoinositol ring at the 3 position, generating PI 3-phosphate from PI, PI 3,4-bisphosphate from PI 4-phosphate, and PI 3,4,5-triphosphate from the 4,5-bisphosphate. Phospho-IRS1 also binds other adapter proteins such as Grb2. The advantage of signaling through IRS proteins is that signal from the insulin receptor can easily be amplified (Sun et al., 1993). This can occur without the constraints that limit membrane-bound receptors that bind SH2-containing proteins directly, as one autophosphorylated insulin receptor can engage multiple IRS proteins. In this manner, the insulin receptor can potentially be linked concurrently to multiple signaling cascades.

Subsequently other members of the IRS protein family (IRS-2, -3, -4, and Gab-1) have been described, all with homology to IRS-1, particularly in the PH and PTB domains. IRS-2 was initially found in lymphopoietic cells (Araki et al., 1993; Patti et al., 1995) but is abundant in hepatocytes and somewhat less so in muscle cells and adipocytes (Sun et al., 1997). IRS-3 mRNA is present in murine adipocytes and liver but has not been detected in skeletal muscle (Lavan et al., 1997; Sciacchitano and Taylor, 1997). There is no published information about the tissue distribution of IRS-4, thus it is unclear whether this protein has a role in mediation of the metabolic effects of insulin (Lavan et al., 1997). Gab-1 occurs in mammalian tissues and was cloned by expression screening using a Grb2 probe (Holgado-Madruga et al., 1996). Although a PH domain, is present, Gab-1 lacks a PTB domain which may diminish its coupling to the insulin receptor and thus its importance in the insulin signaling pathway is uncertain.

As insulin receptor mutations have been clearly shown not to be present in typical Type 2 diabetes, IRS genes have been scrutinized as potential culprits. To summarize the work from different populations, most studies have been unable to identify polymorphisms that segregate with insulin resistance (Almind et al., 1993; Imai et al., 1997; Almind et al., 1998; Bernal et al., 1998). When sequence variations are identified, little correlation with the degree of insulin resistance has been found. One sequence variant of IRS-1 detected in diabetic patients, when transfected into a cultured cell model, was associated with impaired insulin medi-

ated IRS-1 associated PI 3-K activation, although this is clearly the exception (Almind et al., 1996). In conclusion, most typical Type 2 diabetes is not associated with genetically altered IRS proteins.

C. Phosphatidylinositol 3-Kinase

PI 3-K is a heterodimeric protein composed of a 110-kDa catalytic and various regulatory subunits, which include the widely expressed forms p85 α and p85 β as well as the truncated forms p55 α , p50 α and p55 $^{\text{PIK}}$ (Alessi and Downes, 1998). An SH2 domain that can interact with tyrosine phosphorylation sites on various signaling proteins is common to all forms of the regulatory subunit. The activation of the enzyme results in phosphorylation on the d-3 position of the inositol ring of phosphatidylinositol; the lipid products are important intracellular signaling molecules that mediate large numbers of processes. PI 3-K can be activated by several different growth factors as well as insulin and likely produces both mitogenic and metabolic effects. The functions of the different isoforms are under investigation; they may subserve different pathways although little has been published in this regard (Inukai et al., 1997).

The importance of PI 3-K in the metabolic effects of insulin has been shown by a number of different experimental techniques mainly in cultured cells. Expression of a mutant p85 resulted in failure of insulin-mediated glucose uptake (Hara et al., 1994), as did inclusion of chemical inhibitors (e.g. wortmannin) of PI 3-K (Okada et al., 1994). Conversely, expression of constitutively active forms or over-expression of PI 3-K, via enhancing Glut4 translocation, results in enhanced glucose uptake without requiring insulin stimulation (Martin et al., 1996).

However, although PI 3-K is necessary for insulin-mediated glucose uptake, its activation is not sufficient since other tyrosine kinase receptor ligands such as platelet derived growth factor (PDGF) can activate PI 3-K but fail to effect Glut4 translocation or glucose uptake (Quon et al., 1996). A leading possibility to explain this paradox is that insulin can somehow gain access to a specific pool of PI 3-K that is physically separate from the PI 3-K activated by other growth factors. Alternatively, molecules on parallel pathways activated by the insulin receptor are required in addition to PI 3-K for glucose uptake. There is some preliminary evidence that certain atypical isoforms (λ and ζ) of the protein kinase C (PKC) are involved in insulin-mediated glucose uptake (Kotani et al., 1998).

D. Akt

Akt, also known as protein kinase B because of its structural similarity to previously identified protein kinases C and A, is activated when phosphorylated on a threonine and a serine residue (Kohn et al., 1996). One of the upstream kinases has been identified (PDK1) but the exact sequence of phosphorylation events is unde-

terminated (Alessi et al., 1997; Cohen et al., 1997). Akt can bind directly to inositol 3-phospholipids through its PH domain. Multiple experiments using constitutively active Akt in cultured cells place Akt upstream of the Glut4 glucose transporter in the signaling cascade (Kohn et al., 1996). It has also been shown, through a number of *in vitro* experiments using wortmannin or dominant negative p85, that Akt is downstream of PI 3-K (Burgering and Cocher, 1995).

However, the importance of the role played by Akt in glucose uptake in whole animals as opposed to cultured cells is unclear and the data are conflicting. In several models of rodent diabetes in which insulin-mediated PI 3-K and glucose uptake are inhibited, Akt can be maximally stimulated (Kim et al., 1999; McCowen et al., 1998). One possible explanation is that only a small fold increase in PI 3-K activity is required to stimulate Akt completely, another that PI 3-K-independent mechanisms to activate Akt exist. Experiments in rat adipocytes demonstrated that cross-linking of integrins on the cell surface resulted in activation of IRS1, PI 3-K, and Akt, whereas glucose uptake was unaltered (Guilherme and Czech, 1998). In addition, a dominant negative construct of Akt that abolished up to 95% of Akt activity did not affect insulin-stimulated glucose uptake, whereas protein synthesis was inhibited in CHO and 3T3 L1 cells (Kitamura et al., 1998). Thus, although Akt is clearly a constituent of the insulin signaling cascade, whether Akt is an upstream mediator of glucose transport remains in question.

E. Glut4

Glut4 is a facilitative glucose transporter, one of a family of integral membrane hexose transporters, expressed in skeletal and cardiac muscle and adipocytes (Kahn, 1992). Glut4 undergoes constitutive cycling between LDM and plasma membrane pools. Activation of the insulin signaling cascade increases the rate of translocation to the plasma membrane of Glut4 about 20-fold; this simultaneously inhibits the endocytic limb of the cycle (Cushman and Wardzala, 1980; Hirshman et al., 1990) and enhances intrinsic activity of Glut4. Exercise, hyperosmolarity, hypoxia, and heat shock also serve as stimuli for translocation and activation, although the mechanism for this is not certain but does not involve the insulin signaling cascade (Figure 6). Acute exercise has no effect on IRS protein phosphorylation, PI 3-K activation (Goodyear et al., 1995a), or Akt phosphorylation (Brozinick, Jr. and Birnbaum, 1998). During exercise, it is likely that other protein kinases, perhaps activated by low levels of intracellular ATP (increased AMP/ATP) can effect Glut4 translocation (Hayashi et al., 1998).

However, it is not known exactly how activation of the insulin signaling cascade mediates this translocation/activation step. Subcellular fractionation studies have demonstrated the presence of both activated PI 3-K (Kelly and Ruderman, 1993) and Akt (Calera et al., 1998) in the LDM. There is preliminary evidence that phosphorylation of Glut4, perhaps by either of these kinases, might serve as part of the regulatory mechanism (Kupriyanova and Kandror, 1999). Several vesicle

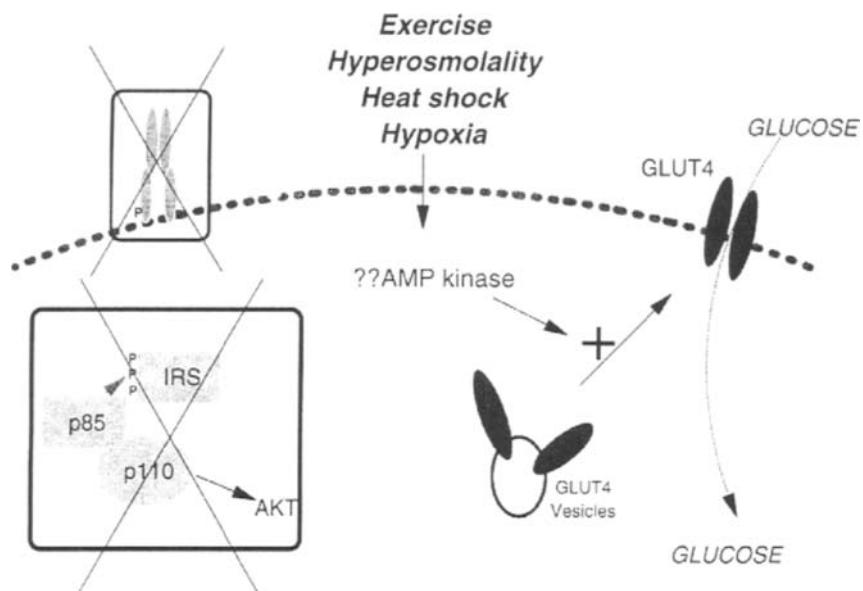


Figure 6. Mechanism of activation of glucose uptake by exercise.

proteins—vesicle-associated membrane proteins (VAMPs) and secretory-carrier membrane proteins (SCAMPs)—have been found that colocalize with Glut4 from the intracellular pool to the cell membrane in response to insulin. VAMPs also fuse with plasma membrane and may play a regulatory role in the trafficking process (Olson et al., 1997).

Glut1, another member of this transporter family, is also moderately abundant in insulin target tissues, although it is present in higher amounts in red blood cells, brain, intestine, and kidney. In response to insulin, Glut1 shows only modest redistribution to the plasma membrane, whereas in the basal state, Glut1 is the major glucose transporter on the cell surface in insulin-sensitive tissues.

IV. INSULIN SIGNALING IN INSULIN-RESISTANT STATES

A. Gene Knockout Models

Gene knockout models have been used to elucidate the function of the insulin receptor (Moller, 1994). Mice with complete inactivation of the insulin receptor are initially viable but are born growth retarded and die shortly after birth with diabetic ketoacidosis (Accili et al., 1996). Tissue-specific deletions of the insulin receptor gene have helped to clarify this. In MIRKO (Muscle Insulin Receptor

Knock-Out) mice, overt diabetes did not occur despite moderate insulin resistance (Bruning et al., 1998). Surprisingly, blood glucose and insulin levels remained normal out to 11 months of age, despite failure of insulin to activate its signaling cascade in muscle. In a different model, expression of a dominant negative kinase-deficient mutant insulin receptor in mouse skeletal muscle did reduce receptor kinase activity and insulin sensitivity; however, glucose uptake was barely affected (Chang et al., 1994). Mice with expression of a similar mutant insulin receptor limited to adipose tissue and muscle, in which hepatic insulin action was normal, also failed to develop diabetes despite evidence of peripheral insulin resistance (Lauro et al., 1998). Thus the conclusions that severe insulin resistance, if limited to skeletal muscle, is insufficient to produce diabetes. This suggests that a more complex interplay between muscle/adipose and the other organs important for glucose homeostasis (liver and pancreas) fails when diabetes develops, reinforced by the preliminary finding that deletion of the insulin receptor from the beta cell alone is associated with a syndrome like Type 2 diabetes (Kulkarni et al., 1999). Also, one must acknowledge that the impact of absent skeletal muscle insulin receptor on total body insulin sensitivity lead to questions of the primacy of muscle as an insulin target tissue in diabetes (Kahn and Rossetti, 1998).

Gene knockout mice also offer insight into the differential roles of the various IRS proteins. Of interest, IRS-1 knockout mice did not have overt diabetes, as it appeared that alternative signaling through IRS-2 was compensatory for glucose regulation, particularly in hepatocytes (Araki et al., 1994; Tamemoto et al., 1994; Yamauchi et al., 1996). The degree of insulin resistance in different target tissues varied in direct proportion to the amount of tyrosine phosphorylated IRS-2 that could be generated, although in adipocytes from the IRS-1 knockout, IRS-3 was the predominant mediator of the insulin signaling response (Smith-Hall et al., 1997). In contrast, disruption of IRS-2 gene in mouse caused a syndrome quite reminiscent of human diabetes, with peripheral insulin resistance accompanied by beta cell failure (Withers et al., 1998). The relative importance of dysfunction of the various IRS proteins in human skeletal muscle has not been determined.

Transgenic mice heterozygous for Glut4 gene knockout have significant reduction in muscle and adipocyte Glut4 protein content, and resultant decrease in insulin-mediated glucose uptake (Rossetti et al., 1997; Stenbit et al., 1997). Surprisingly, mice with homozygous deletion of the Glut4 gene did not have hyperglycemia, either in the fed or fasted state, despite complete absence of the protein from skeletal muscle, heart, and adipose tissue (Katz et al., 1995), although they were hyperinsulinemic, indicating insulin resistance. Exactly how glucose uptake was mediated in these mice is still under active investigation; however, compensation by other members of the Glut family is unlikely, as upregulation of these other transporters was not found.

The most likely explanation for the different relative importances of the IRS proteins and the different Glut4 knockout phenotypes in these models is that

insulin resistance and diabetes in rodents (? and humans) are the end result of a combination of any number of defects in the insulin action pathway. This was shown clearly in a mouse model heterozygous for both IRS1 and insulin receptor deficiency, in which there was approximately 50% reduction in expression of IRS-1 and insulin receptor proteins (Bruning et al., 1997). Following insulin stimulation, receptor autophosphorylation was significantly reduced as was IRS-1 tyrosine phosphorylation. Interestingly, these animals developed a syndrome strongly resembling human Type 2 diabetes.

B. Animal Models of Diabetes and Insulin Resistance

Most animal models of Type 2 diabetes have significant obesity that is either genetic, or induced by either surgical lesions of the satiety centers of the hypothalamus or overfeeding. Skeletal muscle glucose uptake in response to insulin is universally blunted, and this occurs in parallel with a decreased magnitude of activation of components of the phosphorylation cascade.

The Zucker fatty rat and ob/ob mouse are both obese strains with monogenic defects in production of the hormone leptin. In these rodents there is significant reduction in the content of IRS-1 and IRS-2 in skeletal muscle, which may be responsible for impaired insulin signal transduction (Anai et al., 1998; Kerouz et al., 1997). However, it is likely that these defects are secondary to the metabolic milieu associated with obesity. Similarly, feeding a high-fat diet to rats produces obesity, insulin resistance, and skeletal muscle insulin signaling defects. Before obesity becomes marked in these models, while glucose homeostasis is relatively normal, activation of the insulin signaling cascade is unperturbed; increasing degree and duration of obesity is associated with decreased IRS1 content and impaired IRS-1 associated PI 3-K activity (Hansen et al., 1998; Zierath et al., 1997). These authors therefore concluded that abnormalities in the signaling cascade occurred late in the development of insulin resistance, and were likely secondary phenomena.

Pregnancy is another state in which IRS-1 dysfunction has been described in association with insulin resistance. Studies in rats toward the end of gestation have demonstrated reduction in IRS-1 content by over 50% in muscle, associated with diminished tyrosine phosphorylation in response to insulin and decreased association with p85 (Saad et al., 1997). This reduction in flow of signal through the pathway may explain the peripheral insulin resistance; however, whether the changes in IRS-1 content are as a result of the metabolic milieu or are primary features of the insulin resistance associated with the hormonal changes of pregnancy are still unclear, as longitudinal studies have not been performed.

Hyperglycemia itself causes insulin resistance, thus all forms of diabetes—when poorly controlled—are associated with lowered sensitivity to insulin. Rodent diabetes can be induced by injection of the beta cell toxin, streptozotocin (stz), resulting in hypoinsulinemic hyperglycemia. Insulin resistance occurs

consequent to the elevated blood sugars. Signaling abnormalities have been documented in stz-diabetic rats and include, surprisingly, enhanced and prolonged tyrosine phosphorylation of IRS-1 and increased PI 3-K activity (Folli et al., 1993; Giorgino et al., 1992). Despite this, muscle glucose uptake is resistant to insulin stimulation. However, the enhanced activation of PI 3-K is associated with increased expression of genes such as *c-fos* in response to insulin, indicating that although the metabolic actions of insulin are inhibited, signal flux through the mitogenic pathway may be up-regulated.

C. Human Obesity and Type 2 Diabetes

Many of the earliest studies looking at insulin action at the molecular level in humans involved skin fibroblasts and circulating mononuclear cells, tissues that are not typical insulin targets. Initial work was conflicting, with some authors claiming reduced insulin binding in diabetic subjects. When skeletal muscle has been examined, most agree that insulin binding its receptor is normal and that defects associated with insulin resistance are likely to be "postreceptor."

In muscle strips taken from patients with Type 2 diabetes, exposure to physiological levels of insulin resulted in reduced tyrosine phosphorylation of IRS-1 and associated PI 3-K activity than in normoglycemic controls (Bjornholm et al., 1997; Zierath et al., 1998). However, as with the rodent studies, this defect in IRS-1 activation was unlikely the primary problem, as it was reversed with normoglycemia. Similar results have been obtained in obese, nondiabetic subjects. In skeletal muscle strips removed during abdominal surgery, IRS-1-associated PI 3-K activation by insulin, and deoxyglucose uptake were about 50% reduced, compared with lean controls (Goodyear et al., 1995b). In contrast to studies in diabetics, obesity was associated with decreased abundance of the insulin receptor, IRS-1 and p85 proteins which might have contributed to the signaling abnormality.

In diabetes, although a reduction in total cellular Glut4 has been found in rodent models, this has never been demonstrated in human skeletal muscle (Garvey et al., 1992; Pedersen et al., 1990). Abnormal subcellular localization of Glut4 has been described in pregnant women, all of whom have insulin resistance related to changes in the hormonal milieu (Garvey et al., 1993). It is possible that Glut4 can be sequestered in vesicles away from the upstream components of the insulin signaling pathway in states of insulin resistance and unable to be appropriately translocated within the cell (Garvey et al., 1998). Although Glut4 mutations are not the cause of typical diabetes (Choi et al., 1991), functional disturbances in Glut4 activation occur consequent to abnormalities in the insulin signaling cascade. In diabetes, translocation of Glut4 is reduced after an insulin stimulus; again, reversal *in vitro* with normoglycemic medium occurred in 1 study (Zierath et al., 1994). In support of this, an acute bout of exercise activated translocation of Glut4 from LDM to the plasma membrane (Kennedy et al., 1999), even in poorly controlled

diabetes. It is clear that exercise causes muscle glucose uptake independent of insulin activation of the signaling cascade (Hayashi et al., 1997).

V. MECHANISMS OF INSULIN SIGNALING ABNORMALITIES

A. Tumor Necrosis Factor- α

Tumor necrosis factor- α (TNF- α), a cytokine originally described as a product of activated macrophages, has recently been shown to be overproduced in muscle (Saghizadeh et al., 1996) and fat (Hotamisligil et al., 1995) in obese persons. Infusion of TNF- α into normal animals caused resistance to insulin-mediated glucose disposal (Ling et al., 1994), although this was not accompanied by any insulin signaling abnormalities (McCown and Smith, unpublished data). It has been suspected that TNF- α may be one of the obesity-related factors that contributes to insulin signaling abnormalities (Hotamisligil and Spiegelman, 1994). Expression of a TNF- α inhibitor gene in insulin resistant rats improved insulin sensitivity and enhanced tyrosine phosphorylation of the insulin receptor (Figure 7) (Cheung et al., 1998). When adipocytes (Hotamisligil et al., 1996) or hepatoma cells (Paz et al., 1997) in culture are exposed to TNF- α , IRS-1 becomes phosphorylated on serine residues, which inhibits both autophosphorylation and the kinase activity of

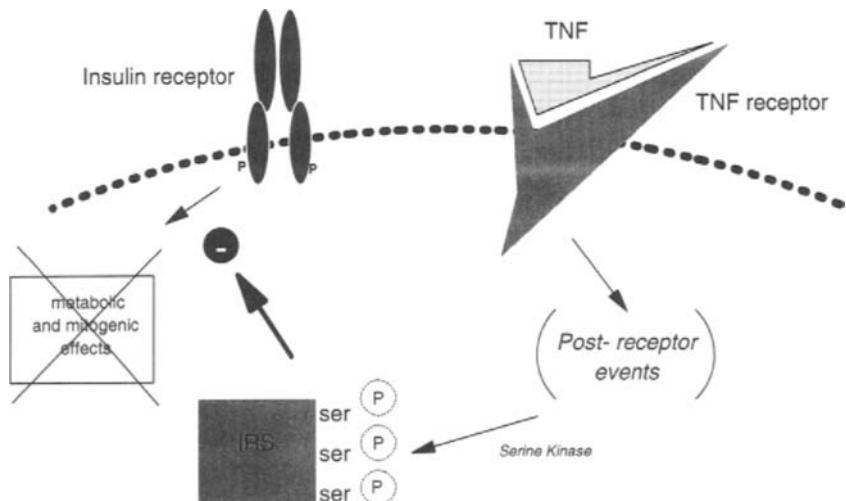


Figure 7. Mechanism of inhibition of insulin receptor kinase by TNF- α . P = phosphorylated residue.

the insulin receptor. It is likely that this mechanism plays an important part in the insulin resistance associated with critical illness and endotoxemia (Fan et al., 1996; McCowen et al., 1998) and burn injury (Ikezu et al., 1997), in which IRS-1 tyrosine phosphorylation by insulin and IRS-associated PI 3 kinase activity are inhibited.

B. Glucosamine

Glucosamine is a metabolite of glucose that is formed through the hexosamine pathway, an alternative route to glycolysis or glycogen synthesis (Figure 8). Under normal conditions, only a small amount of intracellular glucose is metabolized in this way. Glucose flux through this path is determined by the rate-limiting first enzyme, glutamine:fructose-6-phosphate amidotransferase (GFAT); amino groups are transferred from glutamine to fructose-6-phosphate, yielding glutamate and glucosamine-6-phosphate with the ultimate production of the final end-product of the hexosamine pathway, uridine diphosphate-N-acetyl glucosamine, which is used in the synthesis of glycoproteins and peptidoglycans. One putative mechanism, whereby hyperglycemia itself causes insulin resistance in skeletal muscle is by increasing flux through this "overflow" pathway for glucose metabolism with enhanced production of glucosamine (McClain and Crook, 1996). In experiments in cultured cells, overexpression of GFAT or exposure to glucosamine (bypassing GFAT) caused inhibition of insulin-mediated Glut4 translocation and

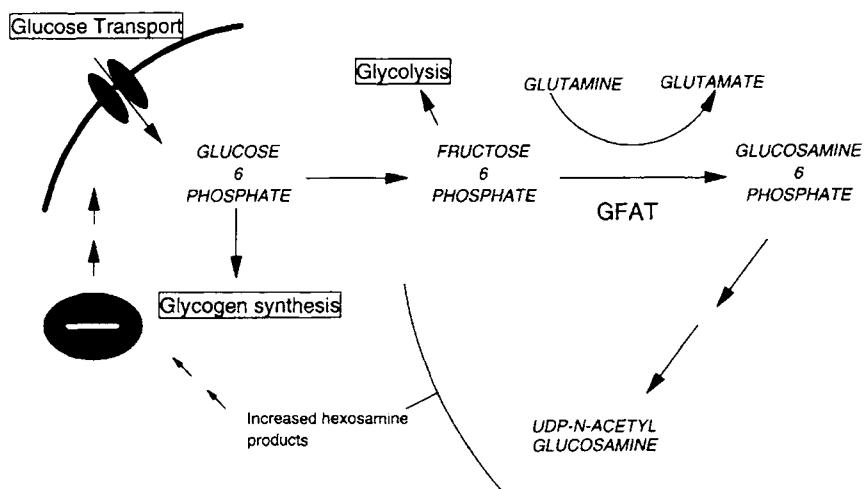


Figure 8. Flux of glucose through the hexosamine pathway. GFAT = glutamine:fructose-6-phosphate amidotransferase.

glucose uptake (Marshall et al., 1991). In whole-animal models, infusion of glucosamine resulted in whole-body insulin resistance (Rossetti et al., 1995) by inhibition of skeletal muscle Glut4 translocation (Baron et al., 1995). Other studies in rodents confirmed decreased skeletal muscle insulin-mediated glucose uptake (Virkamaki et al., 1997) and demonstrated inhibition of IRS-1-associated PI 3-K activation by insulin in parallel with diminished glucose uptake (Kim et al., 1999). In humans this mechanism may also be operative: in insulin-resistant patients with diabetes, GFAT activity was increased in skeletal muscle almost 50% (Yki-Jarvinen et al., 1996). Teleologically, such a desensitization mechanism to increased intracellular glucose might be appropriate, as it prevents further transport of glucose into the cell, although it is unclear which products of the hexosamine pathway can influence the insulin signaling cascade.

C. Phosphatases

Excessive activity of PTPases is an attractive putative mechanism for the decreased IRS-1 phosphorylation that is detected in so many models of insulin resistant diabetes (Byon et al., 1998). It has been demonstrated that both obese nondiabetic and diabetic Zucker rats have increased abundance and activity of the 3 main PTPases in skeletal muscle extracts (Ahmad and Goldstein, 1995). Similarly, increases in basal activity level of particulate phosphatases were demonstrated in skeletal muscle from Pima Indians (McGuire et al., 1991), one population in which Type 2 diabetes and obesity are very prevalent. In insulin-sensitive controls, insulin caused suppression of the activation of soluble phosphatases, but this was severely attenuated in the insulin-resistant subjects. However, these findings could not be replicated in non-Pima patients with diabetes in whom particulate PTPase activity was lower than in controls, along with reduced skeletal muscle content of PTP-1B (Ahmad et al., 1997). PTP-1B serves as a negative regulator of insulin action, as seen by overexpression of an inactive mutated form of the enzyme which enhanced insulin's ability both to autophosphorylate, and to phosphorylate IRS-1 (Kenner et al., 1996). The results in the non-Pima population (Ahmad et al., 1997), which at first seem counterintuitive, might be explained by the action of PTP-1B (located primarily in the endoplasmic reticulum) to deactivate the internalized receptor, permitting it to return to the plasma membrane for re-activation.

In contrast to PTP-1B, there is much less evidence for disturbances in LAR and SHPTP2 being important in insulin resistance. Inhibition of LAR in hepatoma cells caused amplification of insulin-stimulated PI 3-K activity over 300% above control (Kulas et al., 1995) with increased MAPK activation, but the impact on glucose regulation was not examined. SHPTP2 inhibition in fibroblasts using dominant negative transfection techniques resulted in reduced insulin-mediated PI 3-K activity and mitogenesis, indicating that this PTPase might be a positive regulator of the insulin signaling pathway, but effects on glucose uptake were not

examined (Ugi et al., 1996). It is unlikely, based on results from transgenic mouse studies and others, that SHPTP2 has a major role in the glucoregulatory actions of insulin (Kasuga, 1996). Mice heterozygous for SHPTP2 gene knockout with 50% reduction in SHPTP2 protein content (homozygosity was lethal in utero) had completely normal activation of the insulin signaling cascade and glucose homeostasis (Arrandale et al., 1996).

Further evidence supporting the role of PTPases in general in the glucoregulatory effects of insulin comes from work done with vanadium compounds (Fantus et al., 1995). Sodium vanadate is a PTPase inhibitor and multiple studies, both *in vivo* and *in vitro*, have demonstrated its effects both on augmenting tyrosine phosphorylation of the insulin signaling cascade and increasing glucose uptake (Wilden and Broadway, 1995). These compounds are being used in preliminary studies to treat human diabetes (Goldfine et al., 1995).

D. PC1

PC1 is a membrane glycoprotein that inhibits insulin receptor tyrosine kinase activity by an unknown mechanism. It can be detected in many tissues including skeletal muscle, and is located both on plasma membrane and in the endoplasmic reticulum. Its usual physiologic function is unclear and little is known about the PC1 gene or regulation of its expression. PC1 was initially purified from fibroblasts of a patient with severe insulin resistance and subsequently has been shown to be increased in some (Maddux et al., 1995; Frittitta et al., 1998), but not all (Whitehead et al., 1997), insulin-resistant diabetics. Skeletal muscle biopsies in different studies showed a direct correlation between PC1 abundance and degree of obesity (Youngren et al., 1996) as well as an inverse correlation between PC1 content and (1) insulin sensitivity and (2) muscle insulin-stimulated receptor tyrosine kinase activity measured *ex vivo* (Frittitta et al., 1996). In addition, over-expression of PC1 in cultured cells lead to decreased insulin-mediated glucose uptake, although under these conditions, activation of the insulin signaling pathway was unchanged and the results are therefore difficult to interpret (Kumakura et al., 1998).

VI. SUMMARY AND CONCLUSIONS

Thus, there are several candidate mechanisms for the perturbations in insulin signaling that are seen in insulin resistance. It is unlikely that a single lesion of the insulin action pathway will be shown to cause the majority of ordinary cases of diabetes, or the insulin resistance associated with obesity. Instead, as is exemplified by some of the gene-knockout models, these diseases are likely polygenic and multifactorial. In conclusion, normal activation by insulin of its receptor in skeletal muscle results in the tyrosine phosphorylation of cascades of signaling

proteins. This leads to changes in gene expression, increases in synthesis of glycogen, fat, and protein, and activation of a number of enzymes involved principally in glucose regulation. However, in skeletal muscle, probably the most important ultimate effect of insulin is to transport glucose. In Type 2 diabetes, changes occur in the insulin signaling pathway in association with a reduction in glucose uptake. The most consistent change in the various studies is impaired activation of PI 3-K by insulin. In general, although much of the evidence for this comes necessarily from animal and cell culture models of diabetes, signaling abnormalities are often secondary to the prevailing hyperglycemia. Only rarely, as occurs in patients with a defined mutation in the insulin receptor, can we be certain that insulin signaling is the primary defect. However, as understanding of the mechanism whereby IRS-associated PI 3-K activation mediates glucose uptake advances, the ability to develop tools to combat insulin resistance increases, with the eventual aim of treating Type 2 diabetes.

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CYTOPATHIC HYPOXIA IN SEPTIC SHOCK*

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I.	Introduction	66
II.	Evidence for Cellular Hypoxia in Sepsis	67
A.	Aerobic Metabolism: An Overview	67
B.	Lactic Acidosis	69
C.	“Pathological” Supply-Dependency of Oxygen Utilization	70
D.	Regional Measures—Skeletal Muscle	71
E.	Regional Measures—The Gastrointestinal Mucosa	71
III.	“Classic” vs. “Cytopathic Hypoxia”	72
A.	The Debate	72
B.	Tissue pO_2 : <i>In Vivo</i>	72
C.	MTT Assay: <i>In Vivo</i>	73
D.	Tissue O_2 Consumption: <i>In Vitro</i>	74
E.	Cellular O_2 Consumption: <i>In Vitro</i>	74
F.	Cellular Oxidation State: <i>In Vivo</i>	75
G.	“Classic Hypoxia” and “Cytopathic Hypoxia”: A Two-Step Model?	76
IV.	Mechanisms of Cytopathic Hypoxia in Sepsis	76
A.	Overview	76

*Adapted from *Sepsis* 1999, 2, 279–289.

B.	Electron Transport Chain Dysfunction: Role of Reactive Nitrogen Intermediates	77
C.	Failure of Substrate Delivery: PARP and PDH	78
D.	Oxygen Conformance	79
E.	Uncoupling of Oxidative Phosphorylation	80
F.	Mitochondrial Permeability Transition	80
V.	Conclusions	80
	References	80

I. INTRODUCTION

The term sepsis refers to the systemic response of an organism to infection. Septic shock refers to overwhelming sepsis resulting in organ hypoperfusion and decreased blood pressure. The incidence of human sepsis is gradually rising as a result of an aging population, and the increasing severity of acquired forms of immunodeficiency (e.g., the acquired immunodeficiency syndrome (AIDS) and organ transplantation). Results of human trials of targeted therapy for sepsis have been disappointing (Zeni et al., 1997). Although multiple organ failure is the major threat to survival in these patients, the "final common pathway" leading to widespread organ dysfunction and death has been elusive. Although initiation of programmed cell death (apoptosis) by one or more of the myriad mediators of the systemic inflammatory response may play a role (Hotchkiss et al., 1997), and widespread cellular necrosis can be a preterminal finding, signs of reversible or irreversible cellular injury is the rule (Hersch et al., 1990). Although direct cytotoxicity may occur independent of effects on cellular energetics, many believe that de-energization in sepsis may contribute directly or indirectly to dysfunction as well as to apoptosis and necrosis. As most adenosine triphosphate (ATP) is synthesized aerobically, coupled to the reduction of cellular O₂ to H₂O, the role of O₂ kinetics in sepsis has been a major focus. Although evidence for diffuse tissue hypoxia in sepsis is lacking, regional hypoxia has been hypothesized to play a substantial role in the cause of multiple organ dysfunction. As defined by a recent consensus conference, tissue hypoxia refers to "a condition in which the cells of a tissue have abnormal oxygen utilization such that the tissue is experiencing anaerobic metabolism" (Consensus Statement, 1996). Emphasizing the "business end" of the oxygen cascade in this definition allows an addition to the classic supply-side categories proposed by Barcroft (1920), that is, stagnant, anemic and hypoxic hypoxia (referring to low cardiac output states, low blood hemoglobin concentration, and low blood oxygen tension, respectively). The term "cytopathic hypoxia" has been coined to describe the situation, increasingly recognized in sepsis, of impaired oxygen consumption in the setting of normal or supranormal tissue oxygen tension (Fink, 1997).

Low cardiac output, anemia, and arterial hypoxia might occur singly or in combination in sepsis. With adequate resuscitation and respiratory support, however, systemic oxygen delivery (DO_2) is preserved in the vast majority of patients (Parillo, 1990). Human sepsis is characterized by a hyperdynamic circulation with elevated mixed-venous oxygen content in the setting of regional or systemic metabolic acidosis and hyperlactatemia that are felt to be markers of increased anaerobic respiration (Rackow and Astiz, 1991). If cells are indeed hypoxic in the setting of normal or supranormal O_2 supply with an apparent defect in O_2 extraction, one of three general mechanisms could be responsible. There may be a defect in the microcirculation altering the distribution of nutrient flow to the tissue, an abnormality of hemoglobin affinity resulting in impaired oxygen unloading, or metabolic failure of the cell resulting in decreased oxygen utilization, i.e., cytopathic hypoxia. Prior to reviewing the case for the latter in sepsis, we must first address the question of whether cellular hypoxia, as defined above, exists. There is considerable discordance among the results of studies investigating the role of hypoxia in sepsis. Some discrepant results can be explained by differences in experimental models, the duration of the septic process, the adequacy of resuscitation, and the methods of measurement used. Still, it seems likely that there are heterogeneous effects of sepsis on oxygen utilization in different organs or tissues.

II. EVIDENCE FOR CELLULAR HYPOXIA IN SEPSIS

A. Aerobic Metabolism: An Overview

Prior to considering the bioenergetics of the septic state, a brief review of oxidative metabolism may be useful (see Figures 1 and 2). The catabolism of glucose and fat via glycolysis, the Krebs cycle (tricarboxylic acid cycle) and the lipid β -oxidation provides the electrons that generate reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide (FADH_2), molecules with high electron transfer potential (reduction potential). These reducing equivalents are transferred down the electron transport chain (ETC) for the ultimate production of ATP, the cell's energy "currency." Greater than 90% of ATP is formed through this aerobic process of oxidative phosphorylation. The high energy electrons of NADH and FADH_2 are passed down a thermodynamic gradient through 3 enzyme complexes of the ETC that are intrinsic to the inner mitochondrial membrane where they ultimately reduce molecular O_2 to H_2O ($\text{O}_2 + 4\text{e}^- + 4\text{H}^+ \rightarrow 2 \text{H}_2\text{O}$). The enzyme complexes are NADH-Q reductase (complex I), cytochrome c reductase (complex III), and cytochrome c oxidase (complex IV). Ubiquinone (Q) transfers electrons from complex I to complex III and cytochrome c transfers electrons from complex III to complex IV. The electrons from FADH_2 enter the chain a bit downstream, as the enzyme complex that

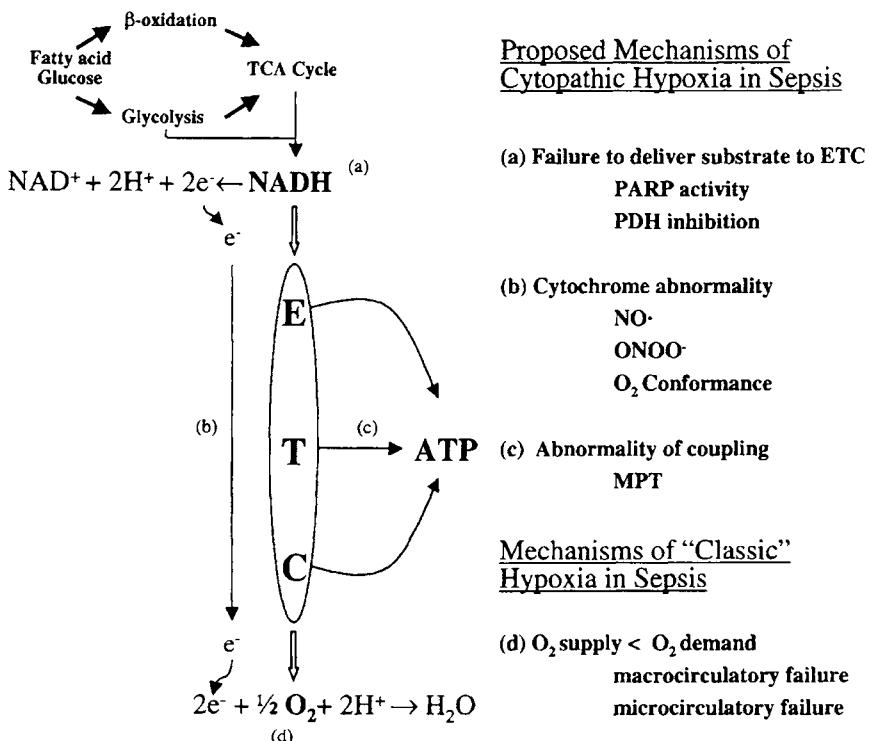


Figure 1. Schematic overview of oxidative metabolism and causes of cellular hypoxia. This figure emphasizes the movement of reducing equivalents down the electron transport chain (ETC). Proposed mechanisms for induction of hypoxia are highlighted: (a), (b), and (c) represent cytopathic hypoxia while (d) represents “classic” hypoxia. See text for details.

produces FADH₂, succinate-Q reductase (complex II), transfers electrons directly to ubiquinone. During electron transfer, the electron motive force is converted into proton motive force by the three enzyme complexes, which pump H⁺ from the mitochondrial matrix across the inner mitochondrial membrane. The membrane potential (E_m) and the chemical potential (ΔpH) created, provide the force for the synthesis of the high-energy anhydride bonds of ATP as H⁺ flow downstream into the matrix through the inner membrane ATP synthase complex (F₀F₁-ATPase). Normal, tight coupling of oxidation and phosphorylation necessitates the formation of ATP from ADP and P_i as electrons are transferred down the chain. The further coupling of ETC flux with cellular energy requirements through the sensing of the ADP level, “respiratory control,” provides a mechanism to efficiently match energy supply and demand.

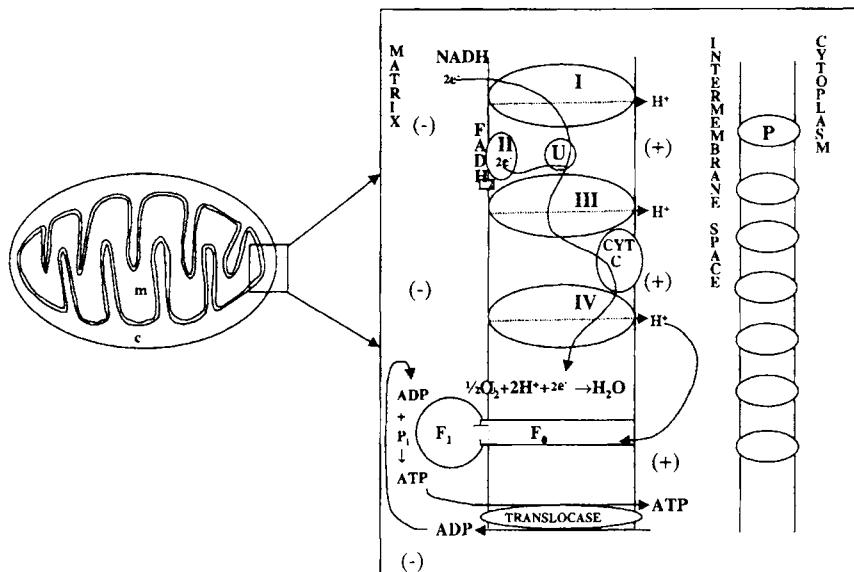


Figure 2. Oxidative phosphorylation. (Left) Schematic of a single mitochondria showing two compartments: (m) = the matrix, functionally separate from the intermembrane space (c) or cytoplasmic side by the inner mitochondrial membrane, which has extensive infoldings. The intermembrane space is in contiguity with the cell cytoplasm through large pores in the outer mitochondrial membrane called porins. (Right) Enlargement of the boxed area. The ETC is represented. Electron flux is shown beginning with NADH, which donates electrons to complex I and ending with the reduction of O_2 to water. FADH₂ is linked to complex II, which donates electrons to ubiquinone (U). Ubiquinone and cytochrome c (cyt c) are mobile electron (e^-) carriers. Protons (H^+) are shown being pumped by complexes I, III, and IV from the matrix to the cytosol and returning through the FoF1 ATPase (ATP synthase). ATP in the matrix is exchanged for cytoplasmic ADP by the translocase. (P) represents a porin in the outer mitochondrial membrane.

B. Lactic Acidosis

Much of the early data supporting the existence of cellular hypoxia in severe sepsis were circumstantial, inferred on the basis of acidemia and hyperlactatemia. The formation of acetyl coenzyme A, a high-energy compound formed through the oxidation-decarboxylation of pyruvate in glycolysis and through the β -oxidation of fatty acids represents a committed step in aerobic respiration. An imbalance between oxygen supply and demand should increase anaerobic metabolism, producing lactate anion (pK_a 3.85) from pyruvate and recycling NAD^+ , a process that neither consumes nor produces H^+ at physiologic pH.

logic pH. In addition, the continuing hydrolysis of ATP ($2 \text{ ATP} + 2\text{H}_2\text{O} \rightarrow 2 \text{ ADP} + 2 \text{ P}_i + 2\text{H}^+$) to satisfy energy consuming processes such as biosynthesis, transport, and mechanical work, generates excess protons. Thus, ongoing substrate level phosphorylation of ADP (in glycolysis) and hydrolysis of ATP (to produce usable energy) promote both excess lactate production and acidosis, albeit by only indirectly linked mechanisms (Hochachka and Mommsen, 1983). Serum lactate and pH measurements in the systemic blood stream are insensitive indicators of regional tissue hypoxia because of multiple confounding variables including dilution, tissue lactate uptake, and utilization and renal compensation (Gutierrez, 1996). There is also some evidence from animal studies that hyperlactatemia may result from a generalized increase in the rate of glycolysis (Gore et al., 1996; James et al., 1996) or changes in the functional activity of pyruvate dehydrogenase (PDH) (Vary et al., 1986) rather than to forced anaerobiosis. Nevertheless, serum lactic acidosis is a strong predictor of mortality in patients with septic shock (Bakker et al., 1996; Bernardin, 1996). In addition, organ specific measures of lactate production and tissue acidosis, that is, gut mucosal interstitial pH (pH_i) or tissue pCO_2 , have been shown to be strong predictors of poor prognosis (Doglio et al., 1991; Friedman and Berlot, 1995; Marik, 1993).

C. "Pathological" Supply-Dependency of Oxygen Utilization

In the recent past, another potential indicator of cellular hypoxia in sepsis was thought to be so-called "pathologic supply-dependent oxygen consumption (VO_2)" (Cain, 1984; Fenwick et al., 1990; Gutierrez and Pohil, 1986; Kaufman et al., 1984; Rhodes et al., 1978). Some studies suggested that changes in systemic VO_2 occurred at higher rates of systemic DO_2 in septic as compared to healthy humans. This finding was felt to indicate occult tissue hypoxia. Much has been written about the potential pitfalls of these observations (Russell and Phong, 1994), and although controversy still exists, it now is generally agreed that pathologic O_2 supply dependence is not characteristic of sepsis in humans (Chittock and Russell, 1997; Ronco et al., 1993). Nevertheless, the ability to increase systemic VO_2 spontaneously or in response to augmentation of DO_2 with fluids or inotropic agents is a strong predictor of survival (Hayes, et al., 1997) and may hint at the presence of a reversible stage of tissue hypoxia that would be amenable to therapy (see below).

D. Regional Measures—Skeletal Muscle

The *sine qua non* of cellular hypoxia of any type is the production of intracellular acid with or without a fall in energy equivalents, i.e., ATP. Hotchkiss and others have provided convincing evidence that in sepsis with adequate fluid

resuscitation, there is not diffuse bioenergetic failure (Hotchkiss and Karl, 1992). Using [^{31}P] nuclear magnetic resonance (NMR) spectroscopy it is possible to monitor changes in average tissue high-energy phosphates, i.e., ATP and phosphocreatine as well as overall energy charge (ATP + 0.5ADP/AMP + ADP + ATP). In addition, because orthophosphate (P_i) has an ionizable hydrogen with a pK_a in the physiologic range, the relative position of P_i on the NMR spectrum is reflective of intracellular pH. In various rat models of sepsis, skeletal muscle and brain were found to have preserved energy charge and intracellular pH or patterns of change inconsistent with cellular hypoxia. Muscle biopsies on human septic patients have yielded conflicting results, without clear evidence of bioenergetic failure (Bergstrom et al., 1976; Liaw et al., 1980; Tresadern et al., 1988). In another study, Jacobs and colleagues (1991) used [^{31}P] NMR to monitor ATP content and related variables in gastrocnemius muscle tissue of rats subjected to "mild" sepsis induced by cecal ligation and puncture (CLP). Although overall ATP, ATP/ P_i , and intracellular pH were unchanged, there was a 20% decline in phosphocreatine. The authors postulated that ongoing sepsis would lead to depletion of phosphocreatine stores and eventual tissue acidosis. In contrast to the data just cited, Astiz and colleagues (1988) studied muscle high-energy phosphate content and tissue lactate in a more severe rat CLP model. In fully resuscitated animals, there was a small but significant drop in total ATP content and energy charge along with an increase in the lactate/pyruvate ratio consistent with cellular hypoxia. Thus, evidence for skeletal muscle hypoxia in sepsis is inconclusive, possibly reflecting the buffer afforded by the storage form of high-energy phosphate, phosphocreatine in this tissue. Additionally, muscle is relatively inactive in critically ill septic patients or animals, and its bioenergetic status may not reflect that of more metabolically active tissues.

E. Regional Measures—The Gastrointestinal Mucosa

The mucosa of the gastrointestinal tract seems to be particularly susceptible to metabolic derangement in sepsis. Anaerobiosis, signaled by a marked increase in gut mucosal hydrogen ion concentration, is probably best assessed by comparing local tissue pCO_2 measured with a balloon tonometer or a CO_2 electrode to arterial pCO_2 (CO_2 gap) (Fink, 1998). An increased CO_2 gap or decreased mucosal pH are frequently seen in sepsis despite aggressive fluid resuscitation and preserved macrocirculatory flow (Fink et al., 1991; VanderMeer et al., 1995). Gut hypoxia not only impairs local function, but breakdown of immune and barrier properties of the mucosa may lead to production or amplification of the multiple organ dysfunction syndrome (Carrico et al., 1985).

III. "CLASSIC" VS. "CYTOPATHIC HYPOXIA"

A. The Debate

If we accept the fact that an O₂ extraction defect exists in certain tissues in sepsis despite a hyperdynamic circulation, we must still attempt to explain it. Impaired O₂ consumption might result from a decreased gradient for diffusion of O₂ at the mitochondrial level because of a low intracellular pO₂, or from the inability to maintain aerobic respiration despite preserved pO₂, i.e., "starvation in the midst of abundance." The remark by Astiz et al. in 1988 that "the relative importance of systemic hypoperfusion and impaired oxidative metabolism in the development of metabolic failure in sepsis remains controversial" is still true 10 years later. Sepsis is believed to represent distributive shock, characterized by a maldistribution of blood flow and volume that results in regional ischemia despite preserved systemic cardiac output. Experimental studies have revealed alterations in all aspects of the microcirculation, including capillary dropout (Lam et al., 1994), anatomic obstruction from fibrin, platelets, or leukocytes (Astiz et al., 1995), extrinsic compression from tissue edema; impaired vasoactivity (Bernsten et al., 1992) and altered red blood cell rheology (Chung et al., 1991). Although there is some evidence that a decreased hemoglobin P₅₀ for oxygen may also be found in sepsis (Lehot et al., 1984), the majority of patients are felt to have relatively normal oxyhemoglobin dissociation curves (Kalter et al., 1982). Although numerous studies support the hypothesis that regional hypoperfusion and consequent "classic" hypoxia occur in sepsis, a body of evidence is accumulating to suggest that organ dysfunction and anaerobiosis may occur even in the absence of hypoxic hypoxia, anemic hypoxia or stagnant hypoxia. Methods to differentiate cytopathic hypoxia from hypoperfusion are manifold. Strategies include direct measurement of tissue oxygen tension (TpO₂); *in vitro* measurement of the oxygen consumption of isolated tissues, cells, or mitochondria, or functional assays of mitochondria by probing their redox state *in vivo* or *in vitro*.

B. Tissue pO₂: *In Vivo*

There is a large gradient of pO₂ beginning in the pulmonary capillary (PaO₂ 100 mmHg) and ending at the mitochondria (est. pO₂ 1 to 5 mmHg). The Pasteur point, representing the critical mitochondrial pO₂ needed to sustain oxidative phosphorylation and reflecting the K_m of the terminal cytochrome enzyme complex of the electron transport chain (cytochrome aa3), may be as low as 1 mmHg (Wilson et al., 1988). TpO₂ is spatially heterogeneous and reflective of the local match of oxygen supply and demand. Wide variations of pO₂ in cells adjacent to the source, i.e., arterioles, or the sink, i.e., mitochondria, make it impossible to talk about a single TpO₂. Instead, a polarographic microelectrode or array of microelectrodes may be inserted directly into, or placed onto, the mucosal or serosal sur-

face on a needle tip or catheter with repeated measurements made in spatially contiguous areas so as to construct a T_pO_2 histogram. If oxygen utilization by tissues is supply-dependent, then the average T_pO_2 will be low. However, if the ability of cells to consume oxygen is impaired, T_pO_2 should be high.

In the study by Astiz et al. (1988) mentioned earlier in support of tissue hypoxia, mean T_pO_2 of the rectus femoris was 23.5 ± 0.6 mmHg 6 hours after rats were rendered septic by CLP, but was raised to 43.5 ± 0.4 mmHg with albumin resuscitation, i.e., not significantly different from sham-operated controls. Boekstegers and colleagues (1994) examined skeletal muscle T_pO_2 by polarographic needle electrodes or implanted catheter probes in three patient groups: intensive care patients with sepsis, limited infection, or cardiogenic shock. Although no effort was made to assess the presence of increased anaerobic respiration, a significantly elevated T_pO_2 was found in the septic patients compared to the other two groups, and the T_pO_2 was directly correlated with sepsis severity. In another study, rats were given intravenous endotoxin and hypodynamic and hyperdynamic states were produced depending on whether the lipopolysaccharide (LPS) was injected as a bolus or a short infusion (Russer et al., 1995). In both settings, surface T_pO_2 of the collapsed urinary bladder was significantly elevated from baseline despite systemic acidosis. Our laboratory has achieved similar results looking at intestinal mucosal pO_2 in a porcine model of endotoxemia. In anesthetized pigs infused with LPS and resuscitated to baseline cardiac output, increased T_pO_2 of the exteriorized ileal mucosa was found despite unchanged mucosal blood flow (Vander Meer et al., 1995). Significant gut mucosal acidosis in the setting of this extraction defect provided convincing evidence for a primary abnormality of oxygen utilization. Although a similar study in endotoxicemic dogs revealed a T_pO_2 histogram in the gut mucosa that was left-shifted, histology in these animals showed marked changes, including frank necrosis and sloughing of the intestinal mucosa that could have accounted for these discrepant results (Vallet et al., 1994). Further evidence against decreased tissue pO_2 in sepsis comes from work by Hotchkiss et al. (Hotchkiss et al., 1992) who used the marker [^{18}F] fluoromisonidazole, which diffuses through cell membranes and accumulates in inverse proportion to cell pO_2 . Studies of the gastrocnemius, muscle, lung, brain, and diaphragm in rats 36 hours after CLP revealed no evidence for reduced intracellular pO_2 .

C. MTT Assay: *In Vivo*

Mitochondrial respiration may be tested indirectly by checking a cell's ability to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The conversion of MTT to a blue formazan derivative (MTT-FZ) requires functional mitochondria. Our laboratory developed an *in vivo* assay to evaluate mitochondrial function in the intestinal epithelium (Unno et al., 1997).

We loaded the lumen of the intestine of endotoxemic and control rats with a solution of MTT and then measured the concentration of MTT-FZ in enterocytes (normalized to DNA content) after a 30-min. incubation. Twenty-four hours after injection of LPS or saline, mitochondrial respiration was significantly impaired in enterocytes from endotoxemic rats compared to enterocytes from the controls.

D. Tissue O₂ Consumption: *In Vitro*

In a further effort to implicate mitochondrial dysfunction as the cause of the extraction defect in sepsis, we studied oxygen consumption directly in isolated tissue, thereby avoiding the confounding variable of perfusion. Our current model has produced highly reproducible results. A strip of tissue is rapidly harvested from the experimental animal and mounted across a ring-shaped plastic support in a temperature-controlled polarographic chamber filled with bicarbonate buffer saturated with air and fitted with a Clarke-type oxygen-sensitive electrode. The consumption of oxygen is measured by monitoring the percent decrease in pO₂ in the solution in the chamber over serial 10-min. periods, with the results normalized to dry tissue weight. We have recently completed a study comparing the oxygen consumption of isolated pieces of liver, lung, and stripped mucosa from the distal ileum of controls ($N = 8$) and rats injected 8 hours earlier with 5 mg/kg of LPS. In these studies, we found direct evidence that O₂ consumption is significantly decreased in gut mucosa and liver (but not in lung) of endotoxemic rats (unpublished observations). Mucosal histology revealed no evidence of necrosis or apoptosis. Moreover, in another experiment in which zero intestinal mucosal blood flow was achieved for 30 min. in control rats by aortic transection, ileal mucosal VO₂ was still significantly greater than that observed in tissue samples from endotoxemic animals. Because the mucosa of the ileum is stripped with the underlying lamina propria intact, total oxygen consumption of the tissue reflects predominantly that of enterocytes, with much smaller contributions from inflammatory cells, fibroblasts, or other cell types.

E. Cellular O₂ Consumption: *In Vitro*

Oxygen utilization by specific cell types can be evaluated by measuring the oxygen consumption of cells in suspension. However, the added complexity of cell harvest and isolation can be a significant obstacle. Several groups have evaluated oxygen consumption of hepatocytes in suspension. Kantrow and colleagues (1997) looked at isolated hepatocytes, mitochondria, and respiratory complexes (ETC components) obtained from rats 16 hours after CLP followed by resuscitation with hetastarch and saline. O₂ consumption was measured with a polarographic electrode in an enclosed chamber. Endogenous respiration by the hepatocytes was significantly decreased in the CLP group compared to con-

trols, whereas the opposite was true for isolated mitochondria. The authors speculated that the latter result may have been artifactual secondary to selective isolation of hyperfunctional mitochondria, implying heterogeneous injury to the organelles among different cells or within the same cell. Dawson and colleagues (1988) also reported that mitochondria isolated from rat liver following lethal doses of *Escherichia coli* endotoxin were hyperfunctional. Using a different method, Rosser and colleagues (1988) studied oxygen consumption continuously in hepatocytes in cell culture following exposure to endotoxin by measuring the O₂-induced quenching of phalloidin phosphorescence. Although early basal oxygen consumption was elevated compared with unstimulated cells, maximal consumption, achieved by inducing uncoupling of oxidation and ATP synthesis with the proton ionophore FCCP, was significantly reduced at 24 hours compared to control cells.

F. Cellular Oxidation State: *In Vivo*

An extremely powerful technique exploits the ability of light in the near-infrared (700–1000 nm) range to penetrate tissue. Accordingly, the *in vivo* oxidation state of various molecules may be determined by the use of transmission spectroscopy in this bandwidth, avoiding potential artifacts related to tissue or cell isolation. The redox state of the copper moiety of cytochrome aa3, the terminal oxidase (complex IV) that accounts for the great majority of normal oxygen consumption, is determined by O₂ availability. Simonson and colleagues (1994) used near-infrared spectroscopy (NIR) to probe the skeletal muscle of baboons for evidence of hypoxia in septic shock. They produced hyperdynamic shock by the intravenous injection of viable *E. coli* in sedated, paralyzed, and mechanically ventilated baboons given fluid resuscitation and antibiotics. Throughout the experiments, systemic VO₂ remained mildly elevated. In control animals, tourniquet placement decreased the availability distally of O₂ as the terminal electron acceptor, and resulted in an increase of the reduced forms of electron carriers, i.e., Cu⁺ > Cu²⁺ in cytochrome aa3. Tourniquet release resulted in rapid reoxidation of the electron carriers. The authors showed that early sepsis (6 hours) was characterized by preservation of rapid cytochrome reduction with iatrogenic limb ischemia implying a steady stream of electrons down the ETC, but slow reoxidation after tourniquet release, implying reduced oxygen delivery. In late sepsis (18 hours) there was a marked decrease in the rate of cytochrome aa3 reduction following tourniquet inflation. This finding suggests an abnormality of mitochondrial function. Possible causes of dysfunction might be an intrinsic defect in cytochrome aa3 preventing its reduction, injury to the ETC proximal to cytochrome aa3, or insufficient delivery of substrate to provide reducing equivalents.

G. "Classic Hypoxia" and "Cytopathic Hypoxia": A Two-Step Model?

Dysfunction of the macro- or microcirculation on one hand, and of cellular oxygen metabolism on the other are not mutually exclusive and may be synergistic. In fact, the dual effects on mitochondrial function implied by the findings of Simonson and colleagues (1994) highlight what is becoming a continuing motif in the study of sepsis pathophysiology. Piecing together data from various methods of investigation, one can paint a picture of a primary, reversible defect in oxygen consumption that may respond to increased DO_2 , and a secondary, irreversible phase that terminates in death. In the single, positive, controlled trial of supranormal oxygen delivery in mixed medical-surgical patients, Gutierrez and colleagues (1992) stratified 260 intensive care patients (moderately ill with systemic signs of inflammation, in many cases from infection) into two groups based on the presence or absence of gut mucosal acidosis on admission ($\text{pH}_i < 7.35$) as measured by gastric tonometry. The group who presented with signs of aerobiosis did not respond to aggressive resuscitation and inotropic support to improve systemic DO_2 (survival, 37% with treatment vs. 36% for controls). In contrast, there was significantly increased survival in the group whose treatment was directed to raising the pH_i when these fell below 7.35 (survival, 58% with treatment vs. 42% for controls). More recently, Hayes et al. (Hayes et al. 1997) analyzed prospective data from another trial of supranormal oxygen delivery in patients with sepsis syndrome and septic shock. Although survivors in this study were able to achieve target increases in oxygen delivery and consumption spontaneously or with support, nonsurvivors showed no increase in VO_2 with increased DO_2 . Although no absolute measure of cellular hypoxia was made in this trial, the dramatic results are consistent with previously discussed data supporting a metabolic basis for the extraction defect seen in sepsis.

IV. MECHANISMS OF CYTOPATHIC HYPOXIA IN SEPSIS

A. Overview

A primary defect in cellular oxygen utilization in sepsis may occur at one or more of several sites. There can be dysfunction of the electron transport chain itself, a decrease in reducing equivalents delivered to the electron transport chain, or a failure of normal coupling between oxidation and the synthesis of ATP (Figure 1). While absolute failure of the first two in the setting of continued ATP hydrolysis should result in anaerobiosis in conjunction with high TpO_2 and decreased energy charge, the latter would be associated with a low TpO_2 and decreased energy charge.

B. Electron Transport Chain Dysfunction: Role of Reactive Nitrogen Intermediates

Nitric oxide (NO^{\cdot}), a free radical gas synthesized from the amino acid L-arginine by a family of enzymes called nitric oxide synthases (NOS), has been implicated in many aspects of the pathophysiology of sepsis. Nitric oxide is a pluripotent signaling and effector molecule produced by a wide variety of mammalian cells (Fink and Payen, 1996). Neuronal or type I NOS (nNOS) and endothelial or type III NOS (eNOS) are constitutively expressed, whereas inducible NOS (iNOS) is expressed only after induction by various cytokines or LPS, generating larger quantities of NO^{\cdot} in response to an inflammatory stimulus. NO^{\cdot} , formed by iNOS in macrophages, vascular smooth muscle cells, and various other cells, including hepatocytes and enterocytes, may contribute to the induction of cytopathic hypoxia in several ways. These effects may be mediated directly by NO^{\cdot} or by the more reactive product of the diffusion-limited reaction with superoxide anion ($\text{O}_2^{-\cdot}$), peroxynitrite (ONOO^{\cdot}). NO^{\cdot} and ONOO^{\cdot} may directly inhibit oxidative phosphorylation through the covalent modification of metal centers of various enzymes, including *cis*-aconitase (the rate-limiting enzyme of the tricarboxylic acid cycle) and components of the electron transport chain (Fisch et al., 1996; Stadler et al., 1991).

The physiologic significance of many of these reactions, characterized *in vitro*, remains to be determined. Pathologic reactions *in vivo* will depend on the location and amount of reactive nitrogen intermediate (RNI) production and the proximity and amount of various oxidant scavengers. For instance, one study observed decreased oxygen consumption associated with the formation of iron-nitrosyl complexes within mitochondria of rat hepatocytes exposed to LPS and mitochondria exposed to an NO^{\cdot} donor. In the *in vivo* model, however, NO^{\cdot} seemed to preferentially react with hemoglobin and had no effect on mitochondrial respiration. In an important study on rat brain submitochondrial particles, Moncada's group was able to clarify the role of NO^{\cdot} and ONOO^{\cdot} on ETC components (Lizasoin et al., 1996). They found that physiologic quantities of NO^{\cdot} reversibly blocked complex IV, while ONOO^{\cdot} irreversibly blocked the first 3 ETC complexes. ONOO^{\cdot} is highly reactive and was easily "detoxified" with low levels of cellular components such as glucose or glutathione. Since it is known that increased $\text{O}_2^{-\cdot}$ is formed at complexes II and III when the terminal cytochrome is inhibited by cyanide, and likely NO , the authors suggested that reversible ETC inhibition by NO^{\cdot} might lead to a local, "protected" production of ONOO^{\cdot} and irreversible mitochondrial failure. NO^{\cdot} has been found to inhibit complex IV by competing with O_2 for the reduced heme of ferrocytochrome a3 (Torres et al., 1995). This mechanism seems be consistent with the NIR findings of Simonsen et al. (Simonsen et al. 1994) described previously. The early defect in reoxidation of cytochrome aa3 with tourniquet release in the septic baboons could be explained by NO^{\cdot} inhibition, raising the functional K_m of the enzyme for oxygen. Any degree of "classic" hypoxia

would augment this reaction as well as promote the production of ONOO⁻, accounting for the defect in cytochrome aa3 reduction seen later in sepsis.

Numerous published studies have reported on the consequences of various NO-inhibitors in sepsis or endotoxemia. The effects of NO· at the mitochondrial level are complex. In the study on isolated hepatocytes after CLP-induced sepsis described above, the cells were incubated with the nonspecific NO inhibitor, L-NAME (N^G -nitro-L-arginine-methyl ester) for 5 min. following isolation. This had no effect on oxygen consumption. In our laboratory, the mitochondrial dysfunction in enterocytes documented by the MTT assay was significantly ameliorated when the endotoxemic rats were treated with the specific iNOS inhibitor, aminoguanidine. We are currently studying the effect of aminoguanidine on the directly measured VO₂ defect in intestinal mucosa and liver described in a previous section. A group from Japan recently reported results of a study of iNOS inhibition on endotoxin-induced cholestasis in rats, which is one manifestation of multiple organ dysfunction syndrome (MODS) (Shiomi et al., 1998). At 2 hours after intraperitoneal injection of LPS, there was evidence for decreased bile formation by the liver in the setting of increased hepatic NO· production, decreased oxygen consumption, and a fall in total ATP. *Ex vivo* perfusion of the liver with aminoguanidine reversed the change in bile formation in conjunction with recovery of baseline O₂ consumption and hepatic ATP levels.

In addition to the possible induction of cytopathic hypoxia, NO· and related RNI's are potent vasodilators and have been implicated in the altered vasoregulation of sepsis. Furthermore, discoveries regarding the relationship between NO· and hemoglobin might shed light on the nature of the microcirculatory derangement. Hemoglobin may not only serve as a passive sink for NO· (NO-Fe²⁺ bond), but the regulated binding of NO· or SNO· (S-nitrosothiol) to a cysteine residue on the B-globin chain recently has been reported (Jia et al., 1996; Stamler et al., 1997). Allosteric control by local oxygen tension may regulate physiologic or pathologic S(NO·) release in the microvasculature. Therefore, significant quantities of SNOHb formed endogenously could conceivably alter O₂ delivery in sepsis.

C. Failure of Substrate Delivery: PARP and PDH

Paralysis of oxygen utilization may also occur due to a failure of electron delivery to the mitochondria despite a functional electron transport chain. Two potential explanations include depletion of NAD⁺, the primary proximal electron donor to the ETC, or the inability to convert pyruvate to acetyl Coenzyme A through deactivation of pyruvate dehydrogenase (PDH).

Poly(ADP)-ribose polymerase (PARP), an enzyme induced only by DNA strand breaks, catalyzes the poly(ADP)-ribosylation of proteins, a process that depletes NAD⁺, and may contribute to cellular de-energization secondary to reduced delivery of reducing equivalents (NADH) to the ETC complex I. In addition to its direct effects on the ETC, ONOO⁻ is an important inducer of

DNA strand breaks, providing an alternative mechanism for RNI-induced mitochondrial failure through the activation of PARP. Support for the importance of PARP comes from studies of inhibition (e.g., by 3-amino benzamide and nicotinamide) and PARP knockout mice, which show relative protection against cellular energetic failure in the face of inflammatory mediators and NO⁻ donors (Heller et al., 1995; Zingarelli et al., 1996). The role of PARP inhibitors and ONOO⁻ scavengers in the treatment of septic shock is under intense investigation.

The irreversible, highly regulated conversion of pyruvate to acetyl Coenzyme A by PDH is crucial to metabolism, committing glucose metabolites to aerobic respiration or fatty acid synthesis. Loss of PDH activity would limit the entry of two-carbon skeletons into the tricarboxylic acid (TCA) cycle and favor the conversion of pyruvate to lactate through mass action. PDH function is regulated by cellular energy stores and mediated by phosphorylation, with PDH kinase inactivating, and PDH phosphatase activating, the enzyme. It has been demonstrated that reduced activated PDH in rat skeletal muscle is partially responsible for sepsis-induced hyperlactatemia (Vary et al., 1986, 1995). The role of PDH modulation in the production of hyperlactatemia and cytopathic hypoxia in humans is controversial, as some studies show that glycolytic flux to oxidation is actually increased in critical illness (Gore et al., 1996; Hotchkiss and Karl, 1992; James et al., 1996).

D. Oxygen Conformance

Oxygen conformance is another mechanism whereby low cellular pO₂ may contribute directly to a subsequent O₂ utilization defect. Oxygen conformance is believed to be an adaptive mechanism whereby cells (or mitochondria) exposed to prolonged hypoxia exhibit reversible, decreased O₂ consumption at a cellular pO₂ which is higher than the range normally associated with O₂ dependent, O₂ consumption (5-10 mmHg for cells, 2-4 mmHg for mitochondria) (Chandel et al., 1995; Schumacker, 1993). These investigators have shown that isolated rat hepatocytes and isolated, coupled mitochondria developed significant suppression of O₂ consumption (up to 40% decrease) following prolonged incubation in hypoxic buffer, a state which promptly reversed on exposure to high pO₂. Taylor et al., (Taylor et al. 1998) postulated that increased oxygen dependence of septic mitochondria might account for the reported oxygen utilization defect. In the rat CLP model, isolated mitochondria from the septic animals showed no evidence for significant O₂ conformance. These mitochondria were hyperfunctional compared to controls, and may not represent the "injured" organelles one might expect to exhibit abnormal O₂ kinetics.

E. Uncoupling of Oxidative Phosphorylation

Loss of tight coupling between mitochondrial electron transport and ATP synthesis would result in cytopathic hypoxia with a low TpO_2 , as mitochondria not "hindered" by respiratory control would increase their ETC flux and oxygen utilization. Studies in isolated "septic" mitochondria have revealed both significantly decreased (Harris and Green, 1968; Mela and Miller, 1971) and increased coupling (Kantrow et al., 1997) as expressed by the respiratory control ratio, O_2 consumption in state 3 (stimulated with excess substrate and ADP)/state 4 (excess substrate, all ADP converted to ATP). Complete uncoupling would have a respiratory control ratio of unity.

F. Mitochondrial Permeability Transition

It has been shown that coupling is highly dependent on an intact inner mitochondrial membrane that can maintain the membrane potential (E_m) and the chemical potential (ΔpH). One specific cause of uncoupled oxidative phosphorylation is activation of the mitochondrial permeability transition (MPT), a phenomenon observed in hepatocytes *in vitro* that has yet to be directly implicated in sepsis. MPT describes the reversible opening of a proteinaceous pore in the mitochondrial membrane(s) resulting in depolarization, uncoupling, mitochondrial swelling, and subsequent cellular necrosis. MPT may be induced by numerous factors including mitochondrial Ca^{2+} accumulation and oxidant stress and has recently been shown to be influenced by local $NO\cdot$. Although postulated to cause bioenergetic failure in ischemia-reperfusion injury, in sepsis MPT may be a link between primary ATP depletion and cell necrosis or apoptosis in the terminal phase. Simbula and colleagues (1997) have shown that loss of ATP stores allowing calcium dysregulation is critical to the induction of the MPT, providing a possible mechanism for tissue death related to cytopathic hypoxia.

V. CONCLUSIONS

As the evidence for cytopathic hypoxia in sepsis is mounting, it compels us to intensify our efforts to describe its physiologic relevance and operative mechanisms *in vivo*. Through better understanding, novel therapy specifically targeted to these metabolic defects should emerge.

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BONE HEALING AND THE DIFFERENTIATION OF OSTEOPROGENITOR CELLS IN MAMMALS

Doreen E. Ashhurst

I.	Introduction	84
II.	Animal Models for Investigating Fracture Repair	85
III.	Fracture Healing	86
	A. The Stages of Fracture Healing	86
	B. The Effects of Mechanical Conditions	86
IV.	Healing of Defects	94
V.	Distraction Osteogenesis	96
VI.	Macromolecular Synthesis during Callus Development	99
VII.	Cell Differentiation during Healing	107
	A. Origin and Differentiation of Cells in Fracture Callus	108
	B. Further Differentiation of Osteoblasts and Chondrocytes	109
VIII.	Role of Cartilage in Fracture Repair	110
	A. The Cellular Environment and Cartilage Formation	111

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B. Endochondral Fracture Repair	112
IX. Concluding Comments	112
Acknowledgments	114
References	114

I. INTRODUCTION

The integrity and well-being of the skeleton is of utmost importance to all vertebrates. Any inherent deformity or injury causing a fracture can lead to impaired mobility, and thus a decrease in the quality of life. The skeleton is also affected by metabolic diseases, such as Paget's disease and osteoporosis, which, with aging populations throughout the world, are of great potential economic importance.

Successful bone healing is, therefore, of great importance. Bone has a great capacity for healing. It is constantly remodeled throughout life as part of the mechanism of calcium homeostasis. It is estimated that the adult human skeleton is completely replaced every 7 years throughout life. In general, fractures heal well provided the fragments are accurately reduced and adequately immobilized; this may require the immobilization of the associated joints.

Fracture treatment can be traced back to the third millennium *B.C.* when simple external splintage with wooden boards was used (Elliot-Smith, 1908). This treatment persisted until the introduction of plaster of Paris in the middle of the 19th century enabled casts to be molded around the affected limb (Mathijsen, 1852). Although the bone usually healed, other disorders, such as joint stiffness, muscle wasting and fibrosis, and shortening of the healed bone, which together constitute "fracture disease," frequently followed as a result of the long periods of immobilization required. Despite treatment by physiotherapy, reduced mobility, and therefore permanent disability, were common. Experiments were started on the internal fixation of fractures by means of plates and screws, or medullary nails, in the late 19th century (Hansmann, 1886). Since that time many systems of plates and screws have been developed. They permit more accurate reduction and alignment of the fracture fragments, but with many systems the stability of the fracture is not sufficient without external stabilization with a plaster cast.

To overcome this problem, Danis (1949) experimented with the use of a compressive force across the fragments to increase the stability of the fracture. Despite encouraging results, his ideas were not pursued until the late 1950s when they were investigated by a group of Swiss orthopedic surgeons. The interest of the Swiss arose from two alpine sports—climbing and skiing. These frequently result in fractured bones in young adults and although older patients might tolerate reduced mobility, younger ones do not. Dissatisfaction with the long-term outcome of their treatments led to the formation of a group of surgeons, later to become the *Arbeitsgemeinschaft für Osteosynthesefragen (AO)*, whose aim was

to develop fracture treatment(s) that would lead to full functional recovery. These surgeons developed a system that used compression at the fracture site applied through specially designed plates, or lag screws, which gives complete stability at the fracture site. This removes the necessity for further external stabilization of the fracture and also allows the immediate full mobilization of the surrounding joints. Permanent disability is reduced to a minimum.

As with all types of treatment, the AO methods have their critics, but most orthopedic surgeons would concede that the AO set standards for the longterm outcome of fracture treatment that other techniques, or systems, must also achieve. Many other systems have since been developed and there is now a wide range of "hardware"—plates, screws, medullary nails, external fixators, and so forth—for use in fracture treatment.

Bone healing is important not only after injury, but also after reconstructive surgery of which an extreme example is distraction osteogenesis. This technique was developed about 50 years ago by Ilizarov, a Russian surgeon working in western Siberia, but news of it did not reach the West until 1981 (Einhorn, 1998). To correct a deformity of the lower limb, Ilizarov performed an osteotomy and then, so that the blood vessels, muscles, and nerves would not be damaged by rapid stretching, he attached wires from an external frame to the proximal and distal regions of the bone and distracted the osteotomy by 1 mm per day. To his surprise, bone formed in the enlarging gap, so that the expected bone graft was unnecessary. He subsequently developed the distraction technique for the treatment of many congenital abnormalities. It is now also used to treat large bone loss resulting from severe trauma.

Irrespective of the type of fracture and its method of immobilization, or whether distraction is being applied, bone healing involves the formation of new bony and cartilaginous matrices. The type of matrix and the pattern of the tissues around the healing bone depends on the mechanical environment and hence the method of immobilization.

II. ANIMAL MODELS FOR INVESTIGATING FRACTURE REPAIR

To investigate and develop experimental procedures, animal models must be used. Some procedures require the use of large numbers of animals, others few animals. Because most experiments are designed to provide information that can be applied to human treatments, one criterion is that the bone of the animal used should behave in a similar way to that of man. Other important factors are the costs, especially those of large animals. The long bones of dogs, goats, and sheep have been used; sheep bone has properties very similar to those of human bone. The high costs associated with these animals precludes their use in many experiments, such as those to determine the effects of growth factors and other agents on fracture

repair, in which large numbers of animals are required. For most experiments rabbit or rat models are used. The rat is obviously the cheaper alternative, but there are two disadvantages. First, the size of the bones makes reproducible stabilization difficult and second, the cortical bone of the diaphyses of the long bones is not haversian (Ruth, 1953). Stabilization is frequently by means of a wire through the medullary cavity (Bonnarens and Einhorn, 1984). Nevertheless, the femur or tibia has been used for many experimental studies. The larger size of rabbit bones makes stabilization using different techniques possible (see below) and the cortical bone is haversian.

III. FRACTURE HEALING

A fracture may heal under stable or unstable mechanical conditions, but callus formation and healing are similar in different animals under the same mechanical conditions. Most of the descriptions that follow are of observations of rabbit tibial fractures. The fracture model used enables the fracture to be stabilized with either an AO-dynamic compression plate, so that there are stable mechanical conditions at the fracture site, or with a plastic plate of the same dimensions, designed to leave a gap of 0.5 mm between the fractured surfaces, so that the fracture heals under unstable mechanical conditions (Ashhurst et al., 1982).

A. The Stages of Fracture Healing

Healing occurs in two distinct stages. During the first, or healing stage, bone and cartilage develop on the periosteal surface to create a callus that bridges the fracture. The function of the callus is to immobilize the fracture fragments so that cells and blood vessels can move into the fracture gap, produce bone, and thus unite the cortical bone. Once cortical union is achieved, the second, or remodeling, stage starts. The callus is rapidly resorbed and the remaining callus and cortical bone is remodeled to restore the original structure. Ultimately all signs of the fracture site are obliterated. The first stage is crucial to successful healing and its progress depends on the method used for immobilization of the fracture fragments.

B. The Effects of Mechanical Conditions

When a fracture is immobilized using external means, such as a plaster cast or external fixation device, or by internal fixation with a noncompression plate or medullary nail, some movement of the fragments relative to each other is still possible and the fracture heals under unstable mechanical conditions. In contrast, if a compression plate is used, a compressive force is exerted between the fractured surfaces that keeps them in contact; there is no movement between the fragments and the fracture heals under stable mechanical conditions. It is frequently asserted

that rigid metal plates are essential for compression fixation, but this is not so. Exactly the same patterns of healing are found if flexible carbon fiber reinforced epoxy resin (CFRE) plates are used. The critical requirement is that the fracture surfaces are held together by a compressive force so that they do not move relative to each other.

Irrespective of the mechanical conditions at the fracture site, it is surrounded after the removal of the initial hematoma by a layer of fibrous tissue containing many cells. The regenerating periosteum can be distinguished as a layer of longitudinally orientated fibers. The cells are of periosteal origin; if there is only a narrow gap or compression at the fracture site, cells cannot move from the marrow cavity to the periosteal surface. Most of the cells arise from division of the osteoprogenitor cells in the cambial layer of the periosteum, but in the early stages it is possible that there is a contribution from the surrounding soft tissues (Simmons and Kahn, 1979; Ashhurst, 1986). This is very difficult to prove, but the callus of rabbit tibial fractures tends to develop more rapidly on the surface adjacent to the muscle than on the subcutaneous surface (Ashhurst, unpublished observations). Another factor that might contribute to the more rapid callus formation near the muscle is the proximity of a good blood supply. The periosteal callus is supplied from the surrounding tissues (Rhinelande, 1980). As the callus develops, more cells are recruited from the periosteum.

After the formation of the layer of fibrous tissue, the mechanical conditions at the fracture site have a profound effect on the nature and development of the matrices in the callus. Callus development can, therefore, be used to investigate some of the environmental conditions that influence the differentiation of osteoprogenitor cells.

Mechanically Stable Fractures

Bone formation starts along the whole surface of the cortical bone, and trabeculae grow outward. This means that the fracture site is bridged by bone from the beginning of bone formation. By 10 days after fracture in the rabbit tibia, the layer of bone has reached its full thickness of about 1 mm (Figure 1) (Ashhurst, 1986). Occasional small regions of cartilage are seen within the bone and on the periosteal surface, but there is never a large region of cartilage over the fracture site as there is under unstable mechanical conditions (see below). It is frequently stated that in man and in other large mammals, there is no periosteal callus formed after compression plating (Schenk, 1978). The evidence is primarily from X-rays that might not show a uniformly thin layer clearly.

It was mentioned above that mechanical stability depends on the compressive forces holding the fractured surfaces together, not on the rigidity of the plate. The fractures shown in Figures 2 and 3 were plated with a (CFRE) resin plate with identical dimensions and design as the AO DC plate. This plate is as flexible as a plastic plate. The development of the callus is indistinguishable from that of the

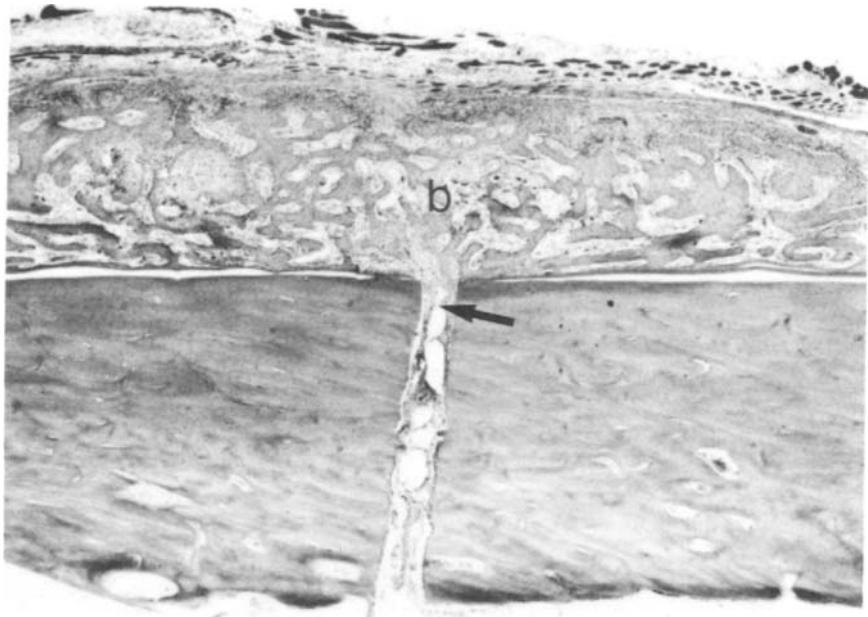


Figure 1. A 10-day fracture healing under stable mechanical conditions. The callus is of approximately uniform thickness and consists entirely of bone (**b**). Some bone is penetrating the fracture gap (arrow); this gap is the sawcut. Magn. X27.

fracture plated with a stainless steel DC plate (see Figure 1); bone grows over the fracture site and no cartilage is formed.

Cortical union in fractures under compression is achieved in three ways (Ashhurst, 1986). Although it is implicit that if there is compression the fractured surfaces are in contact, in practice only parts of the surfaces are in contact, in other areas there are small gaps. With experimental models, a saw cut is made to "start" the fracture and this removes some bone; in the experimental rabbit model the saw cut is approximately 100 μm wide. It was calculated that complete stability is achieved if only 0.5% of the opposing surfaces are in contact (Cordey, personal communication). Thus, there are regions of contact, regions of narrow gaps and regions of wide gaps (Ashhurst, 1986).

Healing of the wide gaps starts at 10 days with the movement of cells and capillaries from the periosteal callus into the gap. A matrix is laid down and some cells differentiate into osteoblasts and start to lay down bone on the fractured surfaces. Within a few days the gap is filled by bone (Figure 4). Occasionally, an osteoclast is seen on the surface of the old bone, but for the most part, resorption of the surfaces does not occur.

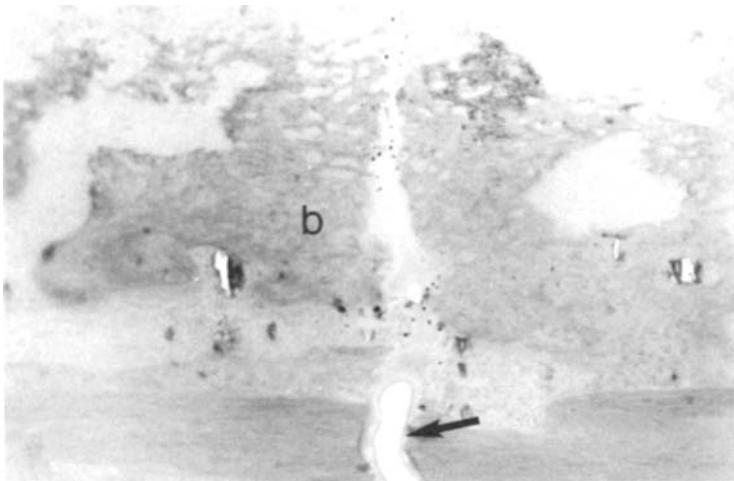


Figure 2. A 6-day fracture stabilized with a CFRE compression plate that gives stable mechanical conditions at the fracture site. Bone (**b**) is forming over the entire periosteal surface of the cortical bone. The narrow fracture gap (**arrow**) contains an air bubble that has disrupted the bone slightly. Magn. X120.

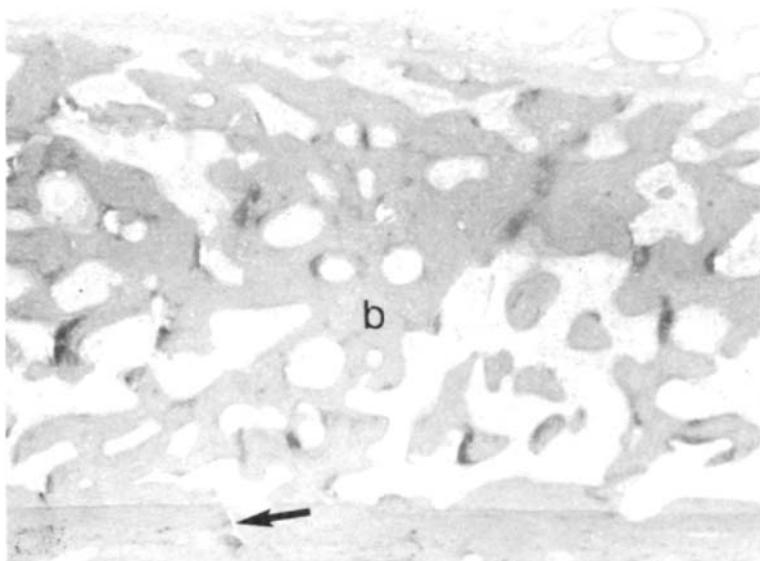


Figure 3. A 14-day fracture stabilized with a CFRE compression plate that gives stable mechanical conditions at the fracture site. The callus is a uniformly thick layer of bone (**b**); no cartilage is present. The fractured surfaces are compressed together in this region (**arrow**). Magn. X38.

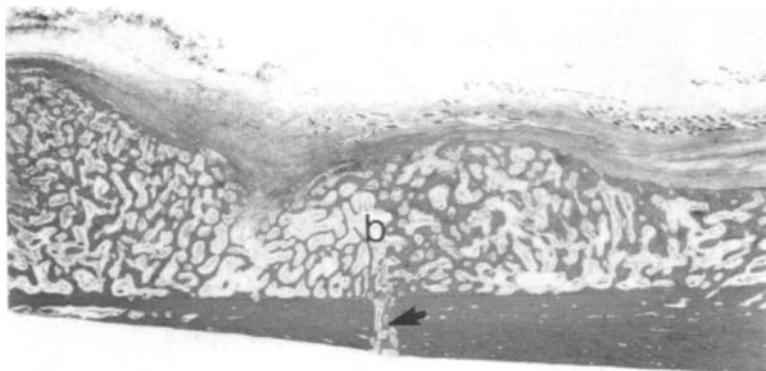


Figure 4. A 14-day mechanically stable fracture (AO-compression plate). The callus consists of bone (**b**) and the gap (**arrow**) is now filled by bone to give cortical union. Magn. X12.

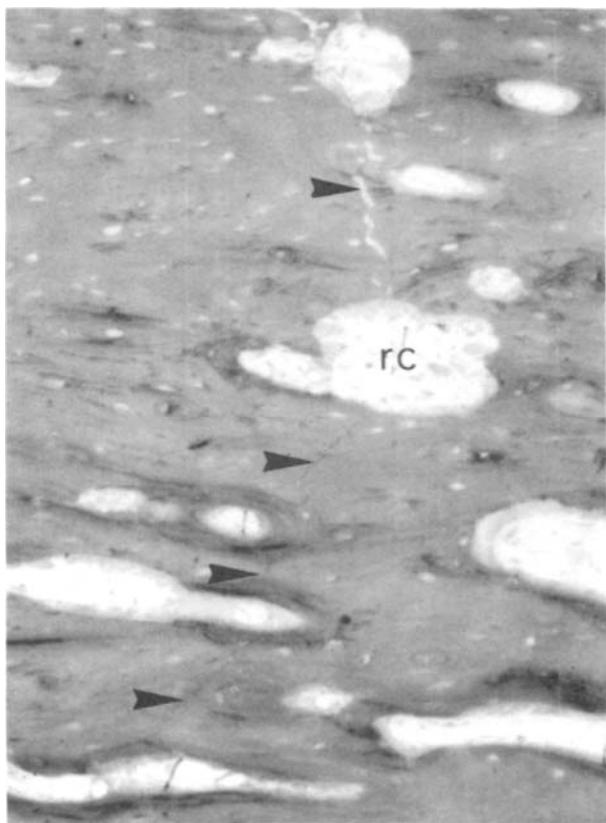


Figure 5. Part of the cortex of a 4-week mechanically stable fracture in which the fragments are compressed together. Cutterheads of remodeling cavities (**rc**) are crossing the fracture line (**arrowheads**). Magn. X135.



Figure 6. Part of the cortex of a 4-week mechanically stable fracture in which there is a narrow gap between the fragments. The gap is being widened by osteoclasts (**arrowhead**) so that osteoblasts and blood vessels (**bv**) can move in and fill the resorbed cavities (**rc**) with new bone (**arrow**). Magn. X170.

The regions of contact and gaps up to 10 μm wide are bridged as a result of direct haversian remodeling (Figure 5); this was termed “primary healing” by Schenk and Willeneger (1967). Remodeling in cortical bone is stimulated by a fracture, but it is usually 2 to 3 weeks before it is apparent. The newly formed remodeling cavities cross the fracture line and new osteons are formed. This process is slow and it may take many months for the whole cortex in the area of the fracture to be remodeled.

The narrow gaps, that is, those between 10 and 50 μm wide, are widened by osteoclasts before cells enter and lay down new bone. These regions are identified as a series of cavities across the cortex at about 3 weeks after fracture (Figure 6) (Ashhurst, 1986).



Figure 7. A 7-day mechanically unstable fracture. Bone (**b**) is forming away from the fracture site. Fibrous tissue (**ft**) is still present between the ingrowing fronts of bone. Small areas of cartilage are developing between the bone and fibrous tissue (**arrows**). Magn. X26.

As soon as cortical union is achieved in part of the cortex, the second phase of healing, and hence resorption of the callus and remodeling of the cortical bone start. Large cavities appear in the callus and also in the cortical bone. By one year after fracture, a single layer of compact bone is present and the fracture site is obliterated.

Mechanically Unstable Fractures

Bone formation starts in the fibrous tissue along the surface of the cortical bone away from the fracture site. This layer becomes thicker, and approaches, but does not reach, the fracture gap. Between 5 and 7 days after fracture in the rabbit tibia, cartilage appears along the boundary of the bone and fibrous tissue (Figure 7). The area of cartilage gradually increases in size until it fills the central region of the callus at about 10 days (Figure 8). Bone grows over the cartilage so that it is enclosed. The callus has now reached its maximal size. Endochondral ossification starts and the callus consists entirely of bone by around 21 days (Figure 9).

The formation of the bony callus stabilizes the fracture completely so that now the stable mechanical conditions at the fracture site permit the ingrowth of cells and blood vessels from the callus into the gap. Any remaining debris is removed by macrophages, a fibrous matrix is produced, and cells differentiate into osteoblasts and lay down bone on the fractured surfaces. Bony union occurs by about 4 weeks (Figure 9).

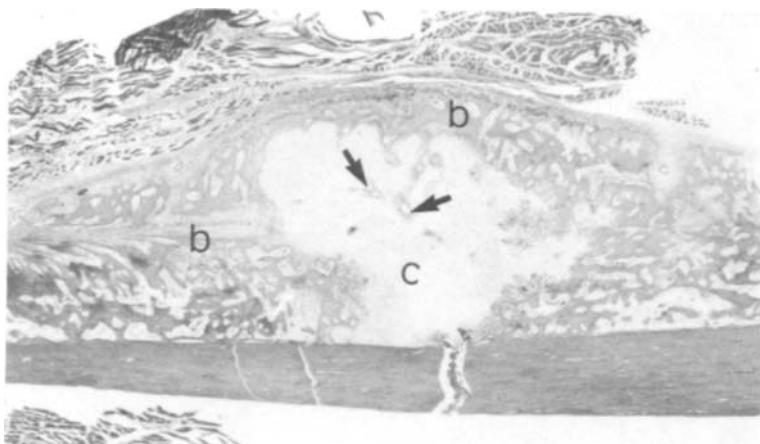


Figure 8. A 10-day mechanically unstable fracture. The callus is fully developed. The region over the fracture gap is filled by cartilage (**c**) and the cartilage is surrounded by bone (**b**). Some small blood vessels are present in the cartilaginous region, but they are surrounded by a thin layer of bone (**arrows**). Magn. X12.

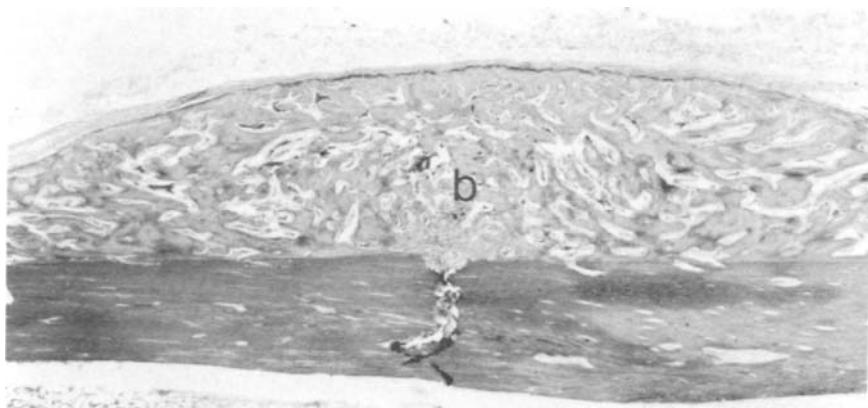


Figure 9. A 21-day mechanically unstable fracture. All the cartilage has been replaced by bone (**b**). Magn. X13.5.

The second stage of healing can now start. The callus is resorbed with the formation of large cavities (Figure 10). The remaining bone in the callus and cortex is remodeled. By one year after fracture there is a single layer of compact bone. It is during this remodeling stage that the bone regains its mechanical strength.

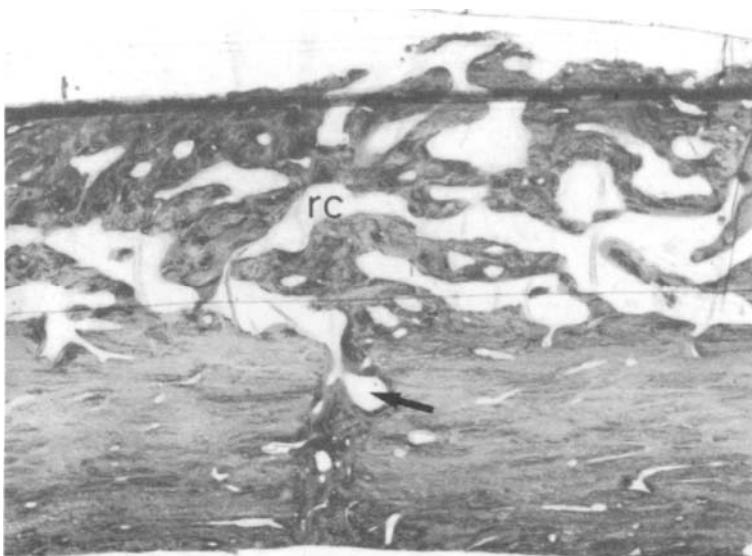


Figure 10. A 6-week fracture that healed under mechanically unstable conditions. The fracture gap is filled by transverse lamellae of new bone that are being invaded by longitudinal cutterheads (arrow). The callus has large resorption cavities (rc). Magn. X26.

The emphasis of this chapter is on the biological rather than the mechanical results of different types of fracture healing. It is, however, pertinent to mention some recent studies of healing osteotomies of sheep metatarsals and tibiae (Claes et al., 1995, 1997). The restoration of the haversian structure in the remodeling stage of the cortical bone was found to be essential for full mechanical strength. Furthermore, it was found that accurate reduction to leave only a small gap lead to better callus formation. In fractures with gaps less than 2 mm wide, the amount of callus was increased by increasing the interfragmentary strain from 7 to 31%, but this did not increase the bending stiffness of 9-week fractures. Healing was inferior when the gap was greater than 2 mm.

IV. HEALING OF DEFECTS

If a segment of bone is removed from the diaphysis of a long bone, healing follows a different pattern. In the rabbit, this occurs when the defect is greater than 2 mm wide. To study healing in defects, segments from 2 to 5 mm wide were removed from the radius, leaving the intact ulna to support the limb. The gap is first filled with loose fibrous tissue (Figure 11). Osteoprogenitor cells from both the perios-

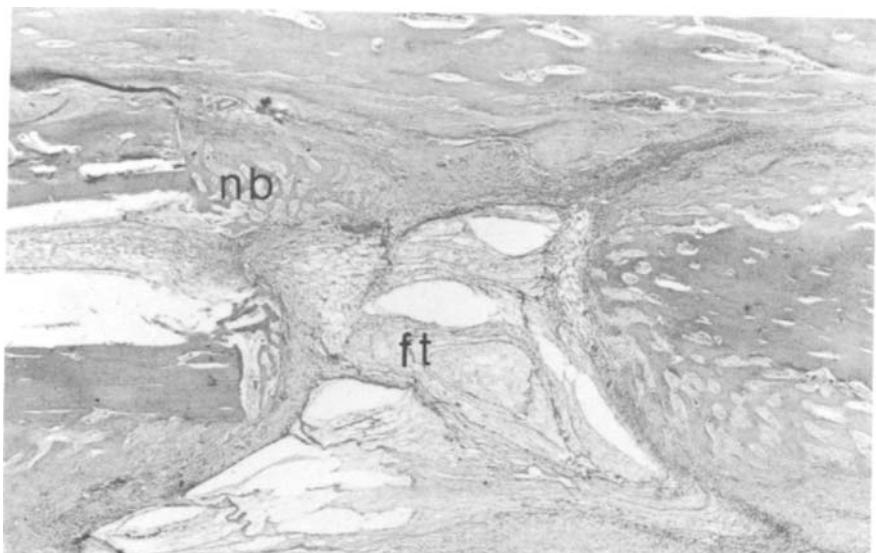


Figure 11. A 5-mm defect in the rabbit radius 1 week after operation. New bone (nb) is forming around both the proximal and distal cut surfaces. The central region of the gap is filled by fibrous tissue (ft). Magn. X17.

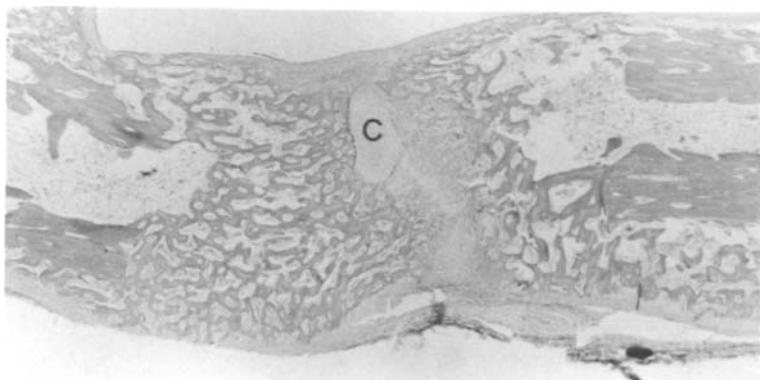


Figure 12. A 4.5-mm defect in the rabbit radius 2 weeks after operation. A small area of cartilage (c) is present in the centre of the new bone. Magn. X15.

teum and stromal cells of the bone marrow differentiate into osteoblasts and lay down bone around the cut surfaces of the bone (Figure 11). These layers of bone increase in size and approach the centre of the gap. As the region between the ingrowing layers of bone decreases, cartilage develops between them (Figure 12);

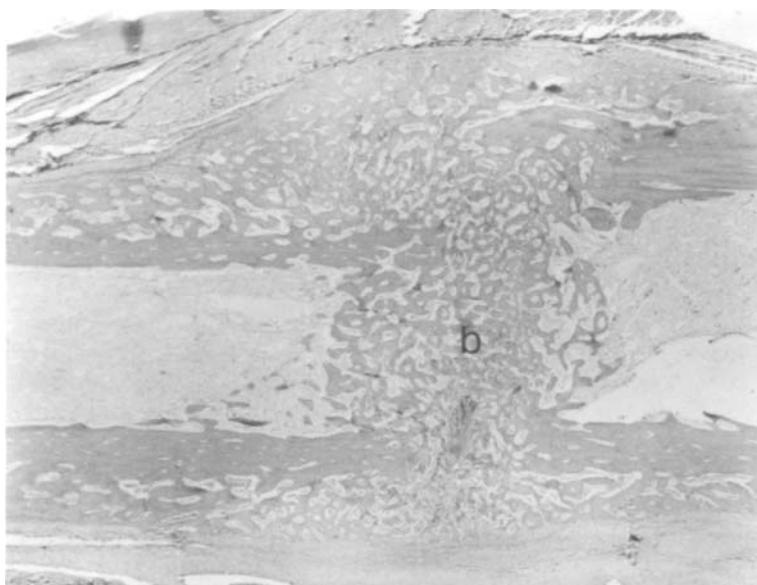


Figure 13. A 3-mm defect in the rabbit radius 3 weeks after operation. The defect is bridged by bone (b). Magn. X12.

this occurs between 14 and 21 days. The cartilage is gradually removed by endochondral ossification and the cortices are united by cancellous bone (Figure 13).

The successful healing of a defect depends on its size. If its length is greater than the diameter of the bone, it does not heal unless a bone graft, or other treatment, is given. The reason for this cutoff point is not understood. In the rabbit radius this occurs if the gap is 6 mm or greater. A small amount of bone may be formed on the cut cortical bone, but an area of fibrous tissue and debris persists centrally (Figure 14).

V. DISTRACTION OSTEOGENESIS

The formation of the new matrices during distraction has been studied in several animal models, including the rabbit. It is more complex than other forms of healing because the formation of the new tissues depends on the rate, rhythm, and number of distractions and the final length achieved. In experimental situations, the lengthening per day varies from 0.3 to over 2 mm. It is usually done in two steps. At the endpoint the bone may be lengthened by several centimeters.

To perform distraction, a dynamic external fixation device is attached to the bone with screws and an osteotomy is made. In most experiments distraction is started 7 days later during which time a normal periosteal callus is developing. Thus, there is bone formation along the cortical bone and, at 7 days, there may also be some cartilage. Endosteal callus may also be forming. The action of the distrac-

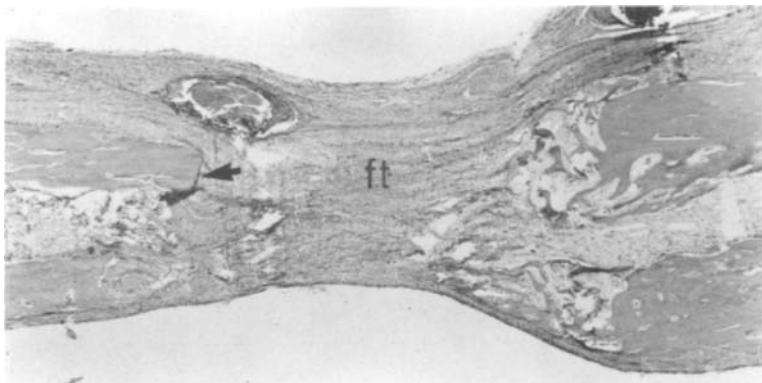


Figure 14. A 6-mm gap in the rabbit tibia 4 weeks after operation. Some new bone has formed around one cut surface, but there is none around the other (arrow). The central region is filled by fibrous tissue (ft) with no blood vessels. Magn. X15.

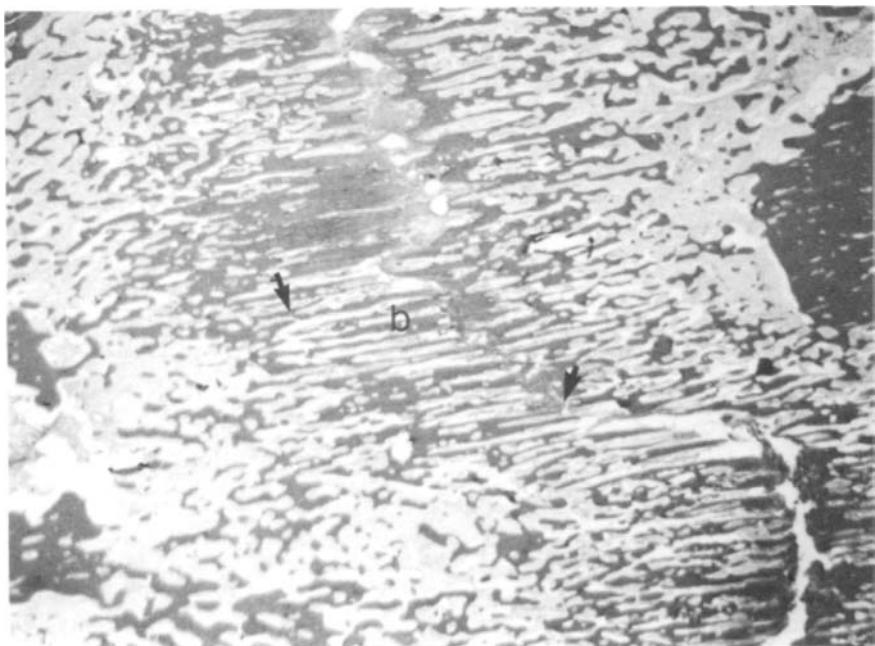


Figure 15. The regenerate after distraction in the rabbit tibia at the rate of 0.3 mm per day. The regenerate consists of bone (b) which centrally is in the form of longitudinal trabeculae. Some of these show small fractures (arrows). Some of the broader trabeculae are fibrocartilaginous. Magn. X6.6.

tive force pulls these tissues apart. Gradually cells enter the enlarging gap and bone, cartilage, and fibrous tissue are formed. The disposition of these tissues depends on the rate, rhythm and amount of distraction.

At distraction rates of 0.3 and 0.5 mm per day in the rabbit tibia, bone is formed at the cut ends of the bone while the central region is filled by longitudinally orientated thick fibres with some rounded, chondrocyte-like, cells along their axes (Figure 15) (Li et al., 1999). This tissue is usually referred to as fibrocartilage. If the distractive rate is 0.7 mm per day or greater in the rabbit, or above 0.3 mm in the rat femur (Jazrawa et al., 1998), bone forms next to the severed cortex, but the central region is filled by fibrous tissue. At higher distraction rates necrotic cysts are formed (Figure 16). Cartilage may be present between the bone and periosteum and very variably in the central regions of the fibrous tissue. The amount of cartilage is usually small. Fibrocartilage, or very small regions of hyaline cartilage, may be present within the bone.

If, after the required lengthening has been achieved, the regenrate is left for a week or longer, cartilage replaces the central fibrous tissue (Jazrawa et al., 1998).

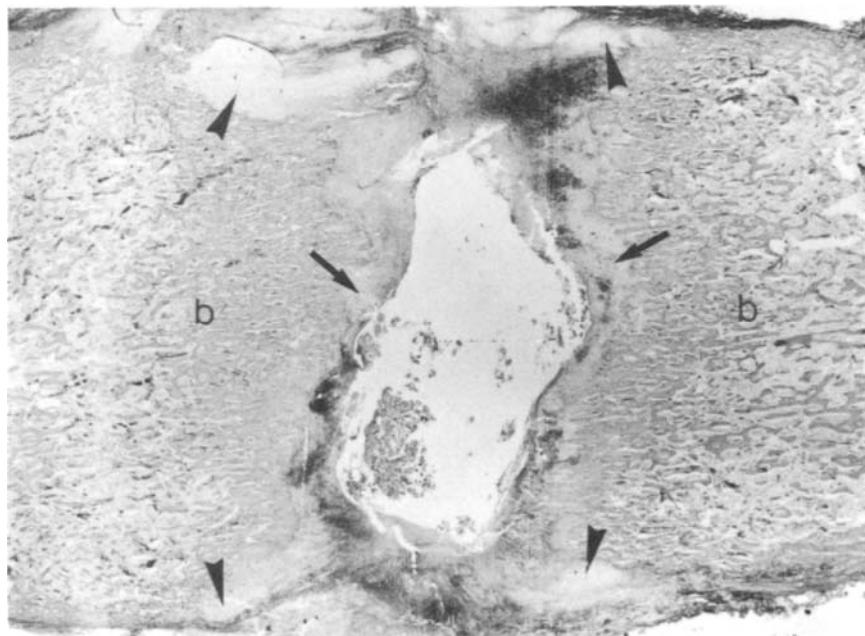


Figure 16. The regenerate after distraction in the rabbit tibia at the rate of 1.3 mm per day. Bone (**b**) has grown from the cut surfaces, but it does not fill the gap. The central region is filled by a large cyst bordered by fibrous tissue (**arrows**). There are small areas of cartilage in the peripheral regions (**arrowheads**). Magn. X6.6.

This suggests that while distraction is ongoing, either the rate of distraction is greater than the rate of capillary growth, or that the mechanical forces restrict angiogenesis so that only fibrous tissue forms. Bone formation requires a good blood supply. The forces exerted in the center are such that only fibrous tissue can form, but when distraction is complete, blood vessels and hence bone can grow into the fibrous tissue. Cartilage formation probably occurs when the bone fronts reach a critical point leaving a gap in which the mechanical stresses in the fibrous tissue inhibit angiogenesis, but favor the formation of cartilage (see below).

The final stages of lengthening involve the formation of a new region of cortical bone. Examination of radiographs of experimental lengthening at different stages indicate that at the early stages there is a zone of mineralization adjacent to the cut cortical bone with a radiolucent central area, but that with time and increasing distraction, the zone of mineralization and the central area remain the same thickness, but are increasingly separated from the original bone by a radiolucent layer. This is explained by the resorption of the cancellous bone and remodeling in the region next to the cortical bone. When distraction is stopped, the central fibrous tissue is replaced by bone, but this, too, is gradually resorbed and remodeled. The gap is finally bridged by a thin cylinder of bone that has developed periosteally from the original callus (Kojimoto et al., 1988).

VI. MACROMOLECULAR SYNTHESIS DURING CALLUS DEVELOPMENT

The major proteins synthesized during callus formation are the collagens. The early studies localized the proteins, but more recently the cells that synthesize the collagens have been identified.

The fibrous tissue laid down in rabbit and rat calluses in the first few days contains types III and V collagens; type I collagen was located in the rat, but not in the rabbit tissue (Lane et al., 1986; Page et al., 1986). The mRNAs for these collagens are expressed by the spindle-shaped fibroblasts (Bland et al., 1999). Membranous bone is laid down within this fibrous matrix and types I, III, and V collagens are located in the bone matrix, but only types III and V collagens are present in the spaces (Lane et al., 1986; Page et al., 1986). Osteoblasts and also osteocytes in the newly formed, but not the mature, trabeculae express types I, III, and V collagen mRNAs (Figure 17) (Sandberg et al., 1989; Hughes et al., 1995; Yamazaki et al., 1997; Bland et al., 1999). The continued expression of collagen mRNAs by osteocytes is confined to intramembranous bone produced in response to injury or other stimulation, for example, growth factors (Critchlow et al., 1995a; Yamazaki et al., 1997). During normal development osteoblasts cease expression of type I collagen mRNA when they enter the bone matrix (Critchlow et al., 1995a).

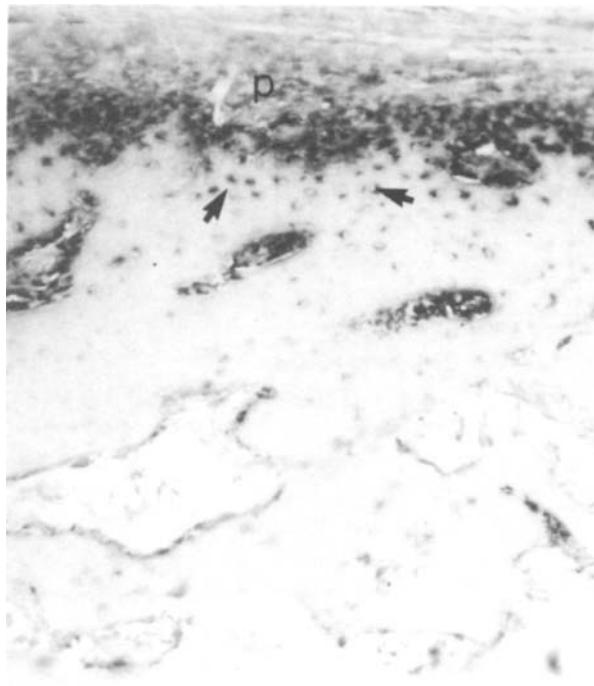


Figure 17. Part of the callus of a 10-day mechanically unstable fracture that has been hybridized with a riboprobe, labeled with digoxigenin, for type I collagen mRNA. The osteoblasts and preosteoblasts in the periosteum (**p**) are expressing the mRNA. It is also expressed by the osteoblasts lining the cavities. Some osteocytes in the newbone under the periosteum (**arrows**) are also expressing the mRNA. Magn. X120.

In mechanically stable fractures, type II collagen occurs only in isolated, discrete sites of cartilage within the bone (Figure 18) (Page et al., 1986; Ashhurst, 1990). In mechanically unstable fractures, type II collagen is found in the cartilage that forms between the ingrowing fronts of bone. Analysis of adjacent sections in which types I and II collagens are localized reveals that in many regions with the morphological appearance of cartilage, type I collagen is also present, particularly around the chondrocyte lacunae (Figures 19 and 20) (Ashhurst, 1990). This suggests that the tissue might more properly be designated chondroid bone (Beresford, 1981).

Cells that express type II collagen mRNA are first seen at the junction between the ingrowing bone and fibrous tissue (Bland et al., 1999). Analysis of serial sections hybridized with types I and II collagen probes reveals that many cells are expressing both collagen mRNAs (Figures 21 to 24) (Sandberg et al., 1989;

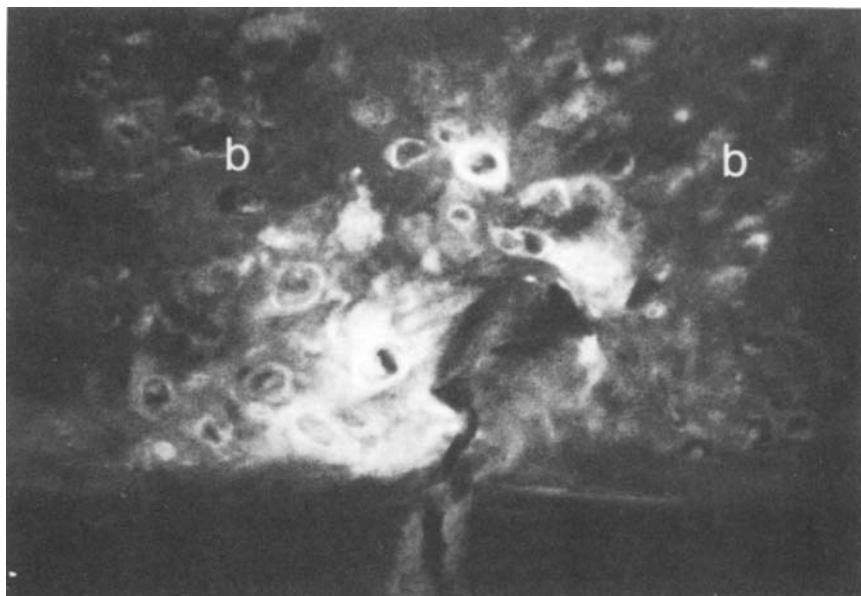


Figure 18. The region immediately above the very narrow gap of an 11-day mechanically stable fracture to show the fluorescent localization of antibodies to type II collagen. This small area of type II collagen-containing tissue is surrounded by bone (b). The tissue has both bone-like and cartilage-like features and is probably chondroid bone. Magn. X343.

Hughes et al., 1995; Bland et al., 1999). This agrees with the localization of the proteins, described above. As the area of cartilage gradually fills the central region of the callus, it is apparent that the cells first express both types I and II collagen mRNAs, but later only type II mRNA (Bland et al., 1999). Expression of type II mRNA ceases and in the rabbit, the hypertrophic chondrocytes do not express any fibrillar collagen mRNA (Figure 25) (Bland et al., 1999). They, therefore, do not synthesize type I collagen at sites of endochondral ossification (see below).

The alternatively spliced type IIA collagen mRNA was located in rat fracture calluses in mesenchymal cells on day 4, in chondrocytes and osteoblasts on day 14, and chondrocytes only on day 21 (Hughes et al., 1995). These observations appear to conflict, first, with the association of type IIA collagen mRNA with precursor or differentiating cells in developing tissues, because at 14 and 21 days the chondrocytes should be mature or hypertrophic and, second, with the assays of Hiltunen et al. (1995) in which the peak levels were recorded at 7 days in mouse fracture callus.

The cartilaginous matrices of the callus also contain types III and V collagens, but these are probably remnants of the original fibrous tissue in which

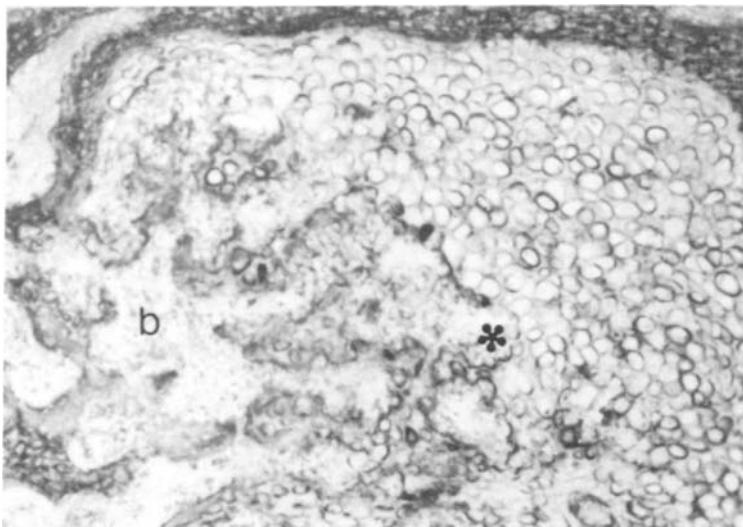


Figure 19

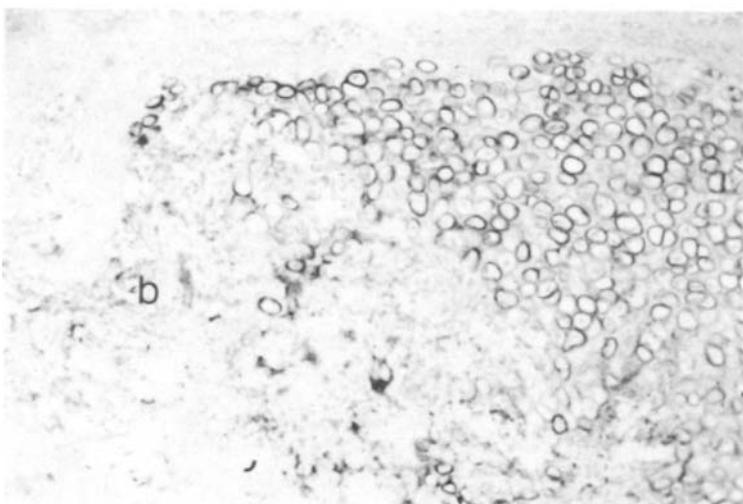


Figure 20

Figures 19 and 20. Adjacent sections of the superficial region of the callus of a 14-day mechanically unstable fracture showing bone (b) next to the cartilage. Endochondral ossification is ongoing along the boundary between the tissues. **Figure 19:** Antibody to type I collagen with alkaline-phosphatase conjugated second antibody—type I collagen is present in the bony trabeculae and in the cartilage, particularly around the lacunae. **Figure 20:** Antibody to type II collagen with alkaline-phosphatase conjugated second antibody—type II collagen is found only in the cartilaginous matrix. The asterisk on Figure 19 indicates the region shown in Figure 25. Magn. X103.

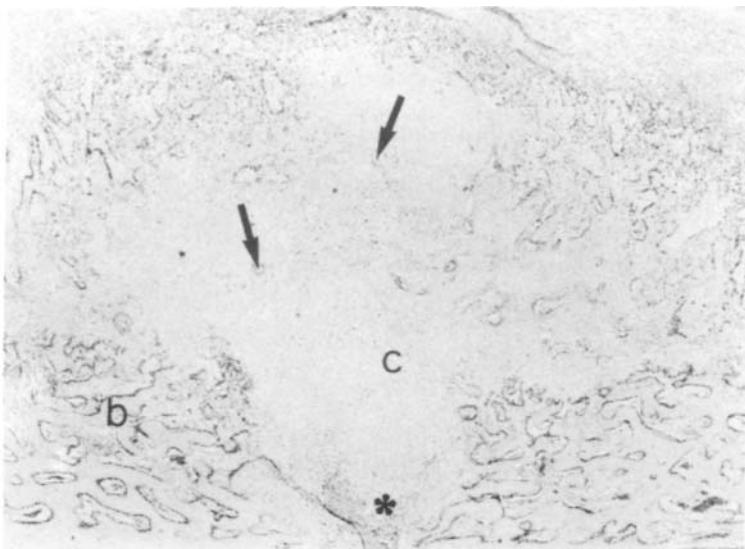


Figure 21

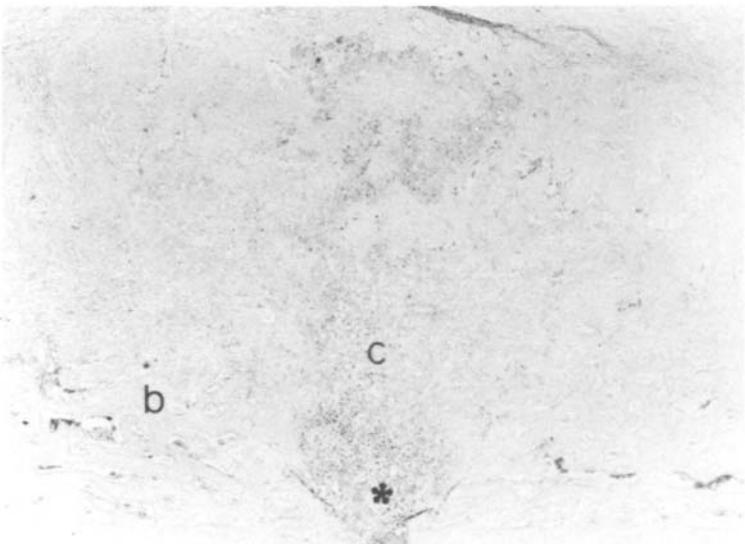


Figure 22

Figures 21 and 22. Adjacent sections of an 8-day fracture callus. A large area of cartilage (c) is surrounded by bone (b). Some small blood vessels (arrows) in the cartilage are surrounded by a layer of bone (see Figure 8). **Figure 21:** Hybridization for type I collagen mRNA (digoxigenin-labeled probe)—the osteoblasts in the bone and around some blood vessels in the cartilage are expressing the mRNA. The asterisk indicates the region shown in Figure 23; here some cells express the mRNA. **Figure 22:** Hybridization for type II collagen mRNA (digoxigenin-labeled probe)—only the chondrocytes are expressing this mRNA. The asterisk indicates the region shown in Figure 24. Figures 21 and 22 magn. X26.

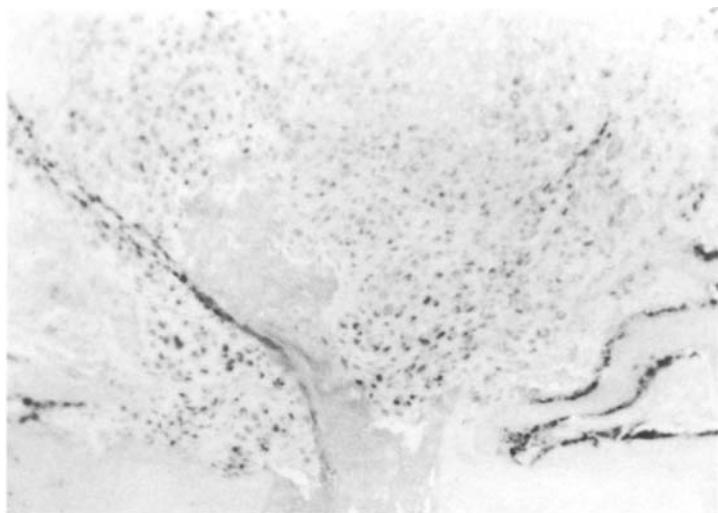


Figure 23

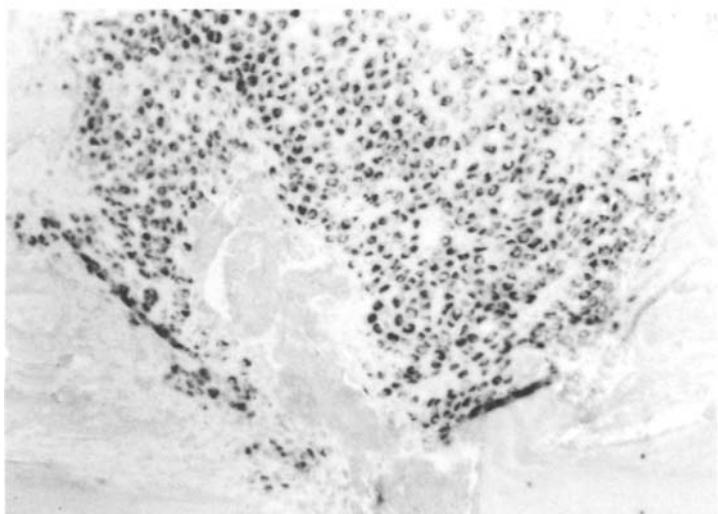


Figure 24

Figures 23 and 24. The regions indicated on Figures 21 and 22, respectively. All the chondrocytes are expressing the mRNA for type II collagen (Figure 24), but some are also expressing the mRNA for type I collagen (Figure 23). **Figure 23:** magn. X103. The regions indicated by asterisks on Figures 21 and 22, respectively. All the chondrocytes are expressing the mRNA for type II collagen. **Figure 24:** However, some are also expressing the mRNA for type I collagen (Figure 23).

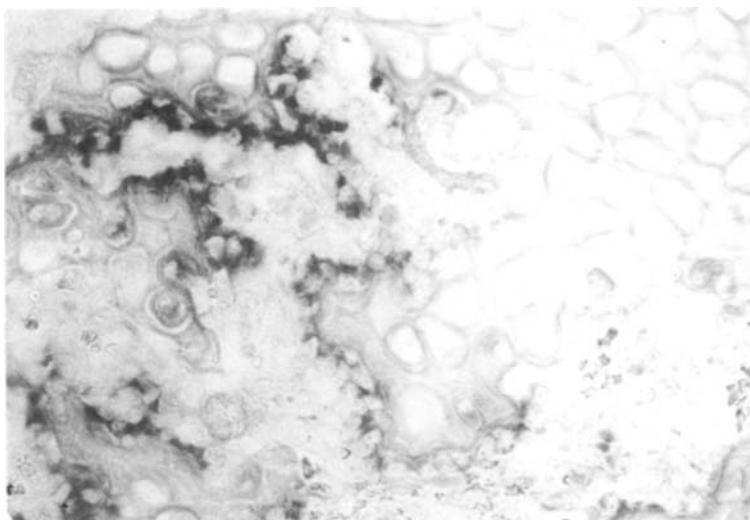


Figure 25. The region of endochondral ossification indicated by an asterisk on Figure 19. Hybridization for type I collagen mRNA (digoxigenin-labeled probe)—only the osteoblasts are expressing the mRNA. None of the hypertrophic chondrocytes is expressing the mRNA. Magn. X305.

the types I and II collagens are laid down (Page et al., 1986). Neither types III or V collagen mRNAs are expressed by the chondrocytes in rabbit and rat callus at the time when type II collagen is being synthesized (Yamazaki et al., 1997; Bland et al., 1999). The heterogeneity of the tissue is confirmed by the presence of bundles of thick banded collagen fibrils and elastic fibers seen in electron micrographs (Ashhurst, 1986).

The minor collagen, type IX, which associates with type II collagen in the fibrils and acts as the core protein of a chondroitin sulphate proteoglycan, is also present in callus cartilage (Page et al., 1986). Type X collagen is present around the hypertrophic chondrocytes in rat fractures (Topping et al., 1994). The other minor collagens have not yet been located immunohistochemically in callus tissue, but some mRNAs have been detected in cells. Rat chondrocytes express type XI collagen mRNA (Yamazaki et al., 1997).

Various bone proteins have been located in developing calluses. Osteocalcin is present in both osteoblasts and bone matrix of rabbits and rats and also in some chondrocytes and cartilage matrices (Figure 26) (Stafford et al., 1994; Hughes et al., 1995). It may be colocalized with type I collagen in cartilage matrix and its mRNA is present in the chondrocytes, which is further evidence for the occurrence of chondroid bone in callus. In contrast, Hirakawa et al. (1994) and Yamazaki et al. (1997) found that osteocalcin mRNA was confined

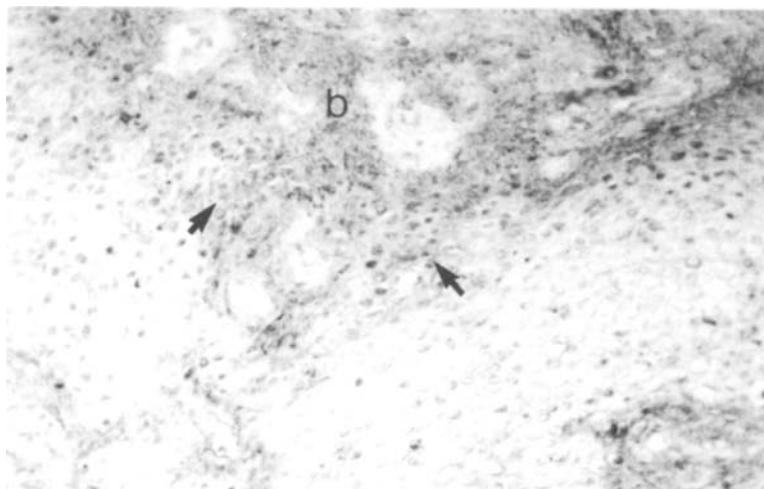


Figure 26. A region of an 8-day callus in which cartilage is forming adjacent to bone (**b**). Antibody to osteocalcin with alkaline-phosphatase conjugated second antibody—the bone contains osteocalcin and some is also present in the cartilage matrix and chondrocytes (**arrows**). Magn. X120.

to osteoblasts. The mRNAs for osteonectin and osteopontin have been located in callus osteoblasts (Hirakawa et al., 1994).

Although a great deal is known about the distribution of collagens in fracture callus, much less is known about the proteoglycans. The glycosaminoglycan (GAG) side chains were initially localized in the 1950s and 1960s long before it was appreciated that they are attached to protein cores; these early investigations were reviewed by Ashhurst (1992). With the refinement of Alcian Blue staining techniques (Scott, 1980) and the production of monoclonal antibodies to the GAGs (Caterson et al., 1983, 1985), it is possible to identify the GAGs precisely. Thus, chondroitin and keratan sulphates are present in the developing bone in rabbit calluses (Page and Ashhurst, 1987); rabbit bone is peculiar in that the bone sialoprotein is the core protein of a keratan sulphate proteoglycan (Diamond et al., 1982; Kinne and Fisher, 1987). The cartilage also contains chondroitin and keratan and sulphate proteoglycans (Page and Ashhurst, 1987). Little has been done to identify the core proteins. Decorin was found in differentiating and mature, but not hypertrophic, chondrocytes, and in osteoblasts and bone matrix in rat callus (Matsumoto et al., 1994). The extracted mRNAs of aggrecan and “proteoglycan core protein” are at peak levels during active cartilage formation between 5 and 14 days in mouse and rat calluses (Jingushi et al., 1992; Hiltunen et al., 1993).

Extraction of mRNA from developing mouse and rat calluses has confirmed the results of *in situ* hybridization studies. The levels of type III collagen mRNA peak first as the fibrous tissue is formed (Hiltunen et al., 1993). The levels of mRNAs for bone proteins, type I collagen, osteocalcin, and osteonectin, and the enzyme alkaline phosphatase increases gradually with increasing intramembranous and endochondral bone formation (Jingushi et al., 1992; Hiltunen et al., 1993). The peaks for types II, IX and XI collagens occur as cartilage formation increases and drops when it ceases; type X expression peaks later (Jingushi et al., 1992; Hiltunen et al., 1993, 1994, 1995). The peak level of the alternatively spliced form of type II collagen, type IIA, occurs earlier than that of normal type II collagen (Hiltunen, 1995) which agrees with its expression in prechondrogenic tissues. Type VI collagen mRNA levels are elevated throughout callus formation (Hiltunen et al., 1995). The only investigations of proteoglycan mRNAs are of aggrecan and "proteoglycan core protein", both of which are at peak levels of expression between 5 and 14 days, that is, during cartilage formation in mouse and rat calluses (Jingushi et al., 1992; Hiltunen et al., 1993). Although these experiments confirm the presence of these mRNAs, they give no information about the activity of the cells.

The turnover of types I and III collagens during fracture repair has been estimated by assaying the amounts of type I collagen carboxyterminal propeptide (PICP), type III collagen amino-terminal propeptide (PIIINP), and type I collagen carboxy-terminal telopeptide (ICTP) in serum of patients with tibial diaphyseal fractures. PIIINP levels rose steadily until week 5, which indicates that type III collagen is being synthesized during this time. ICTP levels increased to 2 weeks and then gradually declined. PICP levels showed an initial slowing of type I collagen synthesis, but by 2 weeks the levels were elevated and remained so for several weeks (Joerring et al., 1994; Kurdy et al., 1998). The value of such data in assessing the progress of healing has yet to be established.

VII. CELL DIFFERENTIATION DURING HEALING

There is a pool of osteoprogenitor cells directly associated with the skeleton. These are in the cambial layer of the periosteum and lining the endosteal surfaces. Normally these cells are quiescent, but on receiving an appropriate stimulus they differentiate and produce skeletal matrices. Bone can also form ectopically and the now classical experiments of Urist (1965) demonstrated that mesenchymal cells in extraskeletal soft tissues can be induced to form cartilaginous and bony matrices by the implantation of demineralized bone matrix. More recently the inducing agents, that is, the bone morphogenetic proteins, have been identified (Wozney et al., 1990).

A. Origin and Differentiation of Cells in Fracture Callus

The osteoprogenitor cells that produce the periosteal callus originate from the cambial layer of the periosteum and possibly also from the surrounding soft tissues (Simmons and Kahn, 1979; Ashhurst, 1986). The stromal cells of the bone marrow are another source of osteoprogenitor cells (Ashton et al., 1980). These certainly contribute to the endosteal osteoblasts and the formation of any endosteal callus, but unless the fracture gap is sufficiently wide for cells to migrate through it, they do not contribute to the periosteal callus.

Osteoprogenitor cells, regardless of their origin, can differentiate into osteoblasts or chondrocytes. This is illustrated *in vivo* by the formation of both bony and cartilaginous matrices in callus and other induced skeletal tissue (Critchlow et al., 1994). To prove this, many experimental studies have been performed. The potential of marrow stromal cells to differentiate into osteoblasts and chondrocytes was shown by culturing rabbit cells in diffusion chambers implanted into the peritoneum of nude mice. After 3 to 4 weeks matrices with the characteristics of bone and cartilage are produced in the chambers (Ashton et al., 1980). Further characterization showed that the cells produced alkaline phosphatase. The bone matrix contains types I and V collagens, whereas the cartilage matrix contains types II and IX collagens; both matrices contain, as appropriate, chondroitin and keratan sulphates (Ashhurst et al., 1990). It has been thought for a long time that pericytes associated with capillaries might possess osteogenic potential. Recently it has been demonstrated that after prolonged culture of bovine retinal pericytes in diffusion chambers *in vivo* bone-like and cartilage-like matrices are formed (Doherty et al., 1998). Thus, pericytes are another possible source of osteoprogenitor cells during fracture repair.

Periosteal osteoprogenitor cells have been isolated and maintained in culture both *in vitro* and *in vivo* in diffusion chambers (Nakahara et al., 1991; Izumi et al., 1992; Iwasaki et al., 1993, 1994). Depending on the culture conditions, both osteoblast-like and chondrocyte-like cells are present in the cultures and cartilaginous and bony matrices are produced. These observations led to the suggestion that committed chondrocyte- and osteoblast-progenitor cells are present in periosteum (Nakahara et al., 1991; Izumi et al., 1992). This possibility is untenable. In both periosteal callus and in sites of TGF- β -induced periosteal bone formation the distribution of bone and cartilage is very variable (Joyce et al., 1990; Critchlow et al., 1994). Committed progenitor cells would have to migrate to specific sites in response to local stimuli. Furthermore, during fracture healing under stable mechanical conditions (see above) chondrocyte progenitor cells would be redundant. To maintain an osteoblast-like or chondrocyte-like phenotype *in vitro* involves specific culture conditions, the discussion of which is outside the scope of this chapter. Given appropriate conditions, osteoblast-like cells produce a bone-like matrix that is mineralized, whereas chondrocyte-like cells produce a cartilage-like matrix.

The membrane bones of the skull and facial region are formed directly from mesenchymal tissue: there are no cartilaginous anlagen. Regions of cartilage and chondroid bone may develop later. It has been demonstrated both *in vitro* and *in vivo* that the osteoprogenitor cells in the periosteum can differentiate into osteoblasts or chondrocytes, or intermediate phenotypes (Fang and Hall, 1997). The evidence suggests that all the osteoprogenitor cells are similar and all have both osteogenic and chondrogenic potential. Thus, these osteoprogenitor cells, too, are not committed to a particular differentiative pathway (Fang and Hall, 1997).

Therefore it seems certain that all osteoprogenitor cells are able to differentiate into either osteoblasts or chondrocytes. The question remains: how is their differentiation controlled? In fracture callus after the formation of the fibrous tissue, the first osteoprogenitor cells to differentiate become osteoblasts and lay down bone, except in precise regions where different mechanical conditions pertain. Bone is a well-vascularized tissue and capillary ingrowth is associated with both endochondral and intramembranous bone formation. Chondrocyte-like cells appear in callus later than osteoblasts and are frequently associated with the interface between bone and fibrous tissue.

B. Further Differentiation of Osteoblasts and Chondrocytes

Differentiated osteoblasts form epithelium-like layers in the surface of bone matrix. The fully differentiated osteoblastic phenotype is defined by the ability of the cells to produce the constituents of bone matrix, that is, type I collagen, osteocalcin, osteopontin, and alkaline phosphatase. When osteoblasts enter the bone matrix, they cease synthesis of type I collagen and other proteins. In the intramembranous bone of callus and other intramembranous bone induced by growth factors, etc., the osteocytes continue to express the mRNA for type I collagen (Critchlow et al., 1995a; Bland et al., 1999). Thus, they differ in this respect from osteocytes in normal bone.

Chondrocytes also undergo further differentiation after assuming a chondrocytic phenotype. Chondrocytes vary considerably between sites in the body. For example, the chondrocyte of the permanent cartilage, that is, articular cartilage and cartilage in the respiratory tree, retain the phenotype of mature cells and are responsible for the slow turnover of their matrix. The chondrocytes of growth plates arise by division of chondrocytes, while those in callus and ectopically-stimulated tissues arise from proliferating, undifferentiated mesenchymal cells and pass rapidly from a mature to a hypertrophic state. From cell division to hypertrophy in callus chondrocytes takes only about 48 hours (Bland et al., 1999). All these regions of cartilage are transitory and are replaced rapidly by bone. This developmental sequence has been reproduced in chicken chondrocytes *in vitro*, but the time scale is measured in weeks, not days (Cancedda et al., 1992).

A question has arisen about the final differentiative stage of chondrocytes in fracture callus. *In vitro*, Cancedda's group found that hypertrophied chicken chon-

drocytes can become osteoblast-like and synthesize type I collagen. This idea was developed by Scammell and Roach (1996) who suggested that hypertrophic chondrocytes in rabbit callus become osteoblasts or osteocytes and synthesize bone matrix. They present evidence for the presence of type I collagen and osteocalcin in the cytoplasm of hypertrophic chondrocytes, but they did not locate type I collagen in the surrounding matrix. Our recent studies (Bland et al., 1999) have shown that the early chondrocytes express both types I and II collagen mRNAs and that both proteins are present in the matrix. As the chondrocytes mature, type I mRNA expression ceases, but type II continues until the cells hypertrophy. The hypertrophic chondrocytes express neither type I, nor type II, collagen mRNAs, and neither collagen protein was located in their cytoplasm (Bland et al., 1999). Thus, the type I collagen that was detected around the chondrocyte lacunae during endochondral ossification in fracture callus (Page et al., 1986) is present from the earliest stages of cartilage formation. The layer becomes thicker as osteoblasts that are expressing type I collagen mRNA enter the opened lacunae and lay down bone matrix. Furthermore, no cells in the lacunae of cartilage spicules surrounded by bone express type I collagen mRNA. None of these observations of type I collagen mRNA and protein localization support the hypothesis that hypertrophic chondrocytes in rabbit fracture callus transdifferentiate into osteoblasts. A similar conclusion, again based on the localization of the cells expressing type I collagen mRNA, was reached for the hypertrophic chondrocytes of the bovine growth plate (Sandell et al., 1994). It has been suggested that transdifferentiation does occur in the avian growth plate (Roach, 1997), but its structure and collagenous composition differ greatly from that of mammals.

VIII. ROLE OF CARTILAGE IN FRACTURE REPAIR

The formation of cartilage in fracture callus is not essential for repair to proceed. This is clearly illustrated by comparing the calluses of fractures healing under stable and unstable mechanical conditions, since the former contain little or no cartilage. Also, the cartilage is replaced very rapidly by bone. In tibial fractures in a type II collagen knockout mouse, cartilage formation was greatly reduced, but at 28 days a bony callus was present (Hiltunen et al., 1994). The mechanical properties of the fractures of the knock-out and control mice were similar. This again illustrates that cartilage is not essential for healing.

This poses questions about the function of cartilage during bone healing. The function of a callus is to stabilize the fracture fragments. The required stability is achieved only when the gap is bridged by bone, that is, when the cartilage over the fracture site has been completely replaced by endochondral ossification. It may be argued that the greater the amount of cartilage over the fracture gap, the longer the time required for ossification to replace the bone, and hence the longer it will take for the fractured cortices to unite. It follows that although under unstable mechan-

ical conditions cartilage is normally present in the callus, immobilization should be designed to minimize, rather than promote, callus formation with large amount of cartilage. In the course of examining many calluses, it was noticed that the fractures with earlier cortical union tended to be those with a thinner callus. Those with exuberant callus and a lot of cartilage took longer to unite. It is also noteworthy that as soon as endochondral ossification is complete and the gap is invaded by cells, resorption of the callus starts and it rapidly becomes thinner.

If cartilage is a temporary tissue that has to be removed for healing to proceed, what is its function and why is it produced during fracture repair?

A. The Cellular Environment and Cartilage Formation

Cartilage develops where there is a sparse blood supply, whereas bone is formed only where the blood supply is good. In a callus developing under unstable mechanical conditions, the region over the gap is subject to mechanical stress and micromovement that will contribute to the inhibition of angiogenesis. Two- and three-dimensional finite element models of callus predict regions of high compressive dilatational and high intermittent stress (Carter et al., 1988; Blenman et al., 1989) that correspond to the regions in which cartilage is formed and that they suggest contribute to the paucity of blood vessels. Under stable mechanical conditions such stresses are not present. The blood supply is good throughout the callus and cartilage is rarely formed. Cartilage in defects develops only when the growing regions of bone are close leaving a region of high mechanical strain between them. Similarly, during distraction osteogenesis, cartilage is found at the junction between bone and fibrous tissue at the center of the regenerating tissue; this is again an area of high strain. In both these examples the regions of cartilage lack blood vessels.

It has been argued recently (Hulth et al., 1990) that this hypothesis is not valid because blood vessels, or rather their basement membranes, were detected in callus cartilage with antibodies to heparan sulphate and laminin. Fracture callus is, however, a very variable tissue and the local environment changes over short distances. In some calluses, large areas are purely cartilage, but in others, the cartilage is permeated by regions of bone with blood vessels, or less frequently, large sinusoid-like vessels may be present (Ashhurst, 1986; Bland et al., 1999).

A similar correlation between cartilage formation and blood supply is found in developing limb buds and also in ectopically formed bone (Caplan and Koutroupas, 1973; Wilson, 1986; Hallman et al., 1987; Critchlow et al., 1994). In addition, *in vitro* chondrocytic differentiation is favoured by reduced oxygen tension (Bassett and Herrman, 1961; Pawelek, 1969) and chondrocytes are sensitive to oxygen-derived substances in culture medium (Tschan et al., 1990).

Cartilage, or rather a chondroid-type bone, is, therefore, produced in regions of the developing callus in which bone cannot form. Its mechanical strength, though low, is greater than that of the fibrous tissue it replaces. It provides suf-

ficient stability to reduce movement between the fracture fragments and allows both endochondral ossification to start along the junction with bone and then bone to grow over the surface. It is, therefore, a very transient tissue that is rapidly replaced by bone. Similar rapid replacement is observed at other sites in which there is induced formation of bone and cartilage (Urist, 1965; Reddi 1981; Critchlow et al., 1994).

B. Endochondral Fracture Repair

The term *endochondral fracture repair* is frequently used and it implies that cartilage is essential to bone healing and that it is formed before bone. This argument presupposes that callus formation equals healing; it does not. Callus formation is merely a mechanism for immobilizing the fracture fragments to allow cortical union. The fractured bone is healed when the cortical bone is reunited. Consequent to the comparison with endochondral ossification, healing has been compared with embryonic limb development (Hiltunen et al., 1994). There are, however, fundamental differences. First, in a callus the cartilaginous collagens are laid down in a pre-existing fibrous matrix containing types I, III and V collagens, whereas only a very little sparse matrix is found in an embryonic limb before chondrogenesis. Second, many of the callus chondrocytes co-express types I and II collagen mRNAs for a short time, but there is no type I collagen in the limb anlagen. The diverse collagens in callus cartilage are visible in electron micrographs (Ashhurst, 1986).

IX. CONCLUDING COMMENTS

That the mechanical conditions at the fracture site have a profound effect on cell differentiation and the composition of the callus is indisputable. The mechanical conditions also affect the size of the callus, that is, the amount of bone and cartilage formed. It was argued that a large callus is not necessarily advantageous because it not only takes longer to develop, but ossification of the cartilage takes longer so that cortical union is delayed. Only the minimal amount of bone necessary to stabilize the fracture fragments is required. After cortical union, the callus is redundant and rapidly resorbed.

This is illustrated by experiments in which exogenous growth factors were applied to fracture sites. Several growth factors, including bone morphogenetic proteins -2 to -7 (BMPs), fibroblast growth factors 1 and 2 (FGF-1 and -2), osteogenic growth peptide (OGP), and transforming growth factors- β 1 and - β 2 are known to stimulate bone and cartilage formation *in vitro* or *in vivo* (Globus et al., 1988; Gazit et al., 1989; McCarthy et al., 1989; Joyce et al., 1990; Mackie and Trechsel, 1990; Wozney et al., 1990; Bab et al., 1992; Critchlow et al., 1994). It was thought that they might accelerate fracture healing and increase the strength

of the callus by increasing the rate and amount of bone and cartilage formation. The results were not dramatic when growth factors were applied to normally healing fractures. In contrast, growth factors, or bone marrow implants, are essential when a defect is too large to heal spontaneously (e.g. Yasko et al., 1992; Gerhart et al., 1993).

To increase the rate of healing, the growth factor should increase the rate of callus formation in the first few days after fracture and so bring forward the time of cortical union. TGF- β s inhibit callus formation in rabbit tibial and rat femoral fractures (Terek et al., 1989; Critchlow et al., 1995b). FGF-1 stimulates cartilage, but not bone formation, in these fractures (Jingushi et al., 1990; Bland et al., 1995). FGF-2, in contrast, stimulated healing so that at 10 days the callus was more mature, but not significantly larger than control calluses (Bland et al., 1995). Kawaguchi et al. (1994) found small increases in the size of rat fibular fractures. Experiments using BMP-2 and OGP have illustrated that these growth factors do not cause an increase in the amount of bone or cartilage produced during healing, but that the rate of callus formation is increased, which leads to earlier cortical union (Sun and Ashhurst, 1998; Bax et al., 1999). Thus, these experiments demonstrate that other factors, in this instance mechanical, modulate the effects of growth factors during callus formation. In some experiments, growth factors have been given over a period of many weeks, that is, into the remodeling period. At that time the amount of bone associated with the fracture region is decreasing, and hence a stimulus to increase bone formation could retard remodeling and the restoration of full mechanical strength. Indeed, remodeling in rat tibial fractures was retarded by prolonged treatment with growth hormone (Mosekilde and Bak, 1993). The acceleration of bone healing remains an area of great interest and it was claimed recently that two Chinese herbal mixtures accelerate healing in rats by raising RNA and protein synthesis (Huang and You, 1997).

A healing fracture provides an insight into the interplay between environmental, mechanical and chemical factors in the control of cell differentiation. It also illustrates that differentiation is not always along well-defined pathways and that when tissues are produced in response to injury, pathways not encountered during normal development may be followed. Healing of skeletal tissues is very important for the general well-being of an animal, but many injuries lead to reduced mobility. It is perhaps perverse that the most dramatic injury, the fracture of a bone, will heal perfectly if treated properly. Injury to cartilage is permanent; there is no regenerative mechanism. Similarly, injuries to the soft tissues, ligaments, and tendons seldom heal with full mechanical recovery because the scar tissue does not have the same structure. The constant remodeling of bone is the key to its ability to achieve full regeneration with full mechanical strength. In this it is unique.

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POLYAMINE-TRAVELED PATHWAYS

SIGNIFICANCE IN HEALTH AND DISEASE

Ulka R. Tipnis

I.	Introduction	118
II.	Structure and Metabolism of Polyamines	118
III.	Inhibitors of Polyamine Synthesis and Degradation	121
IV.	Polyamines and the Cell Cycle	125
V.	Polyamines and Hormone Secretion	126
VI.	Significance of Polyamines in Necrotic and Apoptotic Death	127
VII.	Polyamines and Cancer	131
VIII.	Significance of Polyamines in Immune-Mediated Disorders	135
IX.	Polyamines and Hypertension	138
X.	Polyamines and African Trypanosomiasis	140
XI.	Summary and Conclusions	142
	References	143

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I. INTRODUCTION

Since Antoine van Leeuwenhoek's description of the crystalline material in human semen, polyamines have been source of numerous scientific discoveries that have enhanced our understanding of human health and disease. A glimpse at an historical account published recently (Cohen, 1998), reflects the efforts of innumerable scientists who have contributed to polyamine research, which began two centuries ago with the preparation of quaternary amines, diamines, and triamines (Hoffman, 1851–1863, cited in Cohen, 1998). Other early research included the identification of spermine phosphate as a source of foul odor in prostatic secretion (Schreiner, 1878, cited in Cohen, 1998), and the discovery that prostatic secretion was enriched in an organic base identified as spermine (Fuerbringer, 1881, cited in Cohen, 1998). Since these discoveries in the last century (reviewed in Cohen, 1998), the synthetic and degradative pathways of polyamines have been detailed; their significance in growth processes have been highlighted, and their roles in various disease processes have been emphasized. The synthesis of specific inhibitors of polyamine metabolism and polyamine analogs has confirmed the physiological importance of polyamines in cell growth and differentiation and has opened the possibility of their use in development of drugs to treat hyperproliferative diseases and parasitic infections.

II. STRUCTURE AND METABOLISM OF POLYAMINES

All eukaryotic cells contain polyamines that are small aliphatic cations (Figure 1). At physiological pH, polyamines exist in the protonated form. They are flexible molecules with positive charges distributed along the length of the aliphatic chain. This feature of polyamine structure enables them to interact more efficiently than inorganic cations with negatively charged DNA, RNA, and protein molecules (Pegg and McCann, 1982).

The metabolism of polyamines has been described in many reviews (Pegg and McCann, 1982; Tabor and Tabor, 1984; Pegg, 1988; Janne et al. 1991). Thus, only a brief account of this topic is described herein. The intracellular concentration of polyamines is tightly regulated by their synthesis, degradation, and uptake/release mechanisms. Polyamines are synthesized by all eukaryotic cells except erythrocytes, which accumulate polyamines by an uptake mechanism. As shown in Figure 2, the synthesis of polyamines begins with the decarboxylation of ornithine by ornithine decarboxylase (ODC). This decarboxylation leads to the formation of putrescine. A reaction of adenosine triphosphate (ATP) with methionine forms S-adenosylmethionine that undergoes decarboxylation by s-adenosylmethionine decarboxylase (AdoMetdc) to form decarboxy-adenosylmethionine. In subsequent sequential reactions, a transfer of aminopropyl residues from decarboxylated adenosylmethionine catalyzed by spermidine synthase and spermine

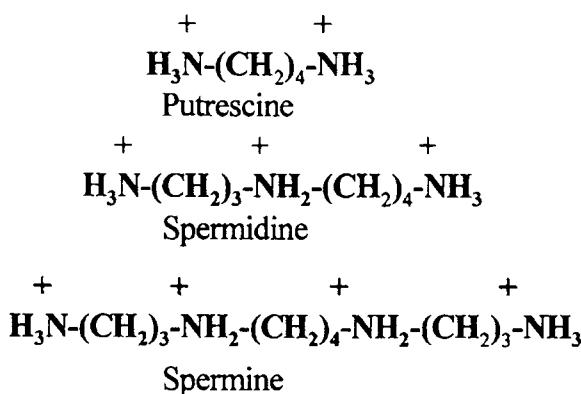


Figure 1. The three major mammalian polyamines in eukaryotes.

synthase results in the formation of spermidine and spermine (Pegg and McCann, 1982).

As shown in Figure 2, spermidine and spermine are degraded *in vivo* by spermidine/spermine N1acetyltransferase (N1SAT) and polyamine oxidase (PAO). N1SAT uses acetyl coenzyme (CoA) to acetylate spermidine or spermine. The acetylated derivatives of spermidine and spermine become the substrates for PAO, which splits them into precursors of spermidine (i.e., putrescine) or spermine (i.e., spermidine), aminopropanal, and hydrogen peroxide. This type of polyamine oxidation pathway, also referred to as a retroconversion pathway, generates putrescine, and spermidine. These polyamines can be reutilized and reconverted into one another either by elimination or addition of the aminopropanal moiety (Seiler et al., 1981).

Putrescine can be oxidized by diamine oxidae yielding γ -aminobutyraldehyde, which can be further oxidized to γ -aminobutyrate. In the terminal oxidation of polyamines, copper-dependent amine oxidases oxidize the terminal amino groups to aldehydes, which are subsequently converted to amino acid and γ -lactam. Polyamines oxidized by this pathway are irreversibly eliminated from the polyamine cycle (Scalabrino and Ferioli, 1982; Pegg and McCann, 1982).

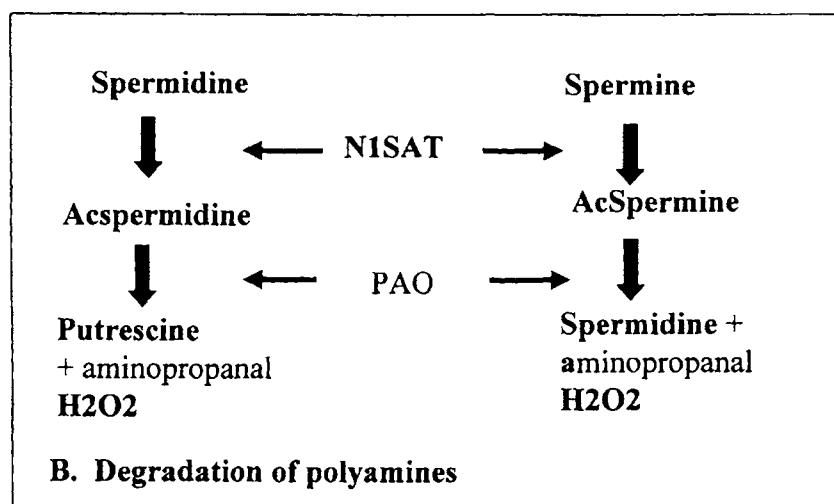
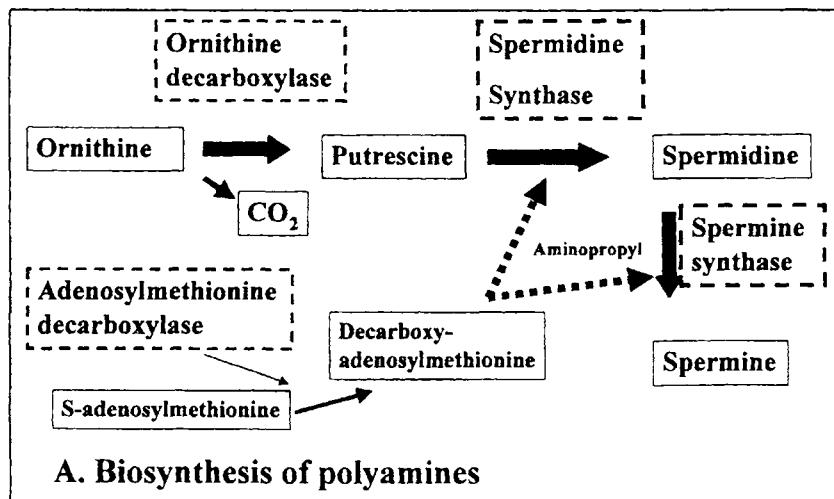


Figure 2. Synthesis and degradation of polyamines showing various enzymes of these pathways. Spermidine/spermine acetyltransferase (N1SAT), polyamine oxidase (PAO), acspermidine (acetylated spermidine), acspermine (acetylated spermine).

ODC, AdoMetdc, and N1SAT are the key enzymes that regulate the metabolism of polyamines. ODC is unique among these three enzymes. In resting tissues, ODC activity is very low. Many hormonal or growth stimuli induce ODC activity, which increases by several fold, peaking at 4 to 6 hours and declining to a basal

level by 12 hours. Even after stimulation, ODC activity remains a small fraction of the total protein. ODC in androgen-stimulated mouse kidney (Seely et al., 1982) and in thioactamide-stimulated liver (Pegg and McCann, 1982) represents approximately 0.01% and 0.0001% of the total protein. Among mammalian enzymes, ODC has the shortest half-life of 15 to 30 min (Russell and Snyder, 1969). It belongs to the family of proteins with short half-lives of less than 30 min that are characterized by regions enriched in proline, glutamic acid, serine, and threonine (Rogers et al., 1986). AdoMetdc is also an inducible enzyme, but its inducibility is not as dramatic as that of ODC. The half-life AdoMetdc spans between 30 and 60 min (Janne et al., 1978). This enzyme is also present in small amounts in tissues. For example, in ventral prostate and liver, the amount of AdoMetdc represents 0.015% to 0.0007% of the soluble proteins. N1SAT activity is rapidly induced after an exposure to hepatotoxins (Pegg and Mccann, 1982) and many other pharmacological and pathological stimuli (Seiler, 1987).

Most cells possess the ability to take up polyamines from blood plasma and to release them from the intracellular pool. The transport system for polyamines is carrier-mediated, energy dependent and saturable (Seiler et al. 1996; Seiler & Dezeure, 1990). The synthesis, degradation, and uptake tightly regulate the intracellular homeostasis of polyamines. Thus, when intracellular polyamines increase, ODC and AdoMetdc activities are repressed and synthesis of polyamines declines; N1SAT activity is increased and degradation of polyamines is enhanced (Pegg, 1988; Casero and Pegg, 1993), and the uptake of polyamines decreased (Pegg, 1988). Loss of this homeostatic control can lead to excessive accumulation of polyamines. An inhibition of ODC or AdoMetdc activities increases the uptake of polyamines. Cells that are genetically defective in the biosynthesis of polyamines (Pilz et al., 1990) or in which biosynthesis is inhibited by drugs (Kramer et al., 1989) maintain homeostasis of intracellular polyamines via increased uptake of polyamines. An abnormal rise in cellular polyamines can cause cytotoxicity or programmed cell death by a polyamine oxidation dependent or polyamine oxidation independent pathway.

III. INHIBITORS OF POLYAMINE SYNTHESIS AND DEGRADATION

Since polyamines have been implicated in cell growth, differentiation, and neoplastic transformation, the synthetic and degradative pathways of polyamines have been targeted for the development of specific inhibitors and polyamine analogs. Using these agents, researchers have been able to manipulate cellular levels of polyamines and define their role in cellular functions. More importantly, research has also focused on examining the potential use of these inhibitors and analogs as clinical tools in the treatment of malignant diseases and parasitic infections. Various inhibitors of these pathways and polyamine uptake

Table 1. Compounds That Inactivate Specific Enzymes in the Synthesis or Degradation of Polyamines and Intervene with the Metabolism of Polyamines

Compounds	Enzyme Inhibited	Reference
DFMO	ODC	Metcalf et al., 1978
α -Methylornithine	ODC	Mamont et al., 1976
MGBG	AdoMetdc	Corti et al., 1974
CCP 35753	AdoMetdc	Renegass et al., 1992
CCP 48664	AdoMetdc	Stanek et al., 1993a, 1993b
AbeAdo	AdoMetdc	Marton and Pegg, 1995
AdoMAC	AdoMetdc	Marton and Pegg, 1995
MDL 72, 527	PAO	Bey et al., 1985

Notes: DFMO- α -difluoromethylornithine; MGBG-dismethylglyoxal bis (guanylhydrazone); CCP 35753-[2,2'-bypyridine]-6,6'-dicarboximidamide; CCP 48664-(4-amidinoindan-1-one 2'amidinohydrazone); AbeADO-[5'-(Z)-4-amino-2-butenyl] methylamino]-5'-deoxyadenosine; AdoMAC-(S-(5'-adenosyl)-1-amino-4-methylthio-2-cyclopentene); MDL 72.527-N,N'-bis(2,3-butadienyl)1,4-butane diamine. Enzymes: ODC-ornithine decarboxylase; AdoMetdc-adenosylmethionine decarboxylase; N1SAT-N1spermidine/spermine acetyltransferase; PAO-polyamine oxidase.

have been reviewed before (Marton and Pegg, 1995). Some of these inhibitors described are shown in Table 1.

The specific inhibitor of ODC activity, α -difluoromethylornithine (DFMO), is recognized by ODC as a substrate that binds to the active site of ODC. The decarboxylation of DFMO converts it into an extremely reactive intermediate that forms a covalent bond with ODC, thus resulting in its irreversible inactivation (Metcalf et al., 1978; Danzin and Mamont, 1987). DFMO reduces the cellular levels of putrescine and spermidine without much effect on those of spermine. This is due to an efficient conversion of residual putrescine into spermine and slow degradation of spermine.

Other enzyme-activated inhibitors of ODC also have been prepared. These include methylester of (E)- α -monofluoromethyldehydroornithine (ρ -MFMO) and (R)- α -ethynyl-(R)- δ -methylputrescine (MAP) (Marton and Pegg, 1995). DFMO is most widely used, as it causes no toxicity except patients given DFMO have thrombocytopenia, leukopenia, and diarrhea. However, these side effects of DFMO are reversible on cessation of DFMO treatment (Janne et al., 1991; Doua and Yapo, 1993; Levin et al. 1992).

Dismethylglyoxal bis(guanylhydrazone) (MGBG), an anticancer agent, inhibits AdoMetdc activity (Corti et al., 1974) and prevents the synthesis of spermidine and spermine. Interest in the synthesis of other inhibitors of AdoMetdc developed after the discovery that MGBG was a nonselective inhibitor of AdoMetdc. MGBG inhibits diamine oxidase and inhibits the degradation of putrescine. More importantly, MGBG is very toxic, and much of its toxicity presumably results from damage to the mitochondrial structural integrity (Pegg and William-Ashman, 1987).

Table 2. Effects of SAMDC Inhibitors on L1210 Cell Polyamine Metabolism Under IC₅₀ Conditions

Treatment (48 h)	Drug Dose ^a (μM)	Enzyme Activities			Intracellular Polyamine Pools (nmol/10 ⁶ cells)		
		ODC	SAMDC	SSAT	PUT ^c	SPD	SPM
Control		18.3 ± 1	3.84 ± 0.85	7.1 ± 1.7	0.37 ± 0.1	2.58 ± 0.2	0.80 ± 0.08
MGBG	0.5	53.5 ± 6	15.0 ± 2.1		4.33 ± 0.5	1.18 ± 0.2	0.16 ± 0.05
CGP 48664	3	54.0 ± 7	0.28 ± 0.05	3.9 ± 0.7	4.95 ± 0.8	0.29 ± 0.1	0.07 ± 0.03
AMA	100	67.4 ± 9	0.47 ± 0.07	9.7 ± 2.0	5.81 ± 0.6	0.61 ± 0.1	0.09 ± 0.02

Notes: ^aCells were treated with drug concentrations that led to 50% inhibition of proliferation within 48 hours.

^bODC and SAMDC (adenosylmethionine decarboxylase) nmol/h/mg; SSAT (spermidine/spermine acetyl transferase) pmol/min/mg.

^cPUT-putrescine; SPD-spermidine; SPM-spermine; AMA-s-(5' deoxy-5' adenosyl) methylthioethylhydroxylamine.

Adapted from Renegas et al. (1994). (Reprinted with permission of American Association for Cancer Research)

Several inhibitors of AdoMetdc have subsequently been synthesized. The enzyme-activated irreversible inhibitors of AdoMetdc include AbeAdo [5'-{[(Z)-4amino-2butenyl] methylamino}-5'-deoxyadenosine and AdoMAC [S-(5'-deoxy-5'-adenosyl)-1-amino-4-methylthio-2-cyclopentene] (Marton and Pegg, 1995). Aryl and heteroaryl analogs of MGBG with selective inhibition of AdoMetdc have also been synthesized (Stanek et al., 1992, 1993a, 1993b). Such competitive and specific inhibitors of AdoMetdc include CGP 39937 [2,2-bipyridine]-6,6'-dicarboximidamide] (Renegass et al., 1992; Stanek et al., 1993a) and CGP-48664 [4-amidinoindan-1-one 2'amidinohydrazone] (Renegass et al., 1994; Stanek et al., 1993a, 1993b). These analogs did not inhibit ODC activity and inhibited diamine oxidase to a far lesser extent as compared to MGBG.

In one of the studies, the efficacy of CGP 48664 in inhibiting AdoMetdc was compared with that of the parent compound, MGBG, and the nucleoside analog inhibitor of AdoMetdc, s-(5' deoxy-5'adenosyl)methylthioethylhydroxylamine (AMA). Table 2 shows the effects of these inhibitors on enzyme activities and polyamine levels in L1210 mouse leukemic cell lines. The dosages indicated in Table 2 caused 50% inhibition of L1210 proliferation. The efficacy of AdoMetdc inhibition was in the order of CGP 48664 > MGBG > AMA. CGP48664 caused 90% inhibition of AdoMetdc activity and caused 90% depletion of spermidine and spermine (Renegass et al., 1994). The antiproliferative activity of MGBG and CGP 48664 was examined in various cell lines (Table 3). Except for the L1210 cell line, the IC₅₀ (μ M) values for CGP 48664 inhibition of cell growth ranged from 0.3 to 3 μ M.

Table 3. Growth Inhibition of Tumor Cell Lines

Cell Line	IC ₅₀ ^a		
	MGBC	CGP 48664	CGP 39937
L1210 mouse leukemia	0.5	3.0	10.0
T-24 human bladder carcinoma	1.1 \pm 0.3	0.6 \pm 0.3	1.4 \pm 1.2
RPMI 7951 human melanoma (melanotic)	0.09	0.28	0.42
Hs 294T human melanoma (amelanotic)	1.8	0.26	0.33
Hs 695T human melanoma (amelanotic)	3.6	0.58	0.46
SK MEL-24 human melanoma	ND ^b	08	ND
HT-144 human melanoma (amelanotic)	6.6	0.26	1.66
KB 31 human epidermoid carcinoma	4.3	0.6	ND
KB 8511 human epidermoid carcinoma MDR+	2.6	0.6	ND

Notes: ^aIC₅₀ drug concentrations leading to 50% of control cell proliferation. Values represent means of control cell proliferation. Values represent means of three or more independent determinations. Growth was 2 days for L1210 and 5 days for all other lines except for the slowly growing SK-MEL-24, which were grown for 10 days.

^bND-not determined.

Adapted from Renegass et al., (1994). (Reprinted with permission of AACR.)

The potent enzyme-activated irreversible inhibitors of flavin–adenine dinucleotide (FAD)-dependent intracellular PAO include N-methyl-N’-(2,3-butadienyl)-1,4-butanediamine (MDL 72521) and N,N’-bis(2,3-butadienyl)1,4-butane diamine (MDL 72527) (Bey et al., 1985). In addition, inhibitors of other amine oxidases (Seiler, 1987) have also been used for elucidating the role of extracellular or intracellular polyamine oxidation in cellular function (Tipnis and He, 1998). Aminoguanidine and semicarbazide are the examples of inhibitors of copper-dependent amine oxidases. These inhibitors attack copper-chelating and carbonyl agents. Aminoguanidine (AG) is a nonselective inhibitor of most amine oxidases (Zeller, 1963), whereas semicarbazide is a specific inhibitor of semicarbazide (SMCB) amine oxidase (Lewinsohn et al., 1984).

IV. POLYAMINES AND THE CELL CYCLE

The cell cycle is divided into S, G₁, M, and G₂ phases. The replication of DNA takes place in the S phase; G₁ is the period between completion of mitosis and the beginning of DNA synthesis; and the G₂ phase is the interval between the end of DNA synthesis and the beginning of mitosis. Studies by analysis of chromatin in synchronized vertebrate cells indicate that the bulk chromatin undergoes structural modifications during the cell cycle. The changes in bulk chromatin occur primarily during DNA replication phase (Moreno et al. 1986; Laitinen et al. 1990; Laitenan and Holtta, 1994).

Many studies have provided evidence documenting the importance of polyamines in normal cell cycle progression, although the exact role of polyamines remains elusive. The activity of ODC peaks in the G₀G₁, G₁/S, and G₂/M phases (Heby et al., 1976, 1982; Laitinen and Holtta, 1994) and leads to increases in the levels of putrescine before the replication of DNA (Heby, 1981). Spermidine and spermine accumulate in S₁, G₀ and mitotic stages (Fredulund et al., 1995). Polyamines bind strongly to DNA and cause alterations in confirmation of DNA. *In vitro* studies indicate that polyamines induce condensation of chromatin prepared from nuclei. They also enhance the stability of isolated core nucleosomes indicating their involvement in structural alterations of chromatin during cell cycle (Makarov et al., 1987; Sunkara et al. 1983; Laitinen et al. 1998). However, the physiological relevance of these findings at a chromatin level is not supported by experiments in Chinese hamster ovary cells deficient in ODC activity (CHO^{odc-}). Polyamine starvation produced by using serum-free medium did not affect nucleosomal organization of chromatin in G¹/S phase of CHO^{odc-} cells synchronized in the G⁰/G¹ phase by serum starvation or of those synchronized in the S phase by hydroxyurea. Polyamine deprivation of these cells by serum starvation, however, arrested 70% of CHO^{odc+} cells in the G₁/S phase and retarded the traverse of those CHO^{odc-} cells, which were already in the first cycle. Supplementing media with polyamines enhanced the progression of the cell cycle in CHO^{odc-}

cells (Laitenen et al., 1998). Other studies, by achieving depletion of polyamines by using inhibitors, also have supported the physiological relevance of polyamines in cell cycle progression. In one of these studies, depletion of polyamines in CHO cells by CGP 48664 was reported to retard the cell cycle through the S-phase (Fredulund and Oredsson, 1997).

A vast amount of information extracted from *in vitro* and *in vivo* investigations has shown that increases in ODC and polyamines are absolutely essential for regulation of cell growth and differentiation. During chick embryogenesis, ODC and AdoMetdc activities increase, leading to increases in polyamines that correlate with proteins and RNA. The biosynthesis of polyamines is enhanced during development and regeneration. One of the changes noted in tissue regeneration or compensatory growth of the organs is the early dramatic stimulation of ODC activity. Increases in the biosynthesis of polyamines are important for many growth processes. Some examples include liver regeneration after partial hepatectomy, compensatory growth after unilateral nephrectomy (Janne et al., 1978), hyperplastic intestinal growth induced by phytohemagglutinin (Sessa et al., 1995) and cardiac hypertrophy induced by isoproterenol (Tipnis et al., 1995). Transcriptional induction of ODC and polyamines have been noted in small intestinal epithelial cells that were stimulated to proliferate by gastrin (Wang et al., 1995). Polyamines regulate transforming growth factor (TGF- β) expression in association with the migration of epithelial cells upon wounding (Wang et al., 1997).

V. POLYAMINES AND HORMONE SECRETION

The studies on localization of ODC and polyamines have been performed to gain an insight of their physiological function. Polyamines have been localized by a cytochemical approach using formaldehyde-fluorescamine and the ortho-phtalaldehyde fluorescence reagents. These studies have shown that the secretory granules of many endocrine and exocrine cells contain polyamines (Hougaard and Larson, 1986). Existing evidence indicates polyamines play a role in protein secretion. For example, in cultured pancreatic islets glucose-induced increases in insulin mRNA are associated with increased polyamine contents. A decrease in polyamine content by exposing the fetal pancreatic islets, to DFMO and ethylglyoxal bis(guanylhydrazone) (EGBG), an AdoMetdc inhibitor, prevents glucose-induced increases in insulin mRNA (Welsh, 1990) and impairs the release of insulin into the media (Sjoholm et al., 1990).

In adult mammalian heart, atria are endocrine glands that contain secretory granules enriched in atrial natriuretic factor (ANF) of 126 amino acids. Upon increases in atrial stretch, ANF is released into the circulation, where it is cleaved into the 14-kDa amino terminus and the 3-kDa carboxy terminus portions. The three-kDa segment binds to the receptors in kidneys, adrenal glands, and smooth

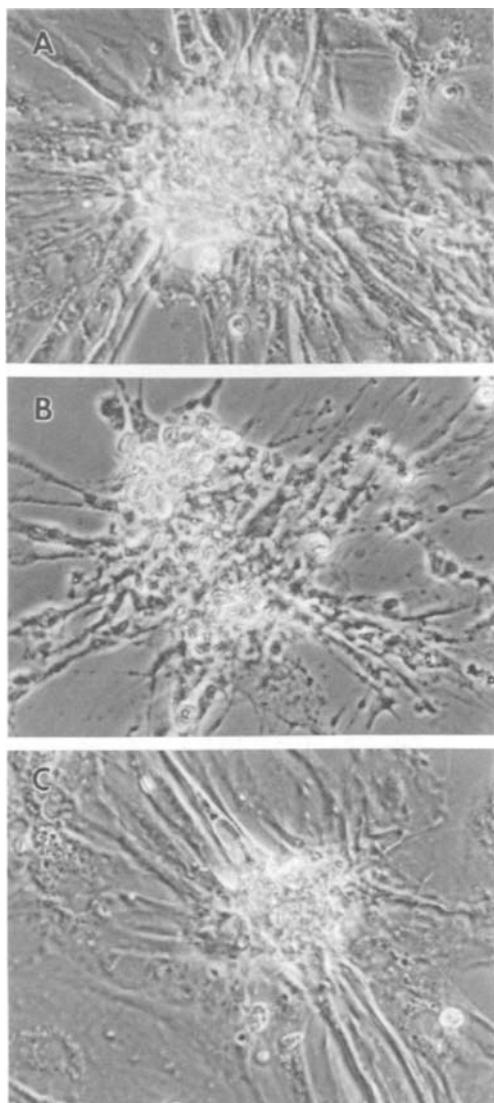
muscles, leading to the induction of natriuresis and diuresis, relaxation of smooth muscles, and an inhibition of renin and aldosterone secretion (reviewed in Ruskoaho, 1992). In the renin-angiotensin II axis, these actions of ANF are believed to have a role in regulation of blood pressure.

To date, it is not known if secretory granules of cardiac atria are enriched with polyamines. However, immunocytochemical studies have shown that in rat cardiac atria, ODC is predominantly present in secretory granules (Tipnis et al., 1989a). These findings were supported by studies showing that DFMO-induced depletion of putrescine and spermidine in rat hearts attenuates the arginine-vaso-pressin-induced secretion of ANF from rat hearts (Tipnis and Boor, 1992). Similarly, *in vitro* studies have shown that DFMO-depletion of polyamines attenuate the constitutive secretion of ANF from cultured rat ventricular cardiocytes (Tipnis et al., 1994). To date, the precise mechanisms by which polyamines may regulate ANF secretion are not known. However, the association of ODC with secretory granules (Tipnis et al., 1989a) has raised the possibility that in atrial myocytes, granule-associated polyamines may be important in mobilization of granules or in their fusion with the membrane. Other studies have also supported the role for polyamines in hormonal secretion.

VI. SIGNIFICANCE OF POLYAMINES IN NECROTIC AND APOPTOTIC DEATH

The cellular death occurs by two pathways: necrotic and apoptotic (Kerr et al., 1972). Polyamines by themselves are not cytotoxic nor do they inhibit cell growth. However, they are potential substrates for intracellular FAD-dependent polyamine oxidases and extracellular copper-containing amine oxidases. Thus, the products produced from their oxidation by amine oxidases have potential cytotoxic effects. Studies have documented the significance of polyamines in necrotic and apoptotic cell death. Cellular necrosis occurs in the face of ischemic or toxic insult. This type of cell death is characterized by cellular swelling, rupture of the cell membrane, and leakage of the cytoplasmic contents.

Ischemic injury to the brain produces many metabolic changes that lead to cerebral edema, infarction, and the breakdown of the blood-brain barrier (Cotran et al. 1994). Current evidence suggests that after cerebral ischemia, ODC activity and polyamine synthesis are enhanced (Dempsey et al., 1988a), predominantly in the areas of infarction (Dempsey et al., 1988b). DFMO-inhibition of ODC activity and prevention of polyamine synthesis has been shown to block the development of edema and the breakdown of blood-brain barrier following ischemic injury (Schmitz et al., 1993). In another study, spermine injected directly into the cerebral hemisphere of mice caused neurotoxicity evidenced by an abnormal gait. Administration of aminoguanidine or MDL 72.527 protected the mice from spermine-induced neurotoxicity (Doyle and Shaw, 1994) suggesting the physiological importance of polyamine oxidation in spermine mediated neurotoxicity.



Note: From Tipnis and He (1998). (Reprinted with permission from Elsevier Science.)

Figure 3. The protective effect of MDL 72,527 on the myocyte structural integrity disrupted by spermidine. The myocytes cultured in a medium containing fetal calf serum were pretreated with MDL 72,527 before exposure to spermidine (50 μM). The unstained cultures of myocytes were examined using a Nikon inverted-phase contrast microscope. (A) Control. (B) Spermidine. (C) MDL 72,527 + spermidine. Magnification X550.

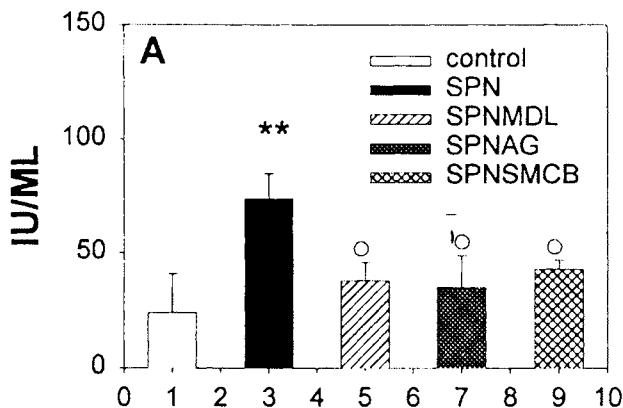
Studies in myocytes cultured from neonatal rat hearts have shown that of the three polyamines, spermidine and spermine caused toxicity to the myocytes. The polyamine mediation of cell injury to myocytes was evidenced by increased release of creatine phosphokinase into media, decreased cell viability, and decline in functional activity of myocytes. Figure 3 shows the effects of spermine on myocytes cultured in a medium containing fetal bovine serum (FBS). The healthy myocytes are large with several long processes that establish contact with neighboring myocytes. The myocytes exposed to spermidine had stringy cell processes and many cells were rounded. The cells exposed to MDL 72,527 and spermidine were healthy with no aberrations in morphology, indicating that inhibition of polyamine oxidation protected myocytes against spermidine toxicity.

Because FBS contains other amine oxidases, horse serum was used in lieu of FBS to elucidate the role of extracellular or intracellular amine oxidases in spermine-mediation of myocyte injury. Horse serum has abundant semicarbazide-sensitive amine oxidase (SSAO) activity and minimal polyamine oxidase activity. Figure 4 shows that aminoguanidine and semicarbazide or MDL 72,527 attenuated the spermine induced increase in media creatine phosphokinase and increased the viability of myocytes exposed to spermine.

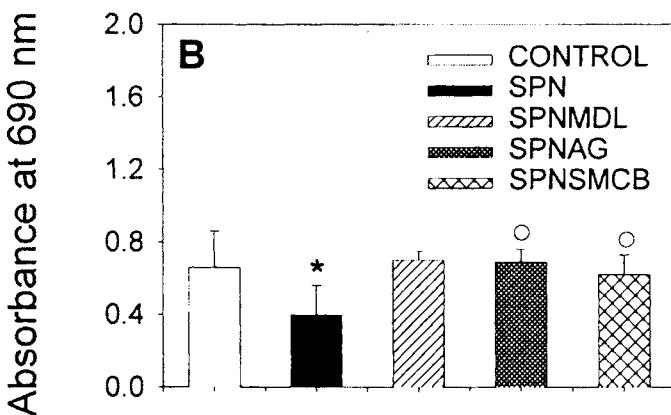
Other investigations in hyperthermia-induced cell toxicity in CHO cells (Harari et al., 1989) have also suggested that polyamine oxidation either by extracellular or intracellular oxidation has a role in the modulation of cell viability. Given these *in vitro* and *in vivo* findings, it is likely that abnormal increases in polyamines or their increased oxidation by intracellular or extracellular amine oxidases have potential of producing adverse effects on the functional and structural integrity of cells and tissues.

Apoptosis is a normal physiological process of cell death characterized by intranucleosomal cleavage of DNA, condensation of the nucleus, and cytoplasm and loss of plasma membrane attachment. Gramzinski and colleagues (1990) provided evidence that polyamine oxidation plays a role in programmed cell death during embryogenesis. Blastocoele fluid was shown to contain hydrogen peroxide that was generated by the oxidation of polyamines. In another study, ODC-deficient CHO cells that have normal feedback control of polyamines were transfected with mouse ODC. A slight increase in ODC activity that occurred in transfected cells had no effect on CHO viability or growth. These cells accumulated high levels of putrescine when polyamine transport was blocked with cycloheximide. An accumulation of putrescine in these cells triggered apoptosis, thereby implicating dysregulated polyamine import as a critical player in apoptotic cell death (Xie et al., 1997). The role of polyamines in apoptotic cell death has also been examined in a variant of L1210 mouse leukemia cells (D-R) selected for overamplification of ODC. Hypoosmotic shock impairs the feedback control of polyamine transport in D-R cells leading to progressive increase in cellular content of spermidine. Overaccumulation of polyamines, either natural or synthetic, in these cells led to chromatin condensation and fragmentation. A polyamine analog (1-methylspermidine)

CPK ACTIVITY



Cell viability



Notes: ** $P < 0.05$ represent the difference as compared with control.

* $P < 0.05$ represents the difference between spermine and spermine + indicated inhibitor. From Tipnis and He (1998). (Reprinted with permission from Elsevier Science.)

Figure 4. The myocytes were initially cultured in DMEM/F12 with 10% FCS. After 2 days in culture, medium was changed to HS (10%) for 24 hours. The cells were exposed to indicated inhibitors (50 μ M) following which creatine phosphokinase (CPK) activity and cell viability were determined. The data represent the mean \pm SD from three independent experiments. SPN, spermine; AG, aminoguanidine; SMCB, semicarbazide.

resistant to oxidation by FAD or copper dependent amine oxidases also efficiently killed the hypoosmotically stressed D-R cells. However, MDL 72,527 did not protect D-R cells from spermidine-induced apoptotic death. These findings suggest involvement of polyamine oxidation-independent pathway in the apoptotic death of D-R cells (Poulin et al., 1995).

Apoptosis is a common occurrence in hematopoiesis in which proliferation and differentiation processes are regulated by several peptide growth factors produced by fibroblasts, monocytes, and lymphocytes. Interleukin-3 (IL-3) is one of the growth factors that has multipotent actions. It enhances the survival rate of hematopoietic cells by suppressing the apoptotic death during hematopoiesis (Williams et al., 1990). The role of ODC in c-Myc-induced apoptosis was examined in murine interleukin-3-dependent clone 32D.3 myeloid cells. It was observed that a withdrawal of interleukin-3 (IL-3) decreases the expression of c-Myc (transcription factor) and ODC and arrests cells in the G₁ phase. An enforced constitutive expression of *c-Myc* induces constitutive expression of ODC and thereby provides a stimulus for entry into the S phase. However, *c-Myc* does not play a role in cell viability that is maintained by IL-3 (Bello-Fernandez et al., 1993, Askew et al., 1991). The 32D.3 cells transfected with *c-myc* expression vector displayed augmented levels of ODC activity relative to the cells transfected with vector alone. Upon IL-3 withdrawal, ODC-transfected cells exhibited apoptotic cell death following G₁ arrest, indicating a rapid initiation of cell death. Furthermore, IL-3 withdrawal of ODC clones derived by transfecting 32D.3 cells with ODC expression vector led to cell death that correlated with the levels of ODC activity. DFMO-inhibition of ODC activity in IL-3-withdrawn 32D.3 cells produced a partial inhibition of apoptotic death of 32 D.3 cells implicating *c-Myc-ODC*-polyamine pathway in the apoptotic death of 32D.3 cells (Packham and Cleveland, 1994).

VII. POLYAMINES AND CANCER

Intense efforts have been devoted toward dissecting the role of ODC/polyamines in cancer and developing drug therapies for treatment of this disease. A malignant cell is distinguished by alterations in the most fundamental codes of cellular behavior. In multicellular organisms, collaborative behavioral efforts of cells enable organisms to sustain a healthy existence. In cancer, the mutated transformed cells proliferate continuously and compete for their own existence with neighboring cells. Normal cells in culture proliferate until they have no room for further growth. At this stage, they cease to proliferate and attach to the substratum. This property of normal cells is known as anchorage dependence. In contrast to normal cells, cancer cells lose the ability of anchorage dependence and proliferate in suspension. Cancer cells invade neighboring sites, enter the circulation, and

lodge themselves at distant sites to form secondary tumors. These characteristics of the disease make cancer a formidable and frightening disease.

The development of malignancies is governed by genetic and environmental factors. At the cellular level, the transformation of a normal cell into a malignant one involves many random events. A vast amount of knowledge obtained from studies on epidermal carcinogenesis suggests that the development of full-blown malignancy involves both initiating and promoting events. For example, a single application of a carcinogen such as benzo[a]pyrene or diaminobanz[a]anthracene induces a dormant mutagenic change that, in response to repeated exposure to certain agents, can promote cells to acquire all the characteristics that are typical of malignant cells.

Studies on carcinogenesis *in vitro* and *in vivo* strongly suggest that polyamines are involved in cell transformation and invasion. Cancer patients have elevated levels of polyamines in their physiological fluids as compared to healthy volunteers, thereby suggesting an association between changes in polyamine metabolism and tumor development (Bachrach, 1992). Earlier studies reported that cancer patients with various types of malignancies have elevated levels of polyamines in their urine. Based on this observation, it was suggested that the polyamine levels could be used for evaluating tumor size, growth characteristics, and prognosis of the disease (Durie et al., 1977). Acetylated derivatives of polyamines are normal components of human urine (Hirmatsu et al., 1995). Lee and colleagues (1998) analyzed polyamines in urine from healthy volunteers and various leukemic patients. The urinary polyamines were mainly in the monoacetylated forms. Among 11 different species of polyamines, N-acetylputrescine was most abundant in urine of healthy volunteers and cancer patients. The levels of a few other acetylated polyamines in urine of cancer patients showed a strong association with the stage of the disease. For example, a noticeable increase occurred in the levels of urinary N¹, N¹²-diacSPN in the malignant stage. In the remission stage, the levels of N¹, N¹²-diacSPN returned to normal levels and increased again during relapse. An examination of the relationship between precursor metabolite and product metabolite showed that in the disease stage, the levels of N¹-acSPD were higher than the levels of N⁸-SPD. The ratio of N¹-acSPD/N⁸-AcSPD increased in the initial disease or relapse stage and decreased in the remission stage. In contrast, the ratio of SPN/N¹ to N¹²-diacSPN ratio decreased during the initial stage of the disease, and returned to normal levels during remission. On the basis of this analysis, it has been suggested that the ratio of N¹-acSPD/N⁸-AcSPD could be used as a diagnostic marker and N¹, N¹²-diacSPN could be used for evaluating the stage of the disease (Lee et al., 1998).

The levels of ODC are consistently elevated in transformed cell lines (Scalabrino and Ferioli, 1982) and in animal tumors (Scalabrino and Ferioli, 1981). The role of ODC and polyamines in carcinogenesis has been examined using *in vivo* and *in vitro* models of carcinogenesis: 12-*O*-tetradecanoylphorbol-13-acetate induced promotion of mouse skin tumors (O'Brien, 1976); 1,2 dimethylhydra-

Table 4. Characteristics of NIH/3t3 Cells Transfected with ODC Expression Vector

Cell line	Contact Inhibition	Doubling Time (hours)	Cloning ^a Efficiency (%)	Tumors/Inoculum ^b	
				4 Weeks	20 Weeks
NLK-2	+	18.2	0.05	0/14	3/14
NLK-3	+	21.6	0.10	0/10	1/10
NODC-1	-	ND ^c	ND	6/6	6/6
NODC-2	-	14.4	53	16/16	16/16
NODC-5	-	12.0	38	16/16	16/16
NODC-6	-	ND	ND	6/6	6/6

Notes: ^aOne \times 10³ cells were plated in 0.3% agarose with a 0.9% agarose underlayer.

^bFive \times 10⁶ cells was injected subcutaneously per mouse flank. Shown are the number of tumors detected 4 and 20 weeks postinoculation.

^cND-not determined.

Adapted from Moshier et al. (1993). (Reprinted with permission from AACR.)

zine-induced mouse colon tumors (Kingsnorth et al., 1983); and 1-methyl-1-nitrosourea-induced rat mammary tumors (Thompson et al., 1984). In recent years, studies have unambiguously supported the role of ODC in cell transformation. Moshier and colleagues (1993) transfected NIH/3T3 cell lines with a construct containing human ODC complementary DNA under the transcriptional control of human β -actin promoter. The cells transfected with a construct containing human ODC had three- to sixfold increase in ODC activity and message levels relative to those transfected with control vectors (NLK1 and NLK2). The transfected cells (NODC) cycled more rapidly, and showed no anchorage dependence or contact inhibition (Table 4). At confluence, the control cells were present as monolayers, whereas ODC transfected cells showed no contact inhibition and formed foci of multiple cells layers (Figure 5). In these experiments, inoculation of transfected NIH/3T3 cells into athymic nude mice caused a rapid and efficient induction of tumors relative to inoculation of cells transfected with a vector (Moshier et al., 1993).

Another evidence to support the role of ODC in cell transformation has come from studies in NIH/3T3 cells that overexpress a translation initiation factor, eIF-4E. An overexpression of this factor in NIH/3T3 cells results in cellular transformation (Lazaris-Karatzas et al., 1990).

To understand the role of increased ODC activity in cellular transformation, Shantz and coworkers (1996) transfected eIF-4E overexpressing NIH/3T3 cells (4E-P2 cells) with a dominant-negative ODC mutant. The mutant ODC was truncated to 450 amino acids and contained lysine 69 and cysteine 360 in lieu of alanine at these active sites. The cells transfected with ODC mutants showed a 60% reduction in ODC activity and abated the transformation of 4E-P2 cells.

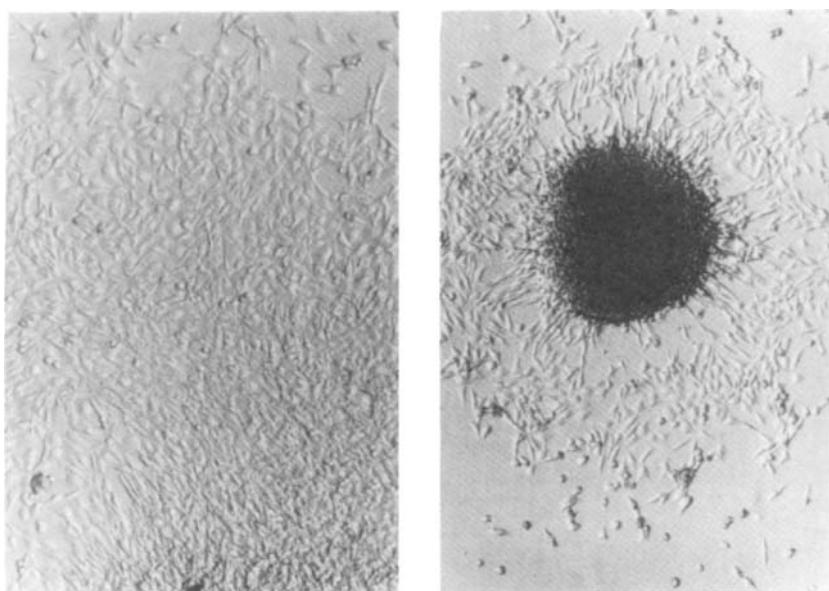


Figure 5. Loss of contact inhibition by transfected NIH/3T3 cells overexpressing ODC. Representative G418-resistant colonies derived from NLK control cells (**left**) and NODC cells overexpressing ODC were grown to confluence. Despite overcrowding the NLK cells remain a monolayer at saturation densities. NODC cells, in contrast, show low of cell-to-cell contact inhibition by forming multilayered foci. From Moshier et al., (1993). (Reprinted with permission from AACR.)

Injection of ODC mutants into nude mice reduced the tumorigenicity of 4E-P2 cells, as shown by a significant delay in the formation of tumors and smaller diameter of tumors.

Further evidence documenting the role of ODC in cell transformation has been provided by studies in epidermal tumorigenesis. In these experiments, primary cultures of mouse epidermal cells possessing an activated *ras^{Ha}* gene were infected with the ODC virus. Injection of epidermal cells infected with ODC virus into nude mice produced tumors that had abundant keratin and hyperplastic epidermis. The epidermal cells had numerous mitotic figures and exhibited dysplasia. Injection of epidermal cells containing the *ras^{Ha}* gene or those of nontumorigenic epidermal cells with ODC virus did not lead to tumor formation in nude mice. These observations suggest that ODC expression enhances the development of premalignant cells. High levels of ODC activity by itself are not sufficient for

tumor formation and ODC expression; acting in concert with other genetic defects such as activation of the *ras* oncogene, plays a role in the growth and progression of tumor (Clifford et al., 1995). In recent studies, Kubota and colleagues (1997) reported that overexpression of ODC associated with activation mitogen-activated protein kinase (MAPK) activity in fibroblasts is responsible for cell transformation and invasion. This brings forth the possibility of polyamine-modulation of MAPK signaling pathway in carcinogenesis.

Various inhibitors of the polyamine biosynthetic pathway and polyamine analogs have been investigated in relation to their antitumor activity. DFMO prevents the development of carcinogen-induced epithelial cancers of many organs (Weeks et al., 1982.; Nowels et al. 1986). In clinical trials, DFMO is being investigated as a chemopreventive agent in colon cancer (Meyskens and Gerner, 1998). CGP-48664, an adenosyl methionine decarboxylase inhibitor (Renegass et al., 1994) is being evaluated as an anticancer agent against solid tumors in phase trials. The synthesis of various polyamine analogs has further spurred interest in attacking the polyamine pathways in the treatment of cancer. These polyamine analogs suppress the synthesis of polyamines (Pendyala et al., 1993). Kramer and colleagues (1997) prepared N11-diethylspermine (DE-333, DENSPN) and a series of modified analogs of the spermine derivative N1. These analogs, which differ in their intramine carbon distances and N-alkyl substituents (methyl, ethyl, and propyl), produced effects on cell cycle progression and apoptosis in MALME-3H human melanoma cells. A prolonged incubation with these diethyl and dipropyl analogs had cytotoxic effects, whereas dimethyl analogs produced cytostatic effects. The cytotoxic analogs blocked cells in the G₀/G₁ phases. They induced apoptosis in melanoma cells at significantly higher rates than those exposed to cytostatic analogs (Kramer et al., 1997).

Besides the use of polyamine antimetabolites in cancer therapy, another approach being investigated in a limited way is through controlling the intake of dietary polyamines. Supplementation of spermine in an enteral diet deficient in spermidine favored the preneoplastic changes in the mouse intestinal tract by an administration of carcinogen, 1,2 dimethylhydrazine. In contrast, a diet deficient in polyamines reduced the contents of putrescine by 40% and decreased the number of abnormal crypt cells in the intestine of mice given carcinogen (Duranton et al., 1997). Thus dietary control of polyamine intake in combination with polyamine antimetabolites may be an effective approach to control the progression and extent of malignancy.

VIII. SIGNIFICANCE OF POLYAMINES IN IMMUNE-MEDIATED DISORDERS

White blood cells (leukocytes) develop from hematopoietic cells and play an important role in defending host against infections or toxins. They are grouped

into granular and agranular leukocytes. Granular leuokocytes develop from bone marrow and are of three types; neutrophils, basophils, and eosinophils. In response to injury or infection, granular leukocytes migrate to the site of injury where they ingest offending organism, degrade necrotic tissue, and also produce damage by releasing chemical mediators and oxygen radicals. Thus, neutrophils contain myeloperoxidase, which in the presence of halide converts H_2O_2 to HOCL, which has antibacterial action. Mast cells release vasoactive substances such as histamine and serotonin, which increase the permeability of venules. Agranular leukocytes develop from bone marrow and lymphoid tissues and include monocytes and lymphocytes. In the early phases of infection, monocytes emigrate to the site of injury and inflammation where they differentiate into macrophages. These cells are involved in phagocytosis and more efficient killing of organisms through release of toxic oxygen species and proteases. Lymphocytes are of two types, B and T lymphocytes. B lymphocytes can differentiate into plasma cells that produce antibodies. T lymphocytes differentiate into effector cells that kill other cells infected with organisms and activate macrophages or B lymphocytes (Cotran et al., 1994).

Polyamines are essential for growth and differentiation of lymphocytes (Seiler and Atanassov, 1994). Activation of ODC has been associated with mitogenic and antigenic activation of lymphocytes (Kay et al., 1973). An increase in ODC activity in T lymphocytes precedes DNA synthesis (Fidelius et al., 1984). An elevation of ODC activity and polyamine synthesis has been observed also in human lamina propria lymphocytes (LPL). Inhibition of ODC activity by DFMO and depletion of polyamines in LPL blocked DNA synthesis by 48%. This inhibition of DNA synthesis was reversed on the addition of putrescine, spermidine, or spermine thereby supporting a role for polyamines in lymphocyte proliferation (Elitsur et al., 1993). ODC activity is also essential for the proliferation and differentiation of B cells into antibody secreting cells. In lymphocytes stimulated with pokeweed mitogen, DFMO inhibited the production of IgM and IgG (immunoglobulin) production. The addition of putrescine or spermidine along with DFMO restored IgM and IgG production in pokeweed stimulated lymphocytes demonstrating polyamine-specific effect on antibody production (Pasquali et al., 1984).

In vitro experiments have shown that spermine inhibits the secretion of 5-hydroxytryptamine (5-HT) from mast cells by the mast cell secretagogue, C48/80. The inhibitory effect of spermine on 5-HT secretion was due to the aldehydes generated by oxidation of spermine by polyamine oxidase. This conclusion was derived from the observation that the effects of spermine on secretagogue-induced secretion of mast cells was blocked either by aminoguanidine, or phenylhydrazine, an aldehyde inhibitor. Furthermore, the metabolic products of polyamine oxidation, N^1 acetylspermine and N^8 acetylspermine, also showed the inhibition of mast cell secretion (Vliagoftis et al., 1992). These studies have led to the suggestion that the polyamine oxidation pathway may be targeted for therapeutic

approach in the pathogenesis of disorders involving the activation of mast cells. Likely diseases include migraine headaches (Theoharides, 1983), interstitial cystitis (Theoharides, 1990; Theoharides and Sant, 1991) or multiple sclerosis (Bayloyannis and Theoharides, 1982).

In contrast to the beneficial role of polyamines in growth and differentiation of lymphocytes, evidence in many immune-mediated disorders supports the immunosuppressive role of polyamines. Serum samples of patients suffering from immune-mediated disorders contain excessive amounts of polyamines as compared to those of healthy volunteers. These include systemic lupus erythematosus (SLE) (Puri et al., 1978), rheumatoid arthritis (Flescher et al., 1989), or acquired immunodeficiency syndrome (Colombatto et al., 1989). SLE is a disease of autoimmune origin characterized by antibodies against a variety of self-antigens. The etiology of SLE is not known, but the existence of autoantibodies in SLE patients indicates that the failure to regulate self-tolerance is the most fundamental defect of this disease. Although most organs are affected, kidneys are involved and glomerulonephritis is the major cause of death in 60 to 70% of the cases.

The role of polyamines in SLE has been examined using MRL-*lpr/lpr* mice. These mice spontaneously develop a disease that mimics human SLE. They carry the autosomal recessive *lpr* gene that is responsible for the development of anti-DNA antibodies, splenomegaly and glomerulonephritis (Theofilopoulos and Dixon, 1985). The spleens of MRL-*lpr/lpr* mice have constitutively higher levels of ODC activity and polyamines as compared to MRL+/+ or balb/c mice (Gunnia et al., 1991; Thomas et al., 1992). Excessive polyamines mediate defects in calcium mobilization as well as in the signaling pathways of splenocytes of lupus-prone mice (Thomas et al., 1993, 1995). DFMO-inhibition of ODC activity and depletion of polyamine levels decreased the production of anti-DNA antibodies against single-or double-stranded DNA and Z-DNA. Furthermore, DFMO ameliorated interstitial and perivascular inflammation in kidneys of lupus-prone mice and increased their life span. The inhibitor had no significant effect on the histopathology of the lungs, thereby suggesting a differential effect of DFMO on renal disease as opposed to pulmonary disease (Gunnia et al., 1991).

The role of polyamines in tumor-induced immune suppression was examined in the mice grafted with Lewis lung cell carcinoma. Spleen cells of grafted mice had elevated levels of polyamines associated with decreased production of interleukin-2 (IL-2) and CD⁴⁺ and CD⁸⁺ lymphocytes. Treatment of mice with polyamine-deficient diet in combination with inhibitors of polyamine synthesis (Chamaillard et al., 1997) restored the production of IL-2 and the population of lymphocytes, thereby suggesting polyamine-mediation of immune suppression in cancer.

IX. POLYAMINES AND HYPERTENSION

Hypertension is a major risk for the development of mortality and morbidity. A sustained increase in blood pressure (systolic >158 mmHg; diastolic > 80 mmHg) can lead to malignant hypertension (systolic, >200 mmHg; diastolic, > 140 mmHg). Hypertension is not a single disease entity but rather represents a consequence of a number of underlying diseases that elevate blood pressure. Although the precise etiology of hypertension is not known, heredity, smoking, stress, dietary factors, or dysregulation of peripheral/central, hormonal, or vascular components are all implicated in the development of hypertension in humans (Cotran et al., 1994). Hypertension is characterized by pathophysiological alterations in the heart and vasculature.

The heart adapts to cardiovascular overload by the development of cardiac hypertrophy and increase in wall thickness. In this adaptive response, the biochemical program of the myocytes is altered, resulting in upregulation of genes that express fetal-specific, contractile, and matrix proteins. Heart hypertrophy is associated with increased expression of atrial natriuretic factor, β -myosin heavy chain (MHC), and fibronectin (Boluyt et al. 1995) or tubulin (Tagawa et al., 1996). Although the precise significance of these changes is not known, some of these changes in long-standing hypertension are thought to contribute toward deterioration of ventricular function. Therefore, heart hypertrophy, although an adaptive process in response to work demand, even when diagnosed in the early stages of hypertension, has a negative prognostic relevance (Roman et al., 1997).

The hallmark of hypertension is an increase in the peripheral resistance of vessels associated with hyperplasia and hypertrophy of smooth muscle cells (Liu et al., 1988) as well as increased deposition of matrix components (Lipke and Couchman, 1991; Lipke et al., 1993, 1997a). These changes in response to pressure alter wall thickness to lumen ratio and decrease arterial compliance (Smith and Neutel, 1997).

The role of polyamines in hypertension has been studied in many rat models. These include genetic models of spontaneously hypertensive rats (SHR), which mimic essential hypertension in humans (Ruskoaho and Raunio, 1987) or deoxycorticosterone-induced, salt-sensitive hypertension (Soltis et al., 1991). In other rat models, hypertension is induced by aortic coarctation (Lipke et al., 1997a, 1997b), angiotensin II infusion (Ibrahim et al., 1995, 1996a, 1996b) or monocrotaline (Orlinska et al., 1988, 1989). Compared to the enormous explosion of information on the functional significance of ODC/polyamines in neoplastic transformation, relatively few studies have been devoted toward understanding the importance of polyamines in hypertension-induced cardiac hypertrophy or arterial modeling.

An elevation of polyamine contents in hearts and vessels of rats is an early and prolonged response to stimuli that induce hypertension and/or heart hypertrophy. Although DFMO has been shown to have no effect on blood pressure elevated in

coarctation model (Lipke et al., 1997a), it has been proven effective in attenuating the blood pressure in other rat models of hypertension.

DFMO-mediated depletion of putrescine and spermidine has been shown to reduce blood pressure of rats elevated by angiotensin II (Ibrahim et al., 1995) or by deoxycorticosterone salt-sensitive hypertension (Soltis et al., 1991). Monocrotaline (MCT) is a plant pyrrolizidine alkaloid that, after a single injection, causes prolonged elevation of polyamines in lungs and pulmonary arteries. This event is associated with progressive pulmonary disease, sustained pulmonary hypertension, and right ventricular hypertrophy. In this model of hypertension, DFMO produced a significant reduction in lung polyamines and attenuated pulmonary arterial pressure elevated by MCT (Olson et al., 1984). Recent findings, which show that the polyamine analog, N1N14-dethyhomospermine (DEHSPM), causes a drastic reduction in blood pressures of normotensive and hypertensive rats, strongly support the functional role of polyamines in hypertension (Bergerson et al., 1995).

Polyamine contents of the rat heart are elevated following the infusion of angiotensin II (Ibrahim et al., 1995) or after administration of isoproterenol (Tipnis et al., 1989a, 1989b, 1995). The latter is a β -adrenergic agonist that causes a marked increase in heart rate, induces cardiac hypertrophy and increases the ventricular expression of atrial natriuretic factor in rat heart (Tipnis et al., 1995). In an isoproterenol model, DFMO-inhibition of ODC activity and depletion of putrescine and spermidine decreased the expression atrial natriuretic peptide in ventricles and attenuated cardiac hypertrophy induced by isoproterenol (Tipnis et al., 1995). Polyamine regulation of other genes expressed in hypertrophied heart has not been reported. However, DFMO-mediated decrease in heart hypertrophy in coarctation (Lipke et al., 1997a) or MCT-induced hypertension (Olson et al., 1984a, 1984b) strongly support the physiological role of polyamines in adaptive growth of the rat heart.

The polyamine contents of the rat lungs and pulmonary arteries increase after monocrotaline administration (Olson et al., 1984a, 1984b). Similarly, polyamine contents of the rat thoracic aorta increase in deoxycorticosterone-salt-sensitive hypertension (Soltis et al., 1991) or coarctation-induced hypertension (Lipke et al., 1997a). In all three experimental models of hypertension, DFMO was effective in causing significant reduction of spermidine contents. The morphometric analysis of the sections of aortas revealed that DFMO caused a significant reduction in the medial thickness increased by hypertensive state. Furthermore, DFMO has been shown to reduce the synthesis of fibronectin/laminin and their deposition in aorta (Lipke et al., 1997a). These findings indicate that hypertension-induced alterations in vessel polyamine metabolism contribute to the cellular processes involved in vascular remodeling.

X. POLYAMINES AND AFRICAN TRYPANOSOMIASIS

African trypanosomiasis, referred to as sleeping sickness, is endemic in 23 African countries. Fifty to sixty million people are at risk of getting this infection. Although 30,000 new cases are reported annually, this estimate does not reflect the severity of the epidemiological problem. Many health centers are not easily accessible to rural populations, and surveillance imposes a major problem in identifying the individuals with the infection (WHO, 1995). The two species of trypanosomes, *Typanosoma brucei gambiense* and *T. brucei rhodesiense* infect humans, whereas *T. brucei brucei*, and *T. congolense* are veterinary pathogen. These protozoa are transmitted by many species of *tsetse* flies (*Glossina*, spp). A fly bites an infected human or animal and ingests trypanomastigotes, which multiply in the midgut of the fly and migrate to the salivary gland. Here they develop into metacyclic trypanomastigotes that are highly infectious. Another bite of the *tsetse* fly transfers infectious trypanomastigotes into lymph or blood vessel of the host. A nodular lesion developed at the site of the *tsetse* bite is called primary chancre (Stage Ia). Replication of trypanomastigotes leads to systemic infection. In the early stages of the disease, lymph nodes and spleens are involved as evidenced by splenomegaly and lymphadenopathy. In late stage of the disease, trypanomastigotes invade the central nervous system and cause somnolence and meningoencephalitis. The latter is characterized by cerebral ataxia causing problems in walking, tremors of the tongue and fingers, and oscillatory movement of the arms, head, neck, and trunk (Merlin et al., 1994).

The patients in the early stage of the disease are treated with trypanocidal agents such as suramin and pentamidine, and those in the late stage of the disease are given melarsoprol. Melarsoprol is a trivalent arsenical that produces serious side effects and increases the risk of mortality in patients taking this drug. Moreover, the organisms develop resistance to this drug, necessitating the alternative treatment protocols particularly in the late stage of the disease (Schechter et al., 1987).

In 1979, Bacchi and co-workers reported that *T. brucei brucei* uses ornithine as a substrate for the synthesis of polyamines, thereby opening the road for DFMO as a therapeutic agent against African trypanosomiasis. They also showed that DFMO is remarkably effective in curing mice against *T. brucei brucei* (Bacchi et al., 1980, 1982). The criteria for accepting DFMO as an effective curative agent were based on survival of mice for more than 30 days and clearance of the parasite from the circulation. In one of these studies, the mice were inoculated with *T. brucei brucei* and the effect of DFMO on survival of mice was examined. DFMO (0.5-4% in drinking water) increased the survival of mice to >28 to 30 days. Thus, DFMO proved to be as effective as the veterinary trypanocidal agents berenil or bleomycin. The advantage of DFMO was that it was nontoxic and, when given in combination with bleomycin, DFMO exerted a synergistic effect in clearing the infection from the mice (Bacchi et al., 1980, Sjoerdsma and Schechter, 1984).

These early experiments in mouse models paved the way for investigating the therapeutic value of DFMO in humans infected with *T. brucei gambiense* (Milrod et al., 1992; Khonde et al., 1997). One of the clinical trials involved 207 patients who were in the early or the late stage of the disease and had received melarsoprol therapy (Milrod et al., 1992). DFMO was given by intravenous administration and by oral route. Some patients exclusively received oral treatment of DFMO. The trypanosomes were cleared from cerebral spinal fluid in 87 out of 187 patients given DFMO by intravenous route. In the follow-up study, 9% of the patients showed a relapse. In this clinical trial, DFMO was not effective in controlling the disease in patients given solely by oral route or in children below 12 years of age. The unresponsiveness of the children to the DFMO regimen was explained by the fact that children have different pharmacokinetics that results in lower DFMO levels in CSF (Milrod et al. 1992). In another study, 47 patients infected with *T. brucei gambiense* and with relapse received intravenous DFMO (100 mg/kg) every 6 hours for 7 days. In a follow-up study, only 6.5% of the patients showed failure. In other patients, DFMO treatment proved successful in curing the disease (Khone et al., 1997).

DFMO has given excellent results in treatment against infection with *T. brucei gambiense*. However, DFMO has not proven successful in curing the infection against *T. brucei rhodesiense*, which is resistant to DFMO, pentamidine, suramin,

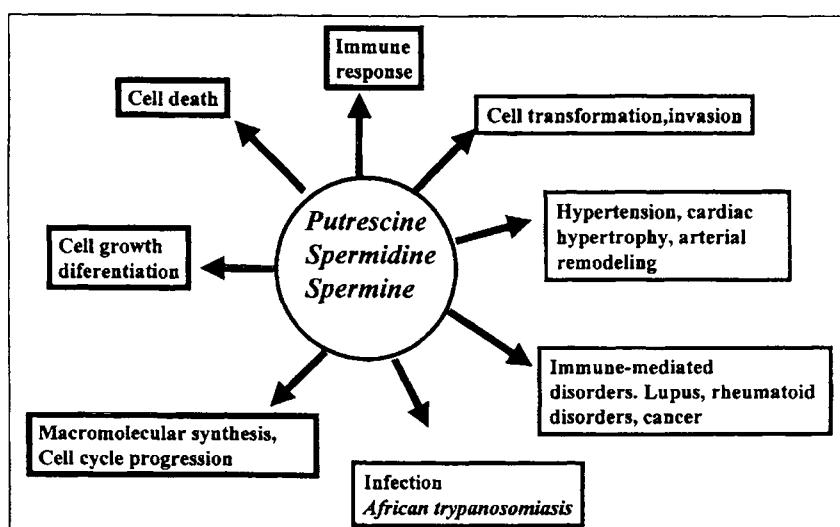


Figure 6. Schematic diagram showing the significance of polyamines in health and disease.

and standard trypanocides (Bayles et al., 1989; Bacchi et al., 1990; WHO, 1995). An examination of efficacy of various MGBG analogs (CGP 35753A, CGP 48664A, CGP35753A, and CGP40215A) *in vitro* and *in vivo* model of trypanosomiasis showed that they were active *in vitro* (Bruns et al., 1996). Although all analogs cleared the infection in mice infected with veterinary isolates of *T. brucei brucei*, CGP 40215A was most potent in clearing the infection.

CGP 40215A was examined for its potency in a mouse model infected with isolates of *T. brucei rhodesiense* (Bacchi et al., 1996) which were resistant to DFMO, pentamidine and melarsoprol (Bacchi et al., 1990). In an acute infection model, the mice were infected with trypanosomes and CGP was given 24 hours postinfection. The untreated mice died within 3 days of infection, whereas the mice treated with 25/kg of CGP 40215A for 3 days were cured. In a chronic infection model, the disease was allowed to progress for 21 days when infection of the central nervous system is apparent. After 21 days of postinfection, CGP 40215A or DFMO + CGP 40215A was given to the mice. CGP 40215A given alone at a dose of 5 to 50 mg/kg/day for 3 days was not effective in curing the infection. In this study, 60 to 80% of the mice were cured when CGP 40215A (5–50 mg/kg/day) was given either in the beginning or middle of DFMO treatment. Thus, a combination of DFMO and CGP 40215A seems to be an alternative approach for further studies on assessing the clinical utility of this compound against trypanosome species resistant to standard treatments or DFMO.

XI. SUMMARY AND CONCLUSIONS

The polyamines research that initiated with their synthesis has progressed to a stage at which their synthesis, degradative pathways have been detailed; their role in cellular growth and differentiation has been defined; and the compounds that inhibit their synthesis or compete for their sites have been synthesized. Figure 6 attempts to summarize our current understanding of the role that polyamines have in some of the diseases. Researchers have used *in vitro* and *in vivo* models to define the role of polyamines in cellular function. The specific inhibitors of polyamine synthesis have allowed researchers to examine the significance of polyamines in various diseases offering hope for using these compounds in clinical trials. One of these inhibitors, DFMO, has been used in clinical studies involving patients infected with African trypanosomiasis. The significance of polyamines in cardiovascular diseases is much less understood. However, as we learn more about the polyamine role in cardiovascular or other diseases, it may someday be possible to adapt a specific therapeutic approach for these diseases using polyamine antimetabolites.

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REACTIVE ASTROCYTES, THEIR ROLES IN CNS INJURY, AND REPAIR MECHANISMS

Jean-Luc Ridet and Alain Privat

I.	Introduction	148
II.	Morphology of Astrocytes	149
III.	Astrocytic Markers	152
	A. Intermediate Filaments and Associated Proteins	152
	B. Cytoplasmic Antigens	157
	C. Markers for Reactive Astrocytes	159
IV.	Reactive Astroglia as a Positive Component in CNS Injury	167
	A. Cytokines and Trophic Factors	167
	B. Membrane-Anchored Molecules	168
	C. Features and Roles of Activated Astroglia in CNS Damage	170
VI.	Reactive Astrocytes and CNS Repair	177
	A. Strategies to Reduce Gliosis	177
	B. Transplantation of Astroglial Cells	178
VII.	Concluding Remarks	179
	Acknowledgments	179
	References	179

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I. INTRODUCTION

Although for a long time considered as an accessory cell type in the central nervous system (CNS), astrocytes constitute one of the most important components in the biology of the brain. Many functions have been attributed to astroglia, including nutritional and structural support during CNS development, ion homeostasis, uptake of neurotransmitters, contribution to the CNS immune system, and neuromodulation. Another role for astroglia is the preservation of the host tissue integrity following injury, which is the property of activated astrocytes, called reactive astrocytes. Reactive gliosis is characterized by the hypertrophy of astrocytes as well as by the proliferation of microglial cells and astrocytes. Although reactive gliosis has long been considered to be the major impediment to axonal regrowth after an injury, the formation of a glial barrier

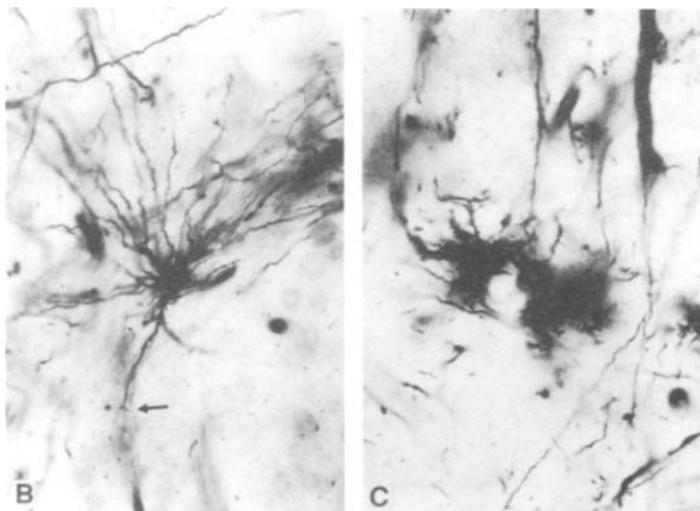


Figure 1. Metallic impregnations in the cerebral cortex gray matter, light microscopy. (B) Golgi-Rio Hortega impregnation showing two protoplasmic astrocytes exhibiting short spiny processes in the cat ($\times 470$). (C) Golgi-Cox impregnation showing a protoplasmic astrocyte exhibiting thin tortuous processes ($\times 760$). (Used with permission from Privat et al., 1995.)

around a lesion site is also a positive event, because it isolates the still intact CNS tissue from secondary lesions. Interestingly, astrocytes exhibit region-specific features, that might reflect functional differences in regional neuroglial interactions. Advances in the structural astrocyte biology might lead to a better understanding of CNS injury and repair mechanisms.

This chapter (1) reviews the current data available on the structure and the biology of reactive astrocytes, and (2) highlights recent concepts, e.g., the permissivity of reactive astrocytes for axonal regrowth, the antioxidant role of (reactive) astrocytes in neurodegenerative and traumatic damages.

II. MORPHOLOGY OF ASTROCYTES

Between 1890 and 1930, a multitude of stainings and metallic impregnations permitted the description of three main types of astrocytes according to their spatial organization: radial cells, fibrous/nonradial astrocytes, and protoplasmic astrocytes (Figures 1 and 2). Radial glial cells are disposed perpendicularly to the axis of the ventricles and span the whole thickness of the white matter in embryos. Fibrous astrocytes, which are located in white matter tracts, extend processes along the main axis of axons, they are not in contact with the pia mater but their processes end up as perivascular end-feet. Protoplasmic astrocytes, which are found in the gray matter, are characterized by short and highly ramified processes extending in all directions and ending on blood vessels and/or pia mater (Figure 2) (see references in Privat et al., 1995). All astrocytes share a number of subcellular features. The major and most characteristic organelle is the intermediate gliofilament (8–10 nm diameter). Gliofilaments are organized in bundles, which are usually more abundant in fibrous than in protoplasmic astrocytes (Figure 3B,C). Astrocyte nucleus is usually regular in shape with rare indentations, and the chromatin is condensed along the nuclear envelope (Figure 3A). The ground cytoplasm of astrocytes is less dense than that of neurons and oligodendrocytes. Astrocytes exhibit specific ultrastructural characteristics, i.e., short-sized cisternae of endoplasmic reticulum, low proportion of free ribosomes, and glycogen granules. Astrocytes exhibit two types of membrane specializations, i.e., gap junctions (Figure 3D), found between astrocytes and composed of connexin 43, and orthogonal assemblies (Figure 4B), consisting of paracrystallin arrays (see references in Privat et al., 1995), which are concentrated on perivascular and subpial end-feet.

Morphological and biochemical features of astrocytes are modified in response to a CNS insult. The so-called activation of astrocytes results in cell hypertrophy and sometimes in cell hyperplasia. A general feature of reactive astrocytes is the abundance of gliofilaments. An increase in the number of gap junctions is frequently observed in glial scar. All astrocytes do not respond in a stereotypical manner to all forms of damage. For instance, after brain trauma,

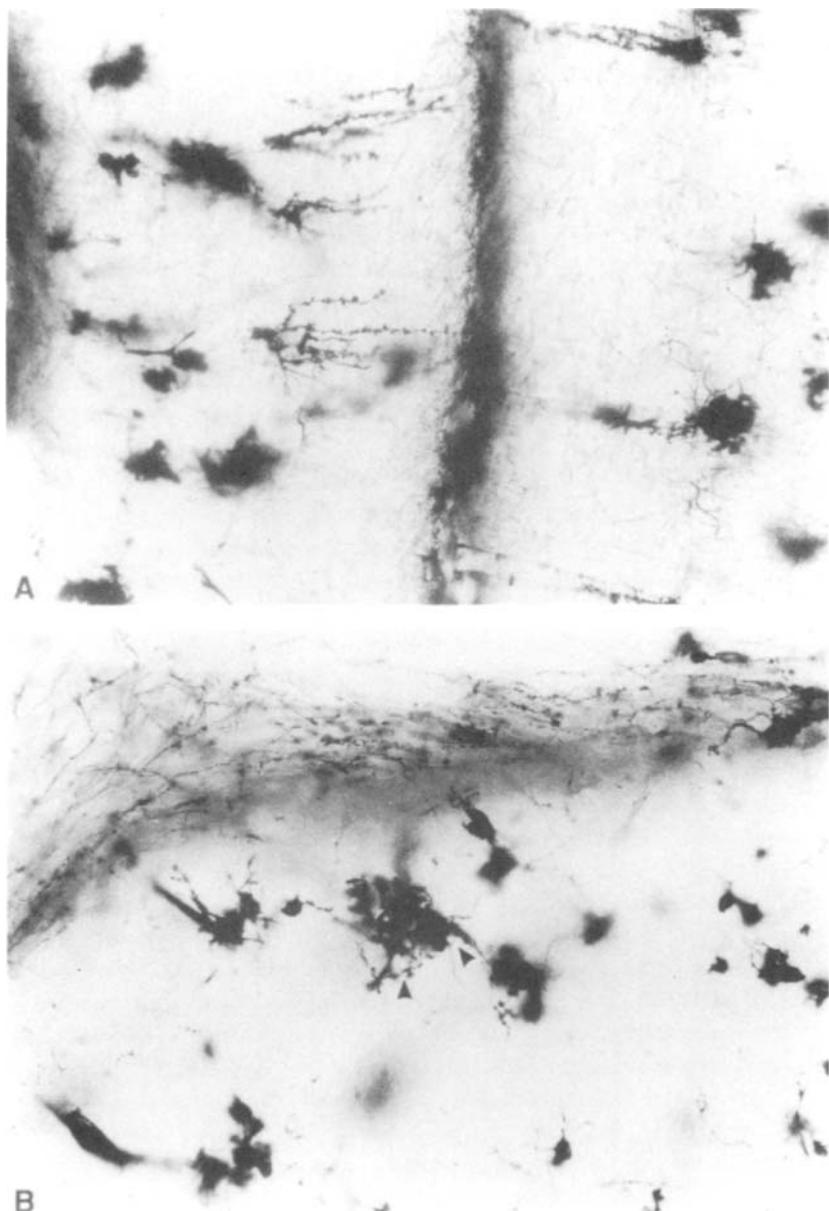


Figure 2. Golgi-Rio Hortega impregnation in the rat cerebellum, light microscopy. (A) Bergmann glial cells ($\times 250$). (B) Velate astrocytes ($\times 500$). (Used with permission from Privat et al., 1995.)

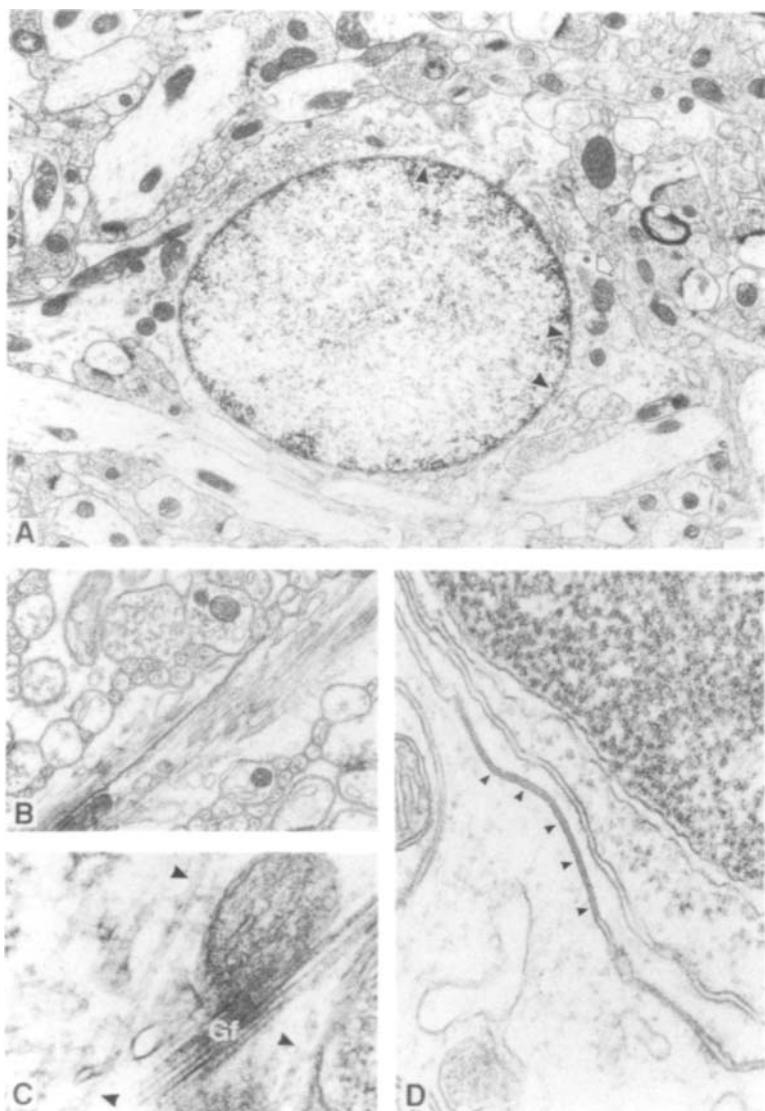


Figure 3. Transmission electron microscopy in the normal rat. (A) Protoplasmic astrocyte in the cerebral cortex ($\times 13,600$). (B) High magnification showing an astrocytic process filled with gliofilaments in the cerebellar cortex (molecular layer, $\times 20,000$). (C) High magnification of astrocytic cytoplasm illustrating isolated microtubules (arrowheads) close to a bundle of gliofilaments in the cerebral cortex ($\times 88,900$). (D) High magnification of a gap junction between astrocytic processes in the cerebellar cortex ($\times 76,700$). (Used with permission from Privat et al., 1995.)

proximal reactive astrocytes are found in the immediate vicinity of the injury and produce ultimately permanent glial scars (anisomorphic gliosis). In contrast, reactive astrocytes, that develop at distance from the lesion, proceed to resolution without the formation of scar tissue since the microenvironment is much less disturbed (isomorphic gliosis).

III. ASTROCYTIC MARKERS

A. Intermediate Filaments and Associated Proteins

GFAP and Vimentin

Intermediate filament proteins constitute a large family of molecules with a central α helix, including keratins, and neurofilaments (see refs. in Eng and Lee, 1995; Rutka et al., 1997). Vimentin and GFAP are the major components of the immature and mature gliofilaments, respectively. During postnatal development, vimentin is replaced by glial fibrillary acid protein (GFAP) (see references in Laping et al., 1994). However, although it is abundantly and predominantly found during development, vimentin is also present in adults in fibrous astrocytes and in reactive astrocytes (Schnitzer et al., 1981; Schiffer et al., 1986; Calvo et al., 1991; Eng and Lee, 1995). Immunocytochemical studies revealed that vimentin may be present in activated astrocytes close to an injury, suggesting that vimentin may be expressed only in proliferating astrocytes (Schiffer et al., 1986). GFAP constitutes the classical marker for astrocytes in the adult CNS (Figure 5). Its molecular weight depends on the species, from 48 kDa in the mouse to 51 kDa in the rat.

To delineate the role of these glial intermediate filament proteins, several studies focused on animals in which GFAP and/or vimentin genes had been inactivated. The first attempt was performed by removing the GFAP gene using antisense oligonucleotides in cultured human astrocytoma (Weinstein et al., 1991). Weinstein and colleagues (1991) thus evidenced a lack of extension of glial processes on neuronal induction. Then, transgenic mice devoid of GFAP (Gomi et al., 1995; Pekny et al., 1995) or of vimentin (Colucci-Guyon et al., 1994) were generated. Interestingly, all these animals develop and reproduce normally and do not exhibit any striking macroscopic abnormality. GFAP gene targeting does not lead to a compensation by vimentin. Similarly, the deletion of the vimentin gene does not lead to a compensation by GFAP. Galou and colleagues (1996) reported that vimentin appears necessary for the formation and/or stabilization of the GFAP network. Neuronal function of GFAP mutant mice has been investigated in the cerebellum. Shibuki and colleagues (1996) showed that long-term depression at the synapse between parallel fibers and Purkinje cells is deficient in mice lacking GFAP. They also reported a significant impairment of eyeblink conditioning in

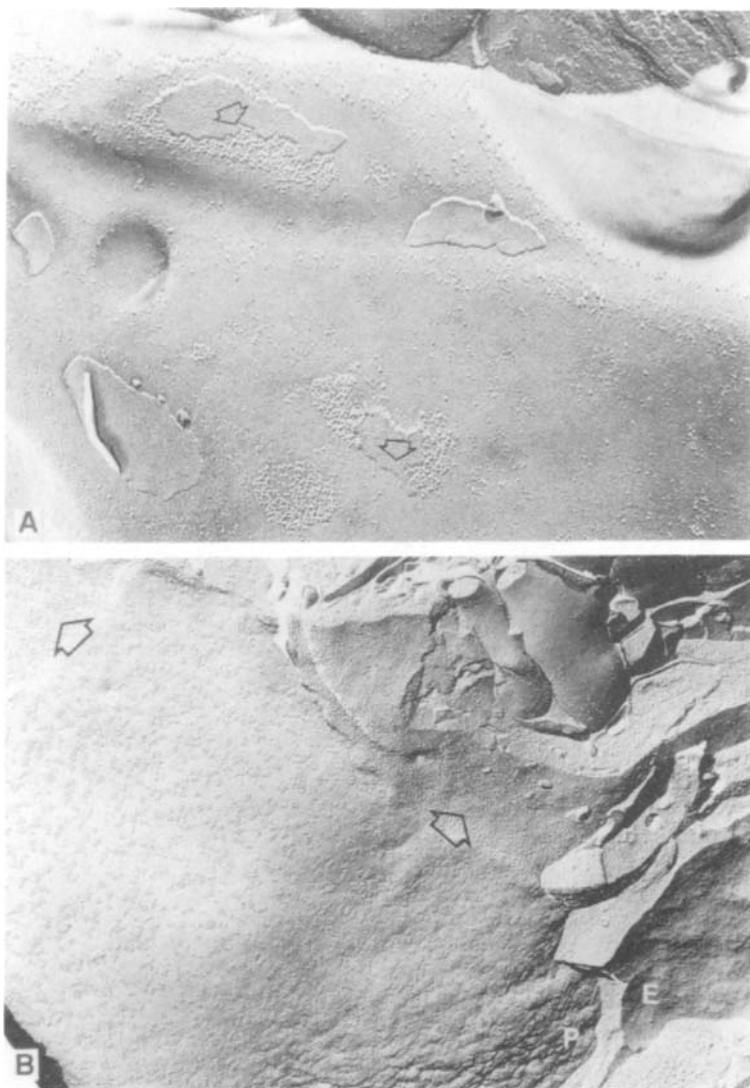


Figure 4. Electron microscopic micrograph of freeze-fracture replica. (A) High magnification of the membrane of a fibrous astrocyte in the normal rat corpus callosum. Note the presence of three macular gap junctions in the membrane. On two of them (**arrows**), patches of attached membranes exhibit complementary pits (x80,000). (B) High magnification of a subpial astrocytic endfoot in the mouse spinal cord white matter. Note the presence of numerous orthogonal assemblies on the surface contacting the basal lamina. The lateral membranes (**arrows**) are almost devoid of assemblies (x60,000). (Used with permission from Privat et al., 1995.)

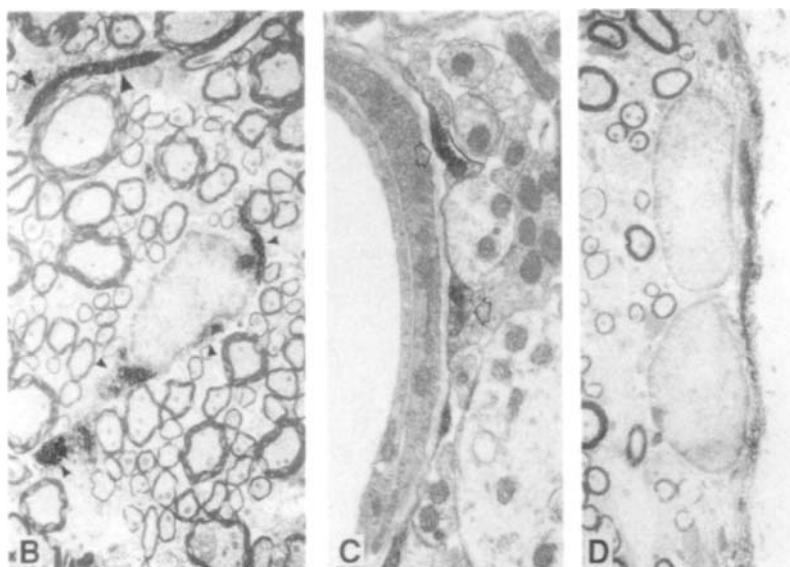


Figure 5. Ultrastructural localization of GFAP in the rat spinal cord. (A) GFAP immunoreactive gliofilaments in the white matter (x4,200). (B) GFAP immunoreactive perivascular astroglial processes (arrows) in the gray matter (x18,400). (C) GFAP-positive gliofilaments in subpial astrocytes, forming the glia limitans at the surface of the dorsal horn (x4,400). (Used with permission from Privat et al., 1995.)

GFAP mutant animals. These findings suggest that GFAP may play an important role in the communication between Bergmann glial cells and Purkinje cells for the induction and maintenance of long-term depression in the cerebellum (Shibuki et al., 1996). More recently, Pekny and colleagues (1998) studied the influence of GFAP absence on the production of intermediate filament proteins and morphological plasticity. Electron microscopic analyses revealed that the amount of gliofilaments were reduced in the GFAP^{-/-} mice, compared to those of GFAP^{+/+} controls. Like normal cultured astrocytes, primary cultured astrocytes from GFAP-deficient mice expressed both vimentin and nestin. In addition, when cocultured with cerebellar neurons, GFAP^{-/-} astrocytes retained their capacity to form processes and were characterized by an increase in cell saturation density (Pekny et al., 1998). Axonal regeneration was studied in GFAP-deficient mice after dorsal hemisection of the spinal cord. No difference in the regrowth of corticospinal axons was observed in mutant mice compared to the wild type (Wang et

al., 1997). The immunostaining of extracellular matrix proteins, i.e., laminin, fibronectin, tenascin, and chondroitin sulfate proteoglycan (CSPG), did not reveal significant differences, except for CSPG, which appeared less intense in the gray matter of GFAP^{-/-} mice. Thus, the lack of GFAP in reactive astrocytes does not alter axonal regrowth (Wang et al., 1997). Recent studies in GFAP-deficient mice strongly suggested that GFAP is not required for an astrocyte to become hypertrophied. Wu and Schwartz (1998) developed interesting tissue culture models to characterize in detail the mechanisms of induction of reactive gliosis as well as the phenotype of activated astrocytes. Recently, GFAP-deficient mice have been shown to be hypersensitive to spinal cord injury following head acceleration in a percussive injury model of head trauma (Nawashiro et al., 1998).

Vimentin-deficient mice were generated by Colucci-Guyon and collaborators (1994). Homozygous knockout animals developed and reproduced without any obvious phenotype. Interestingly, the absence of vimentin was not compensated by the upregulation of another intermediate filament protein (Colucci-Guyon et al., 1994). More recently, these transgenic mice lacking vimentin have been analyzed in detail (Galou et al., 1996; Colucci-Guyon et al., 1999). They present abnormalities in the structure and function of cerebellum (Colucci-Guyon et al., 1999). To see whether vimentin could be implicated in the organization of the GFAP network, Galou and colleagues (1996) examined GFAP expression and network formation in vimentin-deficient mice. It was thus shown that the GFAP network was disrupted in astrocytes that normally coexpressed GFAP and vimentin, such as fibrous astrocytes of the corpus callosum or the cerebellar Bergmann glial cells. Interestingly, after transfection of astrocytes from vimentin-deficient mice with vimentin cDNA, GFAP network aggregated normally. After injury, reactive astrocytes did not exhibit GFAP immunostaining. However, astrocytes that normally express only GFAP retained GFAP labeling. From these findings, it can be hypothesized that vimentin might be necessary for both the formation and stabilization of GFAP network (Galou et al., 1996).

Finally, double mutant mice for GFAP and vimentin have been generated (Ding et al., 1998; Menet et al., 1998) by mating the GFAP-deficient mice (Pekny et al., 1995) with the vimentin-deficient ones (Colucci-Guyon et al., 1994). These transgenic mice have been further investigated anatomically and functionally (Menet et al., 1998). Recently, an *in vitro* study focused on the role of the astroglial intermediate filaments in the maintenance of astrocyte volume. Swelling of normal astrocytes when transferred into a hypoosmotic medium is rapidly reversed in a few minutes. Although astrocytes from GFAP^{-/-} and vimentin^{-/-} animals retain intermediate filaments (Galou et al., 1996; Pekny et al., 1998), astrocytes from animals deficient in both GFAP and vimentin are completely devoid of gliofilaments (Ding et al., 1998; Menet et al., 1998). In an *in vitro* model of homeostasis regulation, Ding and colleagues evidenced a strong reduction of the capacity of volume maintenance in astrocytes from double deficient mice, whereas no modifications were recorded in astrocytes from animals lacking GFAP or vimentin. These biochemi-

cal findings suggest an important role of gliofilaments in astrocyte volume regulation (Ding et al., 1998). In addition, Menet and collaborators (1998) have shown that GFAP/vimentin double knockouts were unable to react to neuronal injury by the formation of a glial scar.

Nestin

During CNS development, intermediate filaments are subjected to sequential modifications. Nestin is an intermediate filament protein that is transiently expressed in multipotential proliferating neuroepithelial stem cells. For that reason, nestin is usually used to label neural precursors in subependymal area (see references in Fisher, 1997). During neurogenesis as well as gliogenesis, nestin is replaced by cell type-specific intermediate filaments, such as neurofilaments and GFAP. In the intact adult CNS, subependymal cells express nestin but not the astroglial and neuronal differentiation markers, i.e., GFAP and neurofilaments. After CNS damage, i.e., ischemic insult, excitotoxic lesion, or mechanical injury, nestin is detected in reactive astrocytes surrounding the damaged area (Clarke et al., 1994; Frisen et al., 1995; Lin et al., 1995; Duggal et al., 1997; Holmin et al., 1997). Nestin expression is induced after spinal cord lesion not only in reactive astrocytes but also in degenerating fibers. Similarly, some cells located in the peri-ependymal area of the spinal cord become nestin-positive within 2 days postinjury (Frisen et al., 1995). After injury, subependymal cells were stained for nestin and GFAP, but never with nestin and neurofilaments. These double-stained cells migrated toward the damaged area. Reactive astrocytes surrounding the lesion expressed nestin, as well as nonactivated astrocytes at distance from the lesion (Holmin et al., 1997). Thus, all these observations pointed out the spatiotemporal localization of nestin label-proliferating cells, located initially in periependymal areas and migrating to the injury site, thus suggesting that at least some proliferating reactive astrocytes might arise from nestin-positive subependymal cells. Such a close relationship between proliferating reactive astrocytes and neuroepithelial precursors was confirmed by lesion experiments performed in transgenic mice carrying the *lacZ* gene under the control of regulatory sequences from the nestin gene (Lin et al., 1995).

B. Cytoplasmic Antigens

The S100 protein family represents a group of small (10–12 kDa) cytoplasmic calcium-binding molecules involved in multiple cellular functions such as cell cycle progression and differentiation (Kligman and Hilt, 1988). S100 β , a 20-kDa protein, is present in astrocytes in the CNS and Schwann cells in the PNS. The S100 β protein can be released, and it has been detected in cerebrospinal fluid. Some authors attributed neurotrophic properties to S100 β , e.g. neurite extension activity (Kligman and Hilt, 1988; Azmitia et al., 1990; Liu and Lauder,

1992). S100 β exerts many biochemical calcium-dependent activities interfering with the regulation of microtubule assembly and tau protein phosphorylation (Kligman and Hilt, 1988). Interestingly, S100 β might regulate the phosphorylation of intermediate filament proteins. Indeed, an inhibitory effect (about 30%) of S100 β on total protein phosphorylation has recently been reported by Ziegler and colleagues (1998). Furthermore, S100 β induces both a stimulation of GFAP expression and a decrease (about 65%) in the phosphorylation of GFAP and vimentin (see references in Ziegler et al., 1998), suggesting an involvement of S100 β in cytoskeletal reorganization. Transgenic mice overexpressing S100 β exhibit spontaneous astrogliosis and axonal sprouting (Reeves et al., 1994). Another calcium-binding molecule, calbindin-D28K, is expressed in reactive astrocytes (Toyoshima et al., 1996).

Glutamine synthetase was at first considered as a specific marker for astrocytes, but some later studies revealed that this enzyme is also detectable in oligodendrocytes (see references in Cammer et al., 1990; Tansey et al., 1991; Wiesinger, 1995). Other studies report the presence of various astrogliant antigens, i.e., enolase $\alpha\alpha$ isoform, $\alpha 2$ glycoprotein, gp190 glycoprotein, and 7B11 antigen (see references in Barbin et al., 1988; Szigeti and Miller, 1993; Malhotra and Shnitka, 1994). However, these putative markers need further characterization, especially *in vivo* as many of them have been identified in cultured astrocytes. A few other antigens have been described in astrogliant cells, such as the C1 antigen, which is preferentially found in cerebellar Bergmann glial cells and in the retinal Müller cells, or the M1 antigen protoplasmic and fibrous astrocytes (see references in Privat et al., 1995).

C. Markers for Reactive Astrocytes

Various Antigens are Upregulated After Injury

Although various molecules have been shown to be modulated in astrocytes after injury (Ridet et al., 1997), their specificity for reactive astrocytes remains to be demonstrated. Reactive gliosis is characterized by a hypertrophy of astrocytes, with an upregulation of several structural components, e.g., vimentin, GFAP (Figure 6), S100 β , glutamine synthetase (see references in Brenner et al., 1994; Laping et al., 1994; Ridet et al., 1997; Morin-Richaud et al., 1998). Various interactions between cytokines, steroids, growth factors are involved in GFAP modulation (see refs. in Laping et al., 1994; Ridet et al., 1997). Transformation of astroblasts/astrocytes into reactive astrocytes is accompanied by changes in the arrangement of microfilaments. It has been shown *in vitro* that astrogliant differentiation is paralleled by an increase in GFAP-containing intermediate filaments, and a downregulation of F-actin and actin-binding proteins such as α actinin, myosin, tropomyosin, caldesmon, vinculin, talin, and spectrin, leading to a decrease in cell motility (Abd-El-Basset et al., 1991). After stab wound injury in

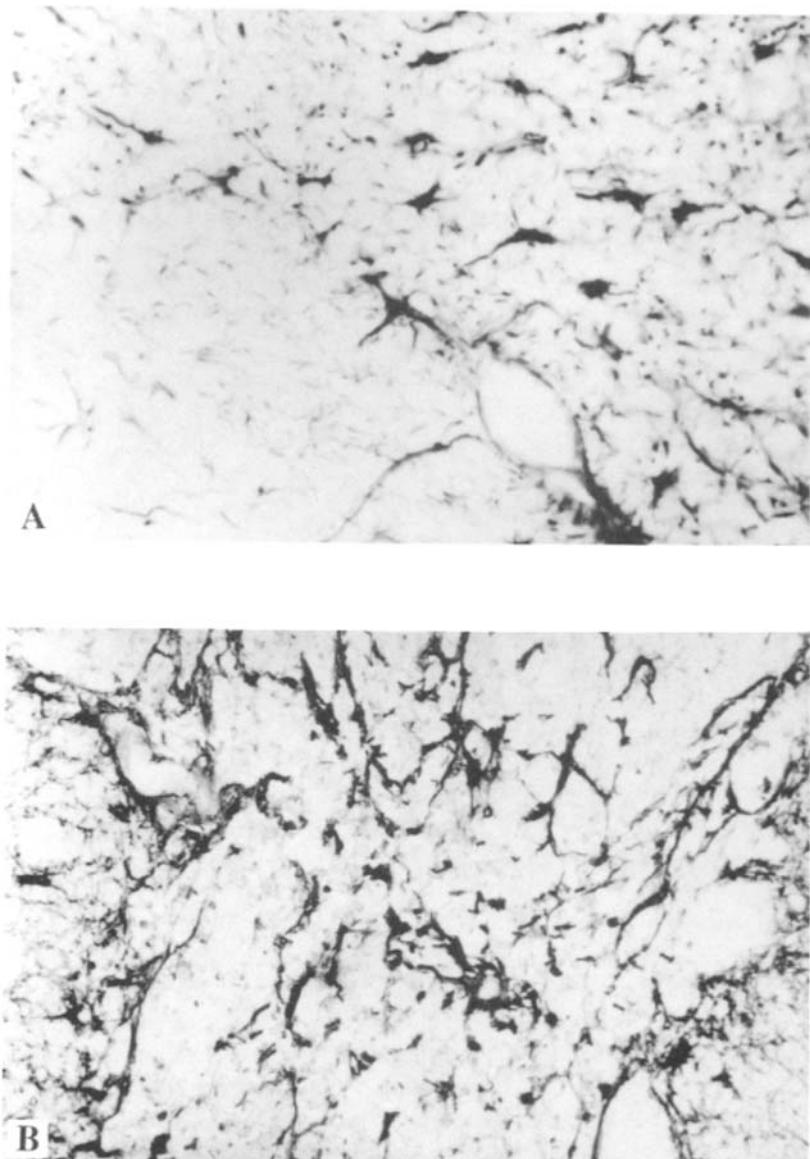


Figure 6. GFAP and vimentin immunostainings of reactive astrocytes in the rat spinal cord. (A) GFAP-positive reactive astrocytes (anisomorphic gliosis) within the damaged area after spinal cord compression (x800). (B) Vimentin-positive reactive astrocytes. An isomorphic gliosis develops within the dorsal column after degeneration of severed ascendant axons after spinal cord lesion (x1,600).

the brain, F-actin and α actinin are upregulated in reactive astrocytes. When cultured, reactivelike astrocytes also express α actinin (Fedoroff et al., 1987; Abd-El-Basset et al., 1997).

Enzymatic metabolism is extensively modified in activated astrocytes (Wiesinger, 1995). Reactive astrocytes are characterized by an increase in oxidoreductive enzyme activities (Malhotra and Shnitka, 1994; Ridet et al., 1997). After a lesion, astrocytes may also modulate neuritic extension by secreting several proteases (Abraham et al., 1993; Nakanishi et al., 1993) and protease inhibitors (Malhotra and Shnitka, 1994; Ridet et al., 1997), which may be involved in (1) the development of reactive gliosis, (2) the degradation of the cellular matrix, leading to an alteration in astrocyte–neuron interactions, and also possibly (3) the pathogenesis of neurodegenerative disorders. Although few studies have focused on early response genes in reactive astrocytes after injury, it has been reported that some *proto-oncogenes* such as HSP27 are increased at the lesion site (Norton et al., 1992; Ridet et al., 1997).

Antioxidant systems are also modulated in astroglial cells after injury. Antioxidant defense systems include superoxide dismutases (SODs), catalase, vitamins C and E, and glutathione and its related enzymes, i.e., glutathione peroxidase (GPx), glutathione reductase, and glutathione transferase. Glutathione-related enzymes are produced by astrocytes, and most of them, i.e., glutathione-S-transferase μ and Yb isoforms, GPx, are increased in reactive astrocytes (Cammer and Tansey, 1989; Cammer et al., 1990; Makar et al., 1994; Knollema et al., 1996; Levison et al., 1996; Wilson, 1997; Lindenau et al., 1998). Although the mitochondrial Mn-SOD is found in cultured glial cells, i.e., microglia, astroglia, and oligodendroglia (Pinteaux et al., 1998), *in situ* Mn-SOD is only detected in neurons, whereas the cytosolic Cu/Zn-SOD isoform is located in astrocytes (Noack et al., 1998). After quinolinic acid-induced lesion, Mn-SOD and Cu/Zn-SOD were upregulated, respectively in neurons and astrocytes. While the excitotoxic injury leads to peroxinitrite formation, which is associated with neuronal death, astrocytes survived (Noack et al., 1998). Astrocytes have also been shown to produce a large variety of growth factors and cytokines, which have been hypothesized to interfere with the enzymatic metabolism of free radicals. Indeed, Mn-SOD is induced by cytokines in astrocytes (Mokuno et al., 1994). Interestingly, it has recently been shown in a neuron/astrocyte coculture model that the decoupling of astrocytic gap junctions enhanced peroxide production after an oxidative insult, suggesting that gap junction communication might decrease neuronal vulnerability to oxidative stress (Blanc et al., 1998). Taken together, these findings suggest that (activated) astrocytes, by increasing antioxidant activities, might constitute one of the lines of defense of the brain against toxic substances, such as reactive oxygen species (Cammer et al., 1989; Makar et al., 1994; Huang and Philbert, 1995; Wilson, 1997; Drukarch et al., 1998).

Interestingly, it has been reported that some *neuronal markers* of the normal brain, i.e., tau protein, neuron-specific enolase (NSE), growth cone-associated

protein 43 kDa (GAP-43), and microtubule-associated protein 2, are expressed in reactive astrocytes after various CNS damages (Geisert et al., 1990; Ikeda et al., 1993; Lin and Matesic, 1994; Frisen et al., 1995; Ridet et al., 1997; Sensenbrenner et al., 1997). It has been reported in progressive supranuclear palsy and in some cases of postencephalitic parkinsonism of Economo that some astrocytes expressed tau protein (Ikeda et al., 1993; Matsusaka et al., 1998). GAP-43 is expressed in stellate (type 2) astrocytes and in reactive astrocytes, but not in protoplasmic (type 1) astrocytes (Sensenbrenner et al., 1997). NSE is induced in reactive astrocytes rapidly after injury when astrocytes undergo morphological changes. This NSE induction might be correlated with a postlesion modulation of the energetic metabolism (Sensenbrenner et al., 1997).

Specific Markers for Reactive Astrocytes

Over the last decade, the development of new cellular and molecular markers for astrogliial cells has advanced our knowledge concerning neuroglial interactions occurring during development and after various types of CNS injury. Vimentin, GFAP (Figures 5 and 6) and S-100 β are the markers most often used for identifying astrocytes in the intact and lesioned CNS (Eng and Lee, 1995; Bignami and Dahl, 1995; Ridet et al., 1997). Most of these molecules are "nonspecifically" increased in reactive astrocytes. Moreover, these molecules are not strictly specific for astrocytes, as some of them are also expressed in ependymocytes, fibroblasts, and meningeal cells.

In the past decade, a few research groups have focused on the characterization of postlesion events in the CNS, especially astrogliosis. Attempts are currently underway to identify additional specific markers for reactive astrocytes.

J1-31 Antigen. The monoclonal antibody (Mab) J1-31 recognizes a protein of 30 kDa isolated from brain tissue of autopsied patients with multiple sclerosis (MS) (Singh et al., 1986; Predy et al., 1987). Immunofluorescent microscopy revealed that J1-31 staining is localized in GFAP-positive cells (Figure 7A,B), i.e., astrocytes, tanycytes, Müller glial cells (Singh et al., 1986; Predy et al., 1987). It has been shown by electron microscopy that the J1-31 antigen is associated with the cytoskeleton, e.g., astrocytic gliofilaments (Figure 7C). Mab J1-31 is upregulated in reactive astrocytes after spinal cord laceration (Predy et al., 1988; Malhotra et al., 1993). Interestingly, J1-31 staining is specifically found in reactive astrocytes which are close to the lesion site (Figure 7A,7B) (Malhotra et al., 1993). In an axotomy model, i.e., hypoglossal nerve transection, no J1-31 staining is observed in the hypoglossal nucleus (Malhotra et al., 1993). Thus, Mab J1-31 constitutes a useful marker for the detection and characterization of trauma-induced glial scar.

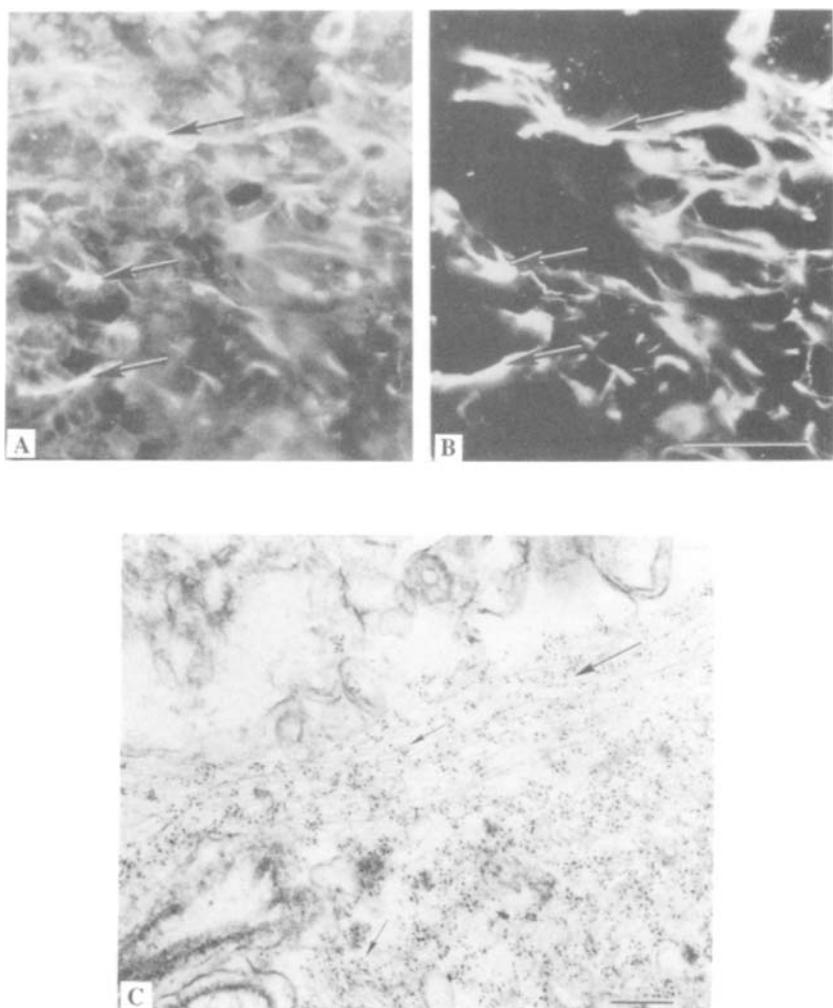


Figure 7. J1-31 immunostaining in the rat CNS. (A–B) Double immunostaining showing the colocalization of J1-31 (A) and GFAP (B) in reactive astrocytes after spinal cord laceration. (C) Electron microscopy micrograph of immunogold labeling (5 nm particles) in the rat cerebellum showing that J1-31 antigen is associated with gliofilaments (arrows) (scale bar = 200 nm). (Used with permission from Malhotra et al., 1993.)

6.17 Antigen. The monoclonal 6.17 antibody, which has been raised against homogenates of embryonic rat muscle (Koenig et al., 1988), was initially used as a maturation marker for the rat neuromuscular junction, and it is suspected of

being produced by Schwann cells in PNS (Koenig et al., 1988). In the normal CNS, few structures are 6.17-immunostained. Multiple immunofluorescent stainings using antibodies recognizing GFAP, vimentin, 6.17, and S-100 β revealed that the large majority of the 6.17-positive profiles were also GFAP-

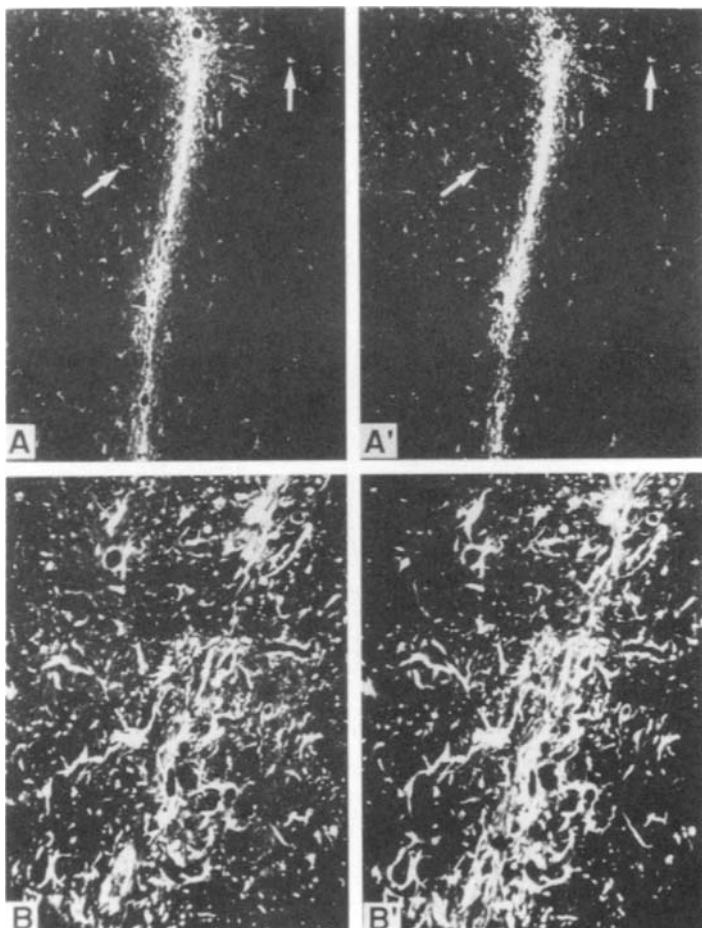


Figure 8. 6.17 immunostaining in reactive astrocytes after stab wound lesion of the adult rat diencephalon. (A–A') Low magnification within the thalamus showing that the glial scar is costained for the 6.17 antigen (A) and vimentin (A'). Note the colocalization of 6.17 antigen (A) and vimentin (B) in isolated reactive astrocytes (**arrows**). (B–B') Higher magnification of hypertrophied astrocytes illustrating the colocalization of the 6.17 antigen (A) and vimentin (A') in reactive astrocytes. (B–B'). (Used with permission from Ridet et al., 1996.)

and vimentin-positive (Figure 8 and 9A,9A') (Ridet et al., 1996). In the white matter, i.e., the corpus callosum, fimbria, fornix, spinal cord tracts, fibrous astrocytes were costained for vimentin and 6.17. Similarly, astrocytes located in the most outer border of the brain, i.e., glia limitans, contained 6.17, GFAP, vimentin, and S-100 β . However, no 6.17 staining was associated with S-100 β - or GFAP-positive astrocytes of the intact central gray matter. Ultrastructural analysis of 6.17 staining sites revealed that the antigen was associated with bundles of gliofilaments (Figure 9B,9C) (Ridet et al., 1996). Ependymocytes bordering the third ventricle were vimentin- and 6.17-positive, whereas those bordering the lateral ventricles were not stained for 6.17 (Ridet et al., 1996). After a CNS lesion, a strong 6.17 staining was associated with S-100 β , GFAP- and vimentin-positive reactive astrocytes (Figure 8 and 9A,9A'). Whatever the damaged region, i.e., cortex, hippocampus, thalamus, hypothalamus, spinal cord, 6.17-stained profiles were detected as far as several millimeters from the injury site, revealing that 6.17 labeling is associated with virtually all reactive astrocytes, regardless of the distance from the lesion site (Ridet et al., 1996). However, whereas J1-31 antigen is well-characterized (Singh et al., 1986), the corresponding 6.17 antigen has not yet been purified. Interestingly, structures immunostained for vimentin (meningeal cells, the spinal central canal area) or GFAP (normal protoplasmic astrocytes) were not 6.17-positive, attesting that the epitope recognized by the 6.17 monoclonal immunoglobulin M (IgM) antibody does not belong to GFAP or vimentin. Thus, the purification of the 6.17 antigen, which is currently in progress, will contribute to further characterize biochemically and molecularly the significance of reactive astrocytes.

Thus, although both 6.17 and J1-31 monoclonal antibodies label reactive astrocytes, 6.17 constitutes a potentially interesting marker, as it's at variance with J1-31 staining it is not restricted to the vicinity of the lesion site (Malhotra et al., 1993).

M22 Antigen. The monoclonal antibody M22 has been raised against the immunodominant domain of HIV gp41, and was subsequently shown to label reactive astrocytes (Yamada et al., 1991; Eddleston et al., 1993a, 1993b, 1996). By means of various CNS aggressions, such as infectious agents, transgenic overexpression of a viral glycoprotein or cytokine, focal trauma, it has been shown that selective induction of M22 was observed or not depending on the type of insult. Scrapie infection induced a strong M22 staining in reactive astrocytes; the M22 staining was restricted to areas with extensive damage. In addition, or focal trauma and HIV gp120 overexpression led to a specific increase of M22 staining in the hippocampus. However, transgenic mice overexpressing interleukin-6 (IL-6) induced M22 staining specifically in cerebellar astrocytes (Eddleston et al., 1996). In addition, M22 was detected in astrocytes of human brain tissue from patients with chronic CNS diseases, i.e., Creutzfeldt-Jakob disease, Gerstmann-Straussler syndrome, multiple sclerosis, and multiple infarct dementia. These findings dem-

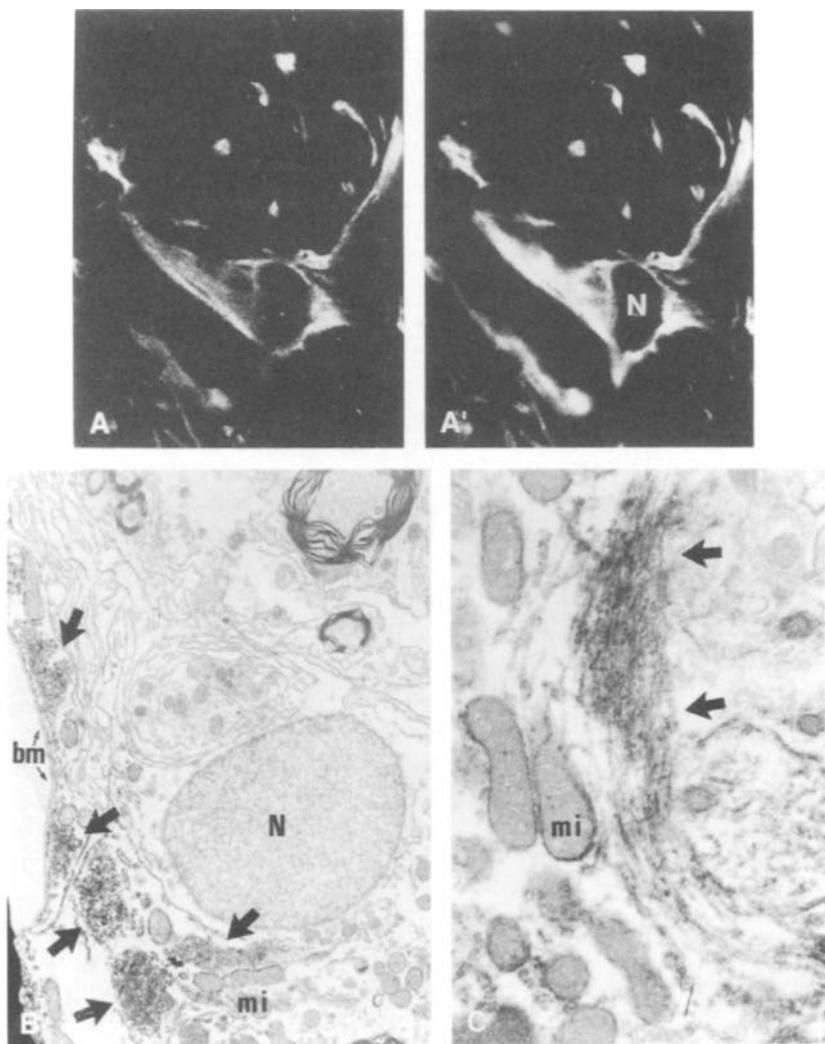


Figure 9. Subcellular localization of 6.17 antigen in (reactive) astrocytes (**A-A'**) Confocal microscopy image (x1,610) of an activated astrocyte colocalizing the 6.17 antigen (**A**) and vimentin (**A'**). Note that 6.17 antigen is immunodetected within the astrogial cytoplasm of processes and cell body. (**B-C**) Electron microscopy micrographs (**B**, x9,500; **C**, x26,650) of astrogial 6.17 immunostaining. Note that the staining is associated with gliofilaments (**arrows**). bm, basal membrane; mi, mitochondria; N, nucleus. (Used with permission from Ridet et al., 1996.)

onstrate that the M22 antigen is overexpressed in a subpopulation of highly activated reactive astrocytes located in areas exhibiting extensive damage (Eddleston et al., 1996). Thus, M22 as well as 6.17 antibodies may provide neuropathologists with good markers for the qualitative and differential analysis of astrocytic response to various insults.

IFAPs. Several novel intermediate filament-associated proteins (IFAPs) have been identified in the CNS (see references in Abd-El-Basset et al., 1989; Yang et al., 1994, 1997; Rutka et al., 1997). Though absent in the adult intact CNS, an IFAP-associated protein (IFAP-70/280 kDa) was shown to be associated with radial glial cells and their derivatives in the developing brain. However, after brain injury in an adult, this IFAP is reexpressed in reactive astrocytes. Depending on the time-course appearance of this IFAP, it seems that reactive astrocytes induced by stab wound injury may be divided into two subtypes: persistent IFAP-70/280 kDa-containing astrocytes proximal to the lesion, and transient distal IFAP-negative cells (Yang et al., 1997). The reappearance of early markers, i.e., vimentin and IFAP-70/280 kDa, in reactive astrocytes may suggest recapitulation of early developmental stages.

Plectin is an IFAP of 500 kDa interacting with intermediate filaments in a large variety of cell types (Rutka et al., 1997; Lie et al., 1998; Wiche, 1998). In the brain, all plectin-positive structures are also GFAP positive (Lie et al., 1998). Plectin is able to bind GFAP and is thus involved in cytoskeletal support of astroglial cells (see references in Rutka et al., 1997). Plectin could play an important role in cytoskeleton network organization, with consequences for viscoelastic properties of the cytoplasm and the mechanical integrity and resistance of cells and tissues. Recently, it has been shown that mutation of the human plectin gene is associated with a hereditary disease, epidermolysis bullosa simplex, characterized by severe skin blistering combined with muscular dystrophy (see references in Wiche, 1998).

Other Antigens AMP1. AMP1, also termed G-CAM, is a membrane-anchored adhesion molecule (106 kDa) present in cultured rat astrocytes and oligodendrocytes (Geisert et al., 1991). AMP1 antigen is upregulated and found in reactive astrocytes after brain injury (Irwin and Geisert, 1993).

pp25 protein. In a recent work focused on postlesional protein phosphorylation, a new phosphoprotein has been identified in hippocampal tissue after excitotoxic lesion by two-dimensional electrophoresis (Lenz et al., 1997). Pp25 was more widespread in lesioned tissue and occurred earlier than vimentin; it might thus constitute an early sensitive marker of gliosis.

Glial hyaluronate-binding protein (GHAP). GHAP is a structural glycoprotein of the white matter extracellular matrix. GHAP is upregulated in isomorphic activated astrocytes, and it has been hypothesized that anisomorphic GHAP-negative

reactive astrocytes are permissive for axonal regrowth, in contrast to isomorphic GHAP-immunostained astrocytes (Mansour et al., 1990).

IGFBP-2. Insulinlike growth factor 1 (IGF-1), which has neuroprotective properties, is known to play an important role during brain development and neurodegenerative and postinjury processes (see references in Connor et al., 1997). IGF-1 and IGF binding-proteins, IGFBP-1–6, are induced after injury (Garcia-Estrada et al., 1992; Walter et al., 1997; Beilharz et al., 1998; Hammarberg et al., 1998). Protein 2 is strongly induced in reactive astrocytes, whereas protein 3 is induced to a lesser extent in activated microglial cells. Protein 5 is also upregulated in reactive astrocytes, more moderately in neurons, and protein 6 is induced in choroid plexus cells, ependymal cells, and reactive glial cells (Walter et al., 1997; Beilharz et al., 1998; Hammarberg et al., 1998). The possible modulation of IGF-1 action by IGFBP-2, produced specifically by reactive astrocytes, might constitute a key mechanism restricting postlesion neuronal death (Beilharz et al., 1998). Interestingly, the analysis of transgenic mice overexpressing IGFBP-1 revealed a reduction of astrogliosis, particularly astroglial proliferation, after injury (Ni et al., 1997).

Cyst fluids. Cyst fluids obtained from patients with cystic gliomas recognize similar antigens (with apparent molecular weights ranging from 50–75 kDa) in reactive astrocytes, microvessels, and perivascular astrocytic processes of the white matter (Brunet et al., 1993). These cystic fluids may be a source of interesting antigens involved in astrocyte activation.

IV. REACTIVE ASTROGLIA AS A POSITIVE COMPONENT IN CNS INJURY

A. Cytokines and Trophic Factors

The production of several cytokines and growth factors is markedly enhanced after CNS damage (see references in Eddleston and Mucke, 1993; Yong, 1996; Ridet et al., 1997; Ghirnikar et al., 1998). Cellular responses to CNS injury include the production of soluble molecules that modulate inflammation and wound healing via complex paracrine and autocrine intercellular loops. Although considered as an immunoprivileged organ, the brain contains resident cells, e.g., astrocytes and microglia, that produce a large variety of immune molecules, e.g., interleukins, TNF, TGF β . Interestingly, one of them—perforin—which is a cytolytic protein commonly present in many immune cells, i.e., cytotoxic T lymphocytes, and killer cells, has recently been detected in (activated) astrocytes (Gasque et al., 1998). Thus, perforin produced by a subpopulation of astrocytes plays a role in CNS inflammation (Gasque et al., 1998; Link, 1998). Inflammation leads to astrogliosis. Reactive astrocytes have been reported to upregulate many growth factors and cytokines, e.g., see

abbreviation list LIF, IGF-I, CNTF, NGF, FGF, NGF, and interleukins (see references in Logan et al., 1994; Schwartz and Nishiyama, 1994; Strauss et al., 1994; Labourdette and Sensenbrenner, 1995; Levison et al., 1996; Merrill and Benveniste, 1996; Ridet et al., 1997; Rossner et al., 1997; Ghirnikar et al., 1998; Goss et al., 1998). Noticeably, most of these secreted molecules, such as the interleukins or CNTF, which are upregulated after injury, are also potent inducers of gliosis (Ridet et al., 1997). For instance, mice overexpressing CNTF have been shown to develop spontaneous reactive gliosis, confirming the role of this factor in the early events leading to the formation of gliosis after a lesion (Winter et al., 1995). In addition, $TGF\beta_1$, which is present in activated astrocytes, in neurological dysfunctions and/or after injury, modulates the expression of cytoplasmic and surface molecules and factors, including NCAM and NGF. The astroglial production of growth factors and cytokines is regulated via complex autocrine and paracrine loops (see references in Aubert et al., 1995; Ridet et al., 1997). IL-1 β and IL-6 produced by reactive astrocytes are likely modulators of astrogliosis (Merrill and Benveniste, 1996; Ridet et al., 1997; Ghirnikar et al., 1998). To explore the role of cytokines in pathophysiological processes affecting the CNS, especially the role of IL-6 in scar formation, IL-6-deficient mice and transgenic mice carrying the IL-6 gene under the control of the GFAP promoter have been produced (see references in Campbell et al., 1993, 1998; Kreutzberg, 1996; Ridet et al., 1997), confirming that IL-6 is a potent inducer of gliosis.

B. Membrane-Anchored Molecules

Many receptors to growth factors, e.g., FGF receptors, p75^{NGF} and *trk* receptors, are upregulated in reactive astrocytes after injury (see references in Morganti-Kossmann et al., 1992; Junier et al., 1994; Reilly and Kumari, 1996; Yong, 1996; McKeon et al., 1997; Ridet et al., 1997; Stachowiak et al., 1997; Goutan et al., 1998), contributing to complex autocrine and paracrine intercellular communication. To further examine the physiological functions of neurotrophic factors, transgenic mice, lacking p75^{NGF}, and *trkB*, *trkC* genes, have been produced over the past 5 years. These recombinant mice are being studied with respect to their neuronal systems, but, surprisingly, gliosis has been investigated in only a few of them (see references in Greensmith and Vrbova, 1996; Levi-Montalcini et al., 1996). Indeed, among FGF receptors, FGFR1 is the major receptor in astrocytes and glioma cells. Interestingly, nuclear FGF-2 is induced in reactive astrocytes. Both Western blot analysis and *in vivo* immunocytochemistry revealed that the majority of FGFR1 is found in the nucleus, whereas very low levels can be detected in the membrane and cytoplasm (see references in Stachowiak et al., 1997).

Besides receptors, astrocytes exhibit membrane specializations involved in interastrocytic communication. Astrocytes are coupled by gap junctions, which are mainly composed of connexin 43 (Cx43) (see references in Giaume and

McCarthy, 1996). This astroglial syncytium allows for the exchange of ions and small molecules, e.g., metabolites, catabolites, and second messenger molecules. These intercellular gap junctions thus play a key role in the maintenance of brain homeostasis (see references in Giaume and Venance, 1998; Wolff et al., 1998). In addition to the former, reflexive gap junctions mediating autocellular coupling have been described (see references in Wolff et al., 1998). Wolff and colleagues (1998) observed Cx43 expression and gap junctions regardless of the presence or absence of intercellular contacts. It has been hypothesized that autocellular coupling is involved in the reorganization of cytoskeleton during cell process genesis. After injury, Cx43 is redistributed and astrocytic gap junctions are modified (Hos-sain et al., 1994; Ochalski et al., 1995; Theriault et al., 1997; Li et al., 1998). The permissive nature of astrogliosis may depend on the amount of gap junctions between reactive astrocytes (Alonso and Privat, 1993); among other functions, gap junctions could coordinate the cytoskeleton of coupled cells (see references in Wolff et al., 1998). Modifications in astrocyte coupling might thus disturb this equilibrium resulting ultimately in neuronal dysfunction and death. Increasing interests are devoted to the influences of growth factors and cytokines on coupling in neural and nonneuronal cells (see references in Reuss and Unsicker, 1998; Reuss et al., 1998). Reuss and colleagues (1998) studied the effects of growth factors on the expression and function of Cx43 in cultured astrocytes. It has been reported that FGF-2 caused a reduction of Cx43 and intercellular communication. These findings highlighted a new FGF-2-dependent mechanism, by which astroglial cell connectivity might be regulated *in vivo* (Reuss et al., 1998).

Reactive astrocytes upregulate various cell surface molecules with facilitating or inhibiting properties on axonal elongation. The production of these adhesion molecules can be modulated by trophic factors and cytokines (Norton et al., 1992; Eddleston and Mucke, 1993; Malhotra and Shnitka, 1994; Aubert et al., 1995; McKeon and Silver, 1995; Merrill and Benveniste, 1996; Ridet et al., 1997). The involvement of NCAMs in synaptic plasticity has been extensively investigated (Fields and Itoh, 1996), whereas still little is known of the role of the same molecules in astrocyte/neuron interactions in axonal regeneration. It has been reported that after intrastriatal implantation of NGF-producing fibroblasts, cholinergic axons expressing L1 regenerate on reactive astrocytic processes expressing NCAM (Chalmers et al., 1996). Reactive astrocytes also reexpress the embryonic polysialylated form of NCAM (PSA-NCAM) (see references in Rutishauser and Landmesser, 1996; Kiss and Rougon, 1997; Ridet et al., 1997; Aubert et al., 1998; Kiss, 1998). Taken together, these findings reinforce the concept of duality of reactive gliosis for axonal regrowth, as previously proposed by Kawaja and Gage (1991).

Among the large variety of cell surface molecules produced by normal astrocytes and reactive astrocytes, which play an important role in the regulation of neuron–astrocyte interactions during CNS development and plastic remodeling, is tenascin, a member of the J1 family of glycoproteins (see references in Faissner

and Schachner, 1995; Deller et al., 1997). Various receptors may mediate tenascin effects, such as integrins and proteoglycans (PGs) (Faissner and Schachner, 1995). Astrocytes synthesize a large variety of PGs, which are able to influence axonal elongation (see references in McKeon and Silver, 1995; Fitch and Silver, 1997; Ridet et al., 1997; Dow and Wang, 1998). A recent study reported a close correlation between tissue distribution and upregulation of CSPGs, inflammation, and altered blood-brain barrier (BBB) after CNS damage (Fitch and Silver, 1997). It has been reported in this study that the presence of degenerating neurons does not constitute a sufficient signal to trigger an increase in PGs. Activated macrophages, their products, or other serum components that cross a compromised BBB may provide a stimulus for changes in ECMs after CNS injury (see references in Fitch and Silver, 1997; Dow and Wang, 1998).

Interestingly, it has been suggested that FGF receptors can be activated by recognition molecules, such as L1, NCAM, PGs, or N-cadherin, and that these receptors may participate in many developmental and regenerative phenomena (see references in Ridet et al., 1997). The precursor of the amyloid β /A4 protein, APP, is upregulated by reactive astrocytes, and APP has been shown to promote neuritic extension by binding extracellular matrix molecules (ECMs) such as PGs or laminin (Aubert et al., 1995; Ridet et al., 1997). In addition, *in vitro* studies have demonstrated that bFGF and TGF β_1 increase messenger expression of APP isoforms (Toulmond et al., 1996; Ridet et al., 1997). The facilitating influence of APP on neuritic extension may be mediated by NGF (see references in Aubert et al., 1995; Ridet et al., 1997). Thus, it can be hypothesized that the balance of cell surface molecules and factors produced is a key determinant for injured axons to degenerate or regenerate. It is likely that the first important requirement for an axon to regrow is that it be surrounded by a permissive environment, which might be modified by molecules produced by reactive astrocytes surrounding the damaged axon.

Since various transgenic mice deficient in certain CAMs and ECMs have been generated, it might be highly interesting to evaluate the regenerating capacities in these animals, as well as to characterize the nature of posttraumatic reactive gliosis. For instance, the formation and features of reactive gliosis might be investigated in detail in NCAM-deficient mice, which are also lacking PSA as a consequence, as NCAM is a major carrier of PSA (Rutishauser and Landmesser, 1996) or in transgenic mice expressing L1 under the control of the GFAP promoter (Wolfer et al., 1998).

C. Features and Roles of Activated Astroglia in CNS Damage

Posttraumatic Glial Scar

Pathophysiological changes occurring after CNS injury include complex acute, subacute, and late processes. The first events are hemorrhage and necrosis, fol-

lowed by the recruitment of inflammatory cells and activation of glial cells, leading to the formation of glial scar. The initial traumatic injury is followed by complex inflammatory responses within the first hours postlesion, involving reduction of blood flow, tissue hypoxia, disruption of the BBB with damage of endothelial cells, recruitment of inflammatory cells, release of cytokines resulting in vasogenic edema, activation of microglia, and astrogliosis (see references in Schwab and Bartholdi, 1996). Reactive gliosis has been considered as one of the major impediments to CNS repair. However, during the last decade, it has been reported that axonal elongation can occur in the presence of reactive astrocytes (Kawaja and Gage, 1991; Alonso and Privat, 1993; Aubert et al., 1995; Li and Raisman, 1995), and even that reactive astrocytes may support neuritic extension in certain circumstances (Kawaja and Gage, 1991). It can be hypothesized that the presence of growth factors might modulate the production of cell surface molecules, as it has been reported that reactive astrocytes provide a permissive substrate for axonal regrowth in the presence of high levels of NGF (Kawaja and Gage, 1991).

Thus, there is increasing evidence that glial cells surrounding the injured axons play a key role in determining CNS regenerative capability. In certain microenvironmental conditions, reactive astrocytes may constitute a necessary requirement for axonal regrowth. There is now strong evidence that reactive gliosis varies qualitatively and quantitatively depending on both the nature of the injury and the microenvironment of the injury site. Although proximal reactive astrocytes show increased expressions of glial filaments, intermediate filament-associated proteins, dehydrogenases, neurotrophic factors, and cytokines, cell surface molecules of distal reactive astrocytes present only moderate modifications (see references in Malhotra and Shnitka, 1994; Ridet et al., 1997). Thus, astrocytes do not respond in a stereotypic manner to all forms of CNS damage (Eddleston and Mucke, 1993; Fitch and Silver, 1997; Ridet et al., 1997). For instance, it has been suggested that the permissive or nonpermissive features of a postlesional gliotic scar is probably reflected on its ultrastructural organization, especially the abundance of gap junctions between reactive astrocytes (Alonso and Privat, 1993). Recent studies concerning Cx43, the most prominent gap junction protein in the CNS, highlighted the important role of Cx3 in the modulation of neuroglial interactions after injury. Cx43 is redistributed in reactive astrocytes in response to injury, closely paralleling the remodeling of gap junctions (Hossain et al., 1994; Ochalski et al., 1995; Giaume and McCarthy, 1996; Theriault et al., 1997; Li et al., 1998).

The adaptive plasticity of reactive astrocytes depends on brain location, the nature of the aggression, the age of the injured animal, and the different microenvironmental conditions, e.g., the combinations of cytokines, growth factors, serum factors, adhesion molecules produced by injured neurons, reactive astrocytes, microglia, endothelial cells and from extravasated blood (see references in Malhotra and Shnitka, 1994; Bignami and Dahl, 1995; Fitch and Silver, 1997; Ridet et

al., 1997). Factors and "injury signals" responsible for the biochemical heterogeneity of reactive astrocytes need to be further investigated.

Reactive Astrocytes and Human Brain Physiopathology

Detection of Activated Astrocytes as an Indicator of Human Pathogenesis. The detection of reactive astrocytes has been a landmark of brain pathology, first with classical stains such as Holzer's and Cajal's, then with the immunodetection of GFAP and/or electron microscopic examination. In cases of neurodegenerative disorders, e.g., tauopathies, Parkinson's and Huntington's diseases, or multiple system atrophy, the anatomopathological examination is usually aimed at detecting abnormal intracellular inclusions and/or glial fibrillary lesions (see references in Lafarga et al., 1991, 1993; Schipper and Cisse, 1995; Castejon, 1998a, 1998b; Goedert et al., 1998). Although it is not a general feature, the development of astrogliosis is often associated with CNS disturbances. For instance, HIV-associated dementia is characterized by a rapid and strong gliosis. It has been hypothesized by some authors that astrogliosis may be responsible for dysfunctions characterizing HIV-associated dementia or aging-related dementia (see references in Sei et al., 1995; Vitkovic and da Cunha, 1995; Unger, 1998). In human brain trauma, light and transmission electron microscopic analysis of astrocytic changes frequently provides useful informations for the neuropathologist to predict both neurological and neurobehavioral deficits. Indeed, deficits are correlated with astrocyte ultrastructural changes and BBB disruption. For instance, swollen, clear, and dense astrocytes are often observed around dark, degenerated, neuronal cells. At an ultrastructural level, reactive hypertrophic astrocytes are frequently characterized by increased amounts of gliofilaments, glycogen, lipofuscin deposits, endoplasmic reticulum, and microtubules. After severe trauma, the ensheathment of synapses by astrocytes is often disrupted, as well as the perivascular astrocytic end-feet and the interastrocyte gap junctions (Castejon, 1998a). In addition, the extent of astrogliosis is a good criterion to assess the effects of neurotoxicity (see references in Malhotra and Shnitka, 1994; O'Callaghan et al., 1995). Thus, reactive gliosis is an important criterion for neuropathological diagnosis. *In vivo*, perforin was not detected in normal adult brain tissue but was present in and around areas of inflammation (white and gray matter) in MS and neurodegenerative brains, strongly suggesting that perforin produced by activated astrocytes plays a role in CNS inflammation (Gasque et al., 1998; Link, 1998). Interestingly, astrocytic changes can also be observed in conjunction with severe metabolic disorders such as liver diseases, e.g., hepatic coma or Wilson's disease. Thus, the immunodetection of astrocytic markers, i.e., GFAP, vimentin, 6.17, J1-31, and M22 antigens, might be considered by the anatomopathologist in investigations of human brain specimens. For instance, J1-31 antigen has been shown to be markedly enhanced in MS plaques (Malhotra et al., 1989).

Since NGF administration has been reported to reduce tissue damage in several neurodegenerative disorders, the detection of *trkA* receptors has been carried out in human neurodegenerative diseases. In normal human brain, the *trkA*, B, and C receptors can be immunodetected in neurons and, to a lesser extent in astrocytes. In the brains of patients suffering from Alzheimer's disease, Huntington's disease, progressive supranuclear palsy, MS, Creutzfeldt-Jakob disease, multifocal leukoencephalopathy, and residual hypoxic encephalopathy, a strong *trkA* immunostaining is detected in reactive astrocytes. The presence of *trkA* receptors in reactive astrocytes from different human neurodegenerative brain diseases confirms that NGF might be involved in the formation of astrogliosis (Aguado et al., 1998).

Glia Tumors. The classification of human astrocytomas is still a matter of debate among neuropathologists (see references in Rutka et al., 1997; Liberski, 1998). The current classification is almost exclusively based on morphologic features. However, efforts to develop new biochemical markers might contribute to the establishment of a universal classification of astrocytoma. Specific developmentally regulated proteins of the IF cytoskeleton, such as GFAP, vimentin, keratins, and an IF-associated protein, IFAP-300 kDa, are used by neuropathologists for astrocytomas diagnosis, and their immunodetection also provides useful tools for investigating the biology of these tumors (Yang et al., 1993, 1994). GFAP is considered as a reliable marker of differentiation of both normal and neoplastic astrocytes (see references in Rutka et al., 1997). It has been reported that, in cases of astroglial tumors, GFAP production is progressively decreased with increasing astrocytic malignancy. The nuclear amount of FGF-2 and FGF-receptor type 1 might regulate the proliferation of neural cells, as the subcellular distribution of FGF-2 differs between quiescent and reactive astrocytes (see references in Stachowiak et al., 1997). Interestingly, low levels of FGF-2 are found in the cytoplasm of quiescent cells, whereas it is abundant in reactive cells in both the cytoplasm and the nucleus. The amount of FGFR1 is reduced in the nucleus concomitantly with a reduction of cell proliferation. Furthermore, in gliomas, large amounts of both FGF-2 and FGFR1, associated with a high kinase activity, are detected within the nucleus of neoplastic cells (see references in Stachowiak et al., 1997). IGF-1 has been suspected of being involved in the growth of human glial tumors, i.e., astrocytomas and glioblastomas (Merrill and Edwards, 1990). In gliomas, e.g., astrocytic tumors (astrocytomas, glioblastomas multiforme) and oligodendrogiomas, *trk* receptors staining was observed exclusively in astrocytic glioma (see references in Aguado et al., 1998; Wang et al., 1998). In oligoastrocytomas, the immunostaining was restricted to the astrocytic component. *Trk* expression was independent of age, sex, or histological grade of the investigated tumors. These observations indicate that in human tumor tissues, *trk* receptors are expressed in a lineage-restricted manner, allowing the distinction between astrocytes and oligodendrocytes (Wang et al., 1998). These data contribute to a

better understanding of the biology of glial tumors and thus improve patient prognosis.

Astrocytes, Aging and Neurodegenerative Processes. There is increasing evidence that glial cells play an important role during aging and neurodegenerative processes. Although no marked global astroglial differences occur in the normal aged brain, specific areas, i.e., the limbic system and neocortex, exhibit strong reactive gliosis associated with neuronal death and synaptic degeneration. In addition, GFAP content and turnover increase during normal aging (Hansen et al., 1987; Beach et al., 1989; Nichols et al., 1993; Yoshida et al., 1996; David et al., 1997). For instance, it has been reported that GFAP increases with age first in the hippocampus and then in the entorhinal cortex of nondemented patients (David et al., 1997). In Alzheimer's disease, senile plaques are complicated lesions composed of amyloid peptides and associated molecules, degenerating neuronal processes, and reactive glia. There is a close association between reactive glial cells and neuritic plaques and neurons undergoing neurofibrillary degeneration (see references in Unger, 1998). Furthermore, S100 β is suspected of being implicated in the pathogenesis of Alzheimer's disease, and Down's syndrome. Age-related increases in S100 β expression have been reported in brain tissue from normal subjects, and this increase is even more prominent in patients with frontotemporal dementia, Alzheimer's disease, and Down's syndrome in which S100 β is present in three copies on chromosome 21 (Griffin et al., 1989; Kato et al., 1991; Sheng et al., 1996; Green et al., 1997), reinforcing the hypothesis that astrocytes might play a key role in age-related neurodegenerative processes.

The correlation between the formation of pathological astrocytic tubular structures and the severity of presenile-onset Alzheimer-type dementia is still a matter of debate. As with intraneuronal neurofibrillary tangles, these abnormal astrocytic tubular structures were tau-positive and stained with Gallyas-Braak's silverimpregnation (Arima et al., 1998).

A recent paper from Tomimoto and colleagues (1997) describes regressive changes in astroglial cells of the white matter in the brains of Alzheimer patients. In this change, termed clastomatodendrosis, astrocytes were characterized by extensive vacuolation and swelling with condensed chromatin. In addition, clastomatodendritic astrocytes express antigens of reactive astrocytes as well as serum proteins, e.g., immunoglobulins and complement proteins (Tomimoto et al., 1997).

Reactive gliosis is a general feature of the neurodegenerating human brain, especially in Alzheimer patients. Extracellular matrix molecules, i.e., PGs and laminins, are abundant in normal aged and Alzheimer brains (Jucker et al., 1996; Dow and Wang, 1998). Interestingly, PGs might play an important role in the pathogenesis of Alzheimer's disease. PGs are associated with senile plaques and neurofibrillary tangles. Senile plaques are composed of APP, which is bound to PGs. It has been hypothesized that the neurite growth-promoting properties of

APP are disturbed by interactions between APP-binding PGs and amyloid plaques, thus contributing to neurite retraction and degeneration (see references in Dow and Wang, 1998).

During normal aging and in Alzheimer's disease, amyloid β protein is the main component of senile plaques observed in the cerebral cortex. Granules containing amyloid β protein are detected in astrocytes and not in microglia or neurons. It has been hypothesized that astrocytes might constitute a way of degradation of amyloid β protein in the aged brain (Funato et al., 1998).

Statin, a 57-kDa nuclear protein, is expressed exclusively in nonproliferating cells. It has been described that a large fraction (~45%) of glial cells is statin-negative in the normal aged brain. This can be correlated with a substantial retention of the proliferative capacity. In brain tissue from Alzheimer patients, there is a significant higher loss of statin (~60%) in neuroglial cells (Schipper et al., 1993), confirming the increased gliosis, i.e., hypertrophy plus hyperplasia, during neurodegenerative processes.

Thus, the fine characterization of gliosis (cytokinetics, up- or downregulation of markers, presence of autofluorescent cytoplasmic inclusions) might indicate the presence and extent of intervening neuropathologic processes.

Imbalance of Glial Cytokines as a Cause of CNS Dysfunctions? Neurons and (reactive) astrocytes produce a large variety of trophic factors and cytokines that play a key role in CNS homeostasis. It has been hypothesized that cytokine-dependent homeostasis disturbances may contribute to the pathogenesis of CNS dysfunctions, such as Alzheimer's disease or HIV encephalopathy (see references in Griffin et al., 1989, 1998; Morganti-Kossmann et al., 1992; Sei et al., 1995; Patel et al., 1996; Mattson et al., 1997; Seilhean et al., 1997). For instance, in Alzheimer's disease, an excess of cytokines/trophic factors might trigger interactions between astroglial cells and β APP metabolism and plaque formation, whereas neuronal deprivation leads to cell death (see references in Patel et al., 1996; Griffin et al., 1998; Unger, 1998). Interactions with amyloid peptides and possibly glycated proteins might induce activated microglia and astrocytes to produce a large variety of molecules, e.g., cytokines, reactive oxygen species, nitrogen intermediates, and proteases, that may be locally toxic to neuronal processes in the vicinity of senile plaques. In addition, activated glial cells produce factors that lead to their reciprocal activation and growth, thus potentiating local inflammatory cascades (see references in Lee et al., 1995; Breitner, 1996; Ridet et al., 1997; Unger, 1998).

MS is associated with an upregulation of proinflammatory (IFN γ , TNFs, IL-12) and immune response-down-regulating (TGF β , IL-10) cytokines. Also IL-6 and the cytolytic molecule perforin are upregulated. Interleukin production, especially that of IL-6 and, to a lesser extent, IL-10, might constitute a common event in inflammatory disease. It has been shown that IL-6 is mostly associated with astrocytes in the brain of MS patients (see references in Maimone et al.,

1997). Perforin was not detected in normal adult brain tissue but was present in and around areas of inflammation (white and gray matter) in MS and neurodegenerative brains (see references in Gasque et al., 1998; Link, 1998). Both pro- and anti-inflammatory cytokines are also produced by microglia and astrocytes, constituting a CNS–cytokine network that interacts with the cytokine network of the immune system (Link, 1998). It is likely that cytokines produced in MS and inflammatory diseases contribute to brain pathogenesis, e.g., inflammation and white matter damage. However, it has been suspected that IL-6 production in MS may also counteract excessive immune responses, thus supporting neuroprotective and remyelinating processes.

Role of Astrocytes in Antioxidant Defenses of the CNS. There is increasing evidence in the literature in favor of the key role of oxidative stress in many CNS pathologies, i.e., neurodegenerative diseases, epilepsy, trauma, stroke (see references in Furuta et al., 1995; Mattson and Furukawa, 1996; Wilson, 1997; Hölscher, 1998; Reiter, 1998). Reduced forms of oxygen such as the hydroxyl radicals are normally produced in the brain, but an acceleration of this production induces extensive cellular damage, e.g., oxidation of proteins, lipids, and nucleic acids. Oxidative stress, which contributes to age-related neurodegenerative dysfunctions, can be defined as an imbalance between the rates of production and elimination of reactive oxygen species. Abnormalities in the cellular regulation and expression of antioxidant enzymes may have a role in mechanisms of CNS aging and neurodegeneration. Various antioxidant systems, e.g., the enzymes SOD, GPx, catalase, as well as reductants such as vitamin E, glutathione, and reduced vitamin C, are produced in (reactive) astrocytes. Enzymes involved in glutathione metabolism constitute a family of cytosolic enzymes involved in the detoxification of electrophilic xenobiotics, but they also participate in the intracellular binding of lipophilic molecules, e.g., glucocorticoids and thyroid hormones. A decrease in the intracellular pool of antioxidant glutathione, a storage form of cysteine which plays a key role in scavenging the reactive oxygen species, is associated with cell death, and astrocytes are known to maintain high levels of glutathione (see references in Cooper and Kristal, 1997; Juurlink, 1997; Drukarch et al., 1998). Glutathione protects against lipid peroxidation and neurotoxicants such as free radicals. Reductions in the pool of glutathione cause cell death. It has been reported that patients with inborn errors of glutathione metabolism often exhibit progressive neurological dysfunctions. In the CNS, astrocytes, by maintaining high levels of antioxidants and regulating the extracellular concentration of excitatory aminoacids, constitute one of the more resistant cell types to oxidative stress, compared to neurons and oligodendrocytes. Astrocytes play a key role in the detoxification of the brain, as glutathione and its related enzymes are abundant in astrocytes (see references in Makar et al., 1994; Bolanos et al., 1995; Cooper and Kristal, 1997; Wilson, 1997; Lucas et al., 1998). Astrocytes might also

play an important role in neutralizing free radicals via the nitric oxide (NO) synthase metabolism. NO is a short-lived molecule involved in many biological activities. NO synthases are present in (reactive) astrocytes (Luth and Arendt, 1997; Cha et al., 1998). NOS inhibitors block neurotoxicity. Superoxide anions react with NO and form cytotoxic peroxynitrite. SOD degrades superoxide anions, thus attenuating neurotoxicity (Skaper et al., 1995). Astrocytes might also be a mediator of brain excitotoxicity, as it has been shown that reactive oxygen species can impair the glutamate uptake system in astrocytes (Sorg et al., 1997).

It has been reported that astrocytes in senile plaques exhibited a strong immunostaining for Mn-SOD in the cerebral cortex and hippocampus of patients with Alzheimer-type senile dementia (Maeda et al., 1997). Another study strongly suggested the role of antioxidant properties of reactive astrocytes in the CNS of patients suffering from familial (FALS) or sporadic (SALS) amyotrophic lateral sclerosis (Blaauwgeers et al., 1996). In this study, Blaauwgeers and colleagues (1996) showed a marked increase of the mitochondrial manganese SOD2 in astrocytes, whereas the expression of the cytosolic copper-zinc SOD1 was unchanged. Such an increase of the manganese SOD2 in reactive astrocytes was also reported in the spinal cord of autopsied patients (Shibata et al., 1996). Interestingly, as astrocytes are strongly SOD1-immunostained in mutant mice overexpressing mutant SOD1, astrocytes are suspected of being an early indicator of ALS progression, and it has been hypothesized that astrocytic damage may promote motor neuron degeneration (Brujin et al., 1997).

Furuta and colleagues (1995) studied the localization of SOD isoforms in the brain of elderly patients and in Alzheimer's and Down's patients. In normal aged patients, Cu/Zn-SOD and Mn-SOD are detected predominantly in neurons and rarely in glial cells. In the cortex and hippocampus of Alzheimer's and Down's patients, Cu/Zn-SOD is abundant in hippocampal pyramidal neurons undergoing degeneration, whereas Mn-SOD is more enriched in reactive astrocytes than in neurons. Amyloid cores, diffuse plaques, and microglia scarcely showed colocalization with SOD-positive structures. The observed changes in the cellular localization of SODs in neocortex and hippocampus in cases of Alzheimer's disease and Down's syndrome support a role for oxidative injury in neuropathological processes. Interestingly, the differential localization of Cu/Zn-SOD and Mn-SOD suggests that cellular responses to oxidative stress is cell- and enzyme-specific and that SOD isoforms might exert different functions in antioxidant mechanisms (Furuta et al., 1995).

It is hypothesized by certain authors that free radical neurotoxicity might constitute the cause of nigral neurons damage in parkinsonians, who may lack antioxidant molecules such as glutathione (see references in Simonian and Coyle, 1996; Ciccone, 1998). Clinical trials involving antioxidant molecules, i.e., selegiline, vitamin E, or lazarooids, are under way (see references in Ciccone, 1998).

VI. REACTIVE ASTROCYTES AND CNS REPAIR

A. Strategies to Reduce Gliosis

Several experimental strategies are currently tested to block posttraumatic gliosis by interfering with the biology of reactive astrocytes. Experimental manipulations of the glial scar, including X-irradiation and injections of antibodies or chemical drugs, have been investigated (see references in Ridet et al., 1997). Some cholesterol derivatives have been shown to reduce reactive gliosis, thus improving the environment for axonal regrowth (Bochelen et al., 1992, 1995; Gimenez y Ribotta et al., 1995; see refs. in Gimenez y Ribotta and Privat, 1998). Promising perspectives include the *in vivo* administration of antibodies raised against specific markers of reactive astrocytes, i.e., 6.17, J1-31, or M22, or that of antibodies raised against gliosis inducers such as TGF- β_1 , which has been shown to attenuate significantly the formation of the astroglial scar (Logan et al., 1994; Toulmond et al., 1996; Ridet et al., 1997). For instance, the application of IL-10, an inhibitor of microglial cytokine synthesis, significantly reduces astrogliosis, likely via the blockage of the postlesion increase of TNF α , a potent inducer of gliosis (Balasingham and Yong, 1996). In fact, any agent raised against astrocytic markers is a potential therapeutic tool to suppress postlesional inflammation and gliotic scar formation. An alternative strategy may be to enhance the permissivity of reactive astrocytes, by the local administration of trophic factors. Reactive astrocytes are involved in the integration of CNS transplants and probably in the recovery of the integrity of a lesioned area. Thus, molecules potentially capable of facilitating this property may be good inducers of axonal regrowth. This could be the case with molecules that reduce secondary excitotoxic damage.

Recently, we, and others, have studied the effects of X-rays with the development of lesion-induced gliosis (Kalderon and Fuks, 1996a, 1996b; Ridet et al., submitted). Surprisingly, it has been reported that a single high dose of 20 Gy of X-rays administered 2 weeks after hemisection of the corticospinal tract yields beneficial effects on the recovery of spinal-injured rats (Kalderon and Fuks, 1996a, 1996b). In order to further characterize X-irradiation influence on post-traumatic gliosis, we assessed whether graded doses of X-rays (from 2 up to 20 Gy) could interfere with the acute/subacute phases, and thus improve the functional recovery of paraplegic animals. From the behavioral analysis performed for 1 month after spinal cord lesion, it appeared that the 2-Gy group exhibited better motor performances than the lesioned nonirradiated animals (LNI) and the 5 to 20 Gy groups. Gliosis was reduced in the 2-Gy group compared to LNI, and increased in the highly (>5 Gy) irradiated animals. The lesion-induced syringomyelia was reduced by ~20% in the 2-Gy group compared to the other groups (LNI and 5–20 Gy). Taken together, these data indicate that low doses of X-rays may interfere with the formation of the syringomyelia and glial scar, thus facilitating recovery of paraplegic animals. These findings suggest that a low-dose irradiation

of the lesion site, in conjunction with other therapy, may be a potentially promising treatment to improve recovery after spinal cord injury.

B. Transplantation of Astroglial Cells

During the last decades, many studies have aimed at exploring the potential of transplantation of neural cells, especially glial cells (see references in Blakemore and Franklin, 1991; Fisher and Gage, 1993; Ridet and Privat, 1995; Fisher, 1997; Franklin and Barnett, 1997; Taylor, 1997; Gage, 1998). In particular, some of them focused on the potential of astrocytes for brain repair strategies (see references in Bernstein and Goldberg, 1989; La Gamma et al., 1993; Wunderlich et al., 1994; Taylor, 1997). For several reasons, astrocytes may be particularly useful for brain repair strategies: they are normal CNS constituents harboring efficient secretory mechanisms and providing support to neurons by releasing factors promoting neuronal survival, differentiation and regeneration; they can easily be expanded in culture, and they survive after transplantation into the brain and integrate into the host environment. More recently, several studies aimed at exploring the therapeutic potential of transplanted astrocytes that have previously been genetically engineered to express foreign transgenes (Cunningham et al., 1991, 1994; La Gamma et al., 1993, 1994; Castillo et al., 1994; Ridoux et al., 1994; Lundberg et al., 1996; Lin et al., 1997; Taylor, 1997; Ridet, et al., 1999). Primary cultures of rat astrocytes genetically engineered to produce levodopa by retroviral-mediated transfer of a human tyrosine hydroxylase gene (hTH) have been shown to ameliorate the rotational behavior induced by apomorphine in a rat model of Parkinson's disease (Lundberg et al., 1996). Astrocytes of human origin engineered to produce therapeutic molecules may emerge as a powerful tool for human gene therapy of neurodegenerative diseases and brain trauma. Recently, human fetal astrocytes derived from legal abortions have been cultured and successfully transduced with a retrovirus driving the expression of active NGF (Lin et al., 1997). Human adult astrocytes may be an even more relevant and promising candidate for human brain repair, as they may open the way to autologous *ex vivo* gene transfer, thus obviating immunological rejection. More recently, we reported the ability of astrocytes derived from human adult cerebral cortex to be expanded and genetically modified *in vitro* (Ridet, et al., 1999). In addition, cells were efficiently transduced by an adenoviral vector encoding hTH under the negative control of the tetracycline-based regulatory system (tet-off). The infected cells synthesized large amounts of active hTH and released levodopa as well as dopamine (Ridet, et al., 1999). Altogether, these data open the way to promising therapeutic strategies based on the use of modified human adult astrocytes for autologous grafting in cases of neurodegenerative and posttraumatic dysfunctions.

VII. CONCLUDING REMARKS

Reactive gliosis, of which astrocytes are only one element, is a very complex phenomenon, involving interactions of several cell types with neurons, leading eventually to neuron survival/death, axonal regeneration/retraction. We have outlined here one aspect of astrocyte complexity and versatility: the diversity of the repertoire of markers, receptors, factors expressed, according to age, location, and extent of the causal injury. There is increasing evidence that astrocytes—or at least some astrocyte subtypes—are able to support axonal regeneration, in certain experimental conditions. Systematic analysis and comparison of markers, receptors, and factors expression in permissive and nonpermissive situations should lead to new advances in the understanding—and monitoring—of the role of astrocytes in CNS dysfunctions, and especially postlesion gliosis.

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PLASTICITY AND RIGIDITY IN THE NERVOUS SYSTEM

LESSONS FROM THE SPINAL CORD

Håkan Aldskogius

I.	The Concept of Neuroplasticity and its Implications for the Spinal Cord	188
II.	Motor Axon Injury Modifies Intrinsic Spinal Cord Connectivity	190
	A. The Response of Motoneurons to Axotomy Is a Combination of Regressive and Regenerative Events	190
	B. Axotomized Motoneurons Lose Presynaptic Terminals	190
	C. Mechanisms Underlying Loss of Presynaptic Motoneuron Terminals	192
	D. Functional Consequences of Loss of Presynaptic Motoneuron Terminals	194
III.	Removal of Primary Sensory Input to the Spinal Cord May or May Not Induce the Formation of New Connections	195
	A. Organization of the Sensory Input to the Spinal Cord	195

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B.	The Response of Peripheral Sensory Nerve Injury Has Markedly Different Effects in the Spinal Cord Compared to Dorsal Root Injury	196
C.	Evaluating Anatomical Changes in Central Projections of Sensory Axons	198
D.	Deafferentation Alone May Be Insufficient to Cause Expansion of Primary Sensory Afferents in the Spinal Cord.	201
E.	Deafferentation by <i>Peripheral</i> Nerve Injury Is Sufficient to Produce Expansion of Primary Sensory Afferent Projections	203
F.	Possible Factors Underlying Differences in Central Plasticity Following Deafferentation by Peripheral but Not Central Sensory Axon Injury.	205
G.	Functional Consequences of Injury to Peripheral Sensory or Dorsal Root Axons	206
IV.	Concluding Comments.	206
	Acknowledgments	207
	References	207

I. THE CONCEPT OF NEUROPLASTICITY AND ITS IMPLICATIONS FOR THE SPINAL CORD

Neuroplasticity is a loose term, commonly used to summarize the responses of the nervous system to changes in its external and/or internal conditions. These responses range from changes in the expression of specific molecules, membrane properties, neural conduction, synaptic transmission, cellular structure, cellular interactions, and changes in the size of cell populations to functional and behavioral changes. The wide range of situations that might generate a neuroplastic “response” obviously makes it unlikely to find a common mechanistic principle for all of them. In fact, the heterogeneity and diversity of these reactions prompted the suggestion already more than 20 years ago that the term be abandoned (Vital-Durand, 1975). On the other hand, loose as it may be, the continued existence of this concept probably attests to its usefulness.

In order to discuss plasticity in a focused manner, some limitations of the general concept are required. Here, we discuss three different situations in which lesions outside the spinal cord provide a potential for the formation of new *anatomical* connections. The limitations in this review are not intended to minimize the importance of other processes that may underlie *functional* plasticity in the spinal cord, such as “unmasking” of “silent” synapses (Wall and McMahon, 1994; Li and Zhuo, 1998), or sensitization (Baranauskas and Nistri, 1998).

Spinal cord circuitry is a useful experimental model for exploring principles of neuroplasticity, and has been the focus of a large number of such studies. Anatomical reorganization of connections in the spinal cord is likely to be an important process for functional restoration after injury and, unfortunately, for the emergence of sensory dysfunctions, the most intriguing and clinically

important one being chronic neuropathic pain (Wall, 1991; Pockett, 1995; Woolf, 1995; Goff et al., 1998). The putative relationship between spinal cord plasticity and neuropathic pain has undoubtedly been a driving force in a large number of experimental spinal cord plasticity studies. Results from studies with modern methods for functional evaluation of central nervous system (CNS) function in humans have also indicated injury-induced plasticity in the motor (Chen et al., 1998; Ziemann et al., 1998) and somatosensory (Borsook et al., 1998; Flor et al., 1998; Tinazzi et al., 1998) system.

Spinal cord anatomical circuitry can be altered by four principally different kinds of lesions: (1) motor axon injury by lesion of spinal ventral roots or peripheral nerves containing motor axons (cf. Figure 1), (2) sensory axon injury by lesion to peripheral sensory nerves (cf. Figure 4A), or (3) spinal dorsal roots (cf. Figure 4B), and (4) injury directly to the spinal cord, with the aim of interrupting ascending or descending spinal cord pathways. Here, we consider the first three of these, which have in common that the lesion is located outside the spinal cord. These lesions can be readily reproduced, and their outcome analyzed with high precision with morphological, physiological, and behavioral methods. Moreover, making the lesion outside the spinal cord implies that the plasticity responses arise

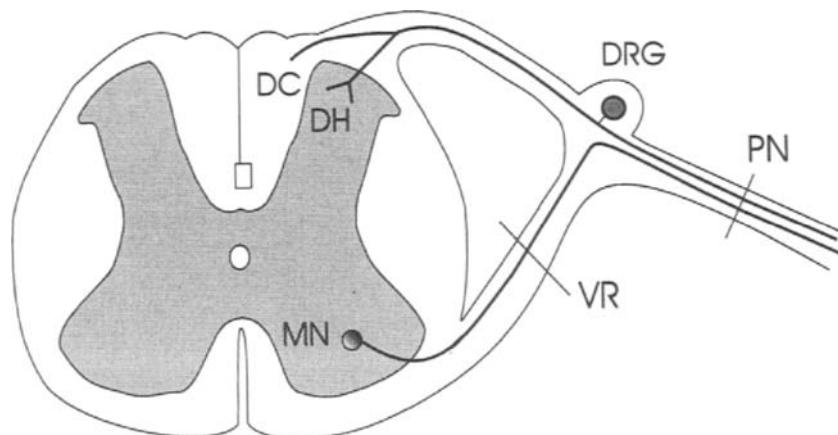


Figure 1. Diagram showing the principles of segmental spinal cord anatomy. Motoneurons (MN) give rise to motor axons, which course via the ventral root (VR) into peripheral nerves (PN). T-shaped sensory axons have their cell bodies in dorsal root ganglia (DRG) from which the centrally directed processes enter the spinal cord dorsal horn (DH), where they all terminate. A fraction of these sensory axons have collaterals that ascend in the dorsal column of the white matter (DC). The two lesion sites where motor axons can be interrupted are indicated (PN and VR).

as a result of "intrinsic" interactions, without possible complications due to mechanical destruction of the blood-brain barrier.

This review will start with a discussion of the structural and connectional plasticity of axotomized motoneurons. This group of neurons has served as a prototype for the study of axotomy responses, and provided substantial principal information relevant also for the reaction of other neurons to axon injury, including sensory neurons.

II. MOTOR AXON INJURY MODIFIES INTRINSIC SPINAL CORD CONNECTIVITY

A. The Response of Motoneurons to Axotomy Is a Combination of Regressive and Regenerative Events

In general, axotomy performed at sites shown in Figure 1 transforms the injured neuron from a transmitting or "secreting" mode to a "growing" mode (Watson, 1974). This is reflected in a downregulation of transmitter-associated molecules, as well as of molecules regulating neuronal stability, in combination with an upregulation of molecules involved in promoting axon growth (reviewed in Aldskogius and Svensson, 1993). In addition to changes in molecules "classically" involved in these two aspects of neuronal activity, recent studies have described profound changes (usually upregulations) in neuropeptides (for review, see Palkovits, 1995) and growth factor receptors (Piehl et al., 1994), but the functional implications of these changes are obscure.

Although the overall response to axotomy appears to be a "productive" process, there are in fact several major regressive components as well. Thus, axon injury places the affected motoneurons in a vulnerable situation, often leading to the loss of a fraction of these neurons. The distal dendrites of axotomized motoneurons retract (Brännström et al., 1992), and there is a loss of axon collaterals from the intraspinal portion of their axons (Havton and Kellerth, 1984, 1990). Paradoxically, at the same time, injured motoneurons generate new branches in the spinal cord (Havton and Kellerth, 1987). These branches may arise from soma, dendrites, or proximal axons (Figure 2). This paradox of simultaneous regressive (or degenerative) and regenerative events appears to be a common accompaniment of axon injury (cf. IIIB).

B. Axotomized Motoneurons Lose Presynaptic Terminals

Concomitantly with the intraneuronal modifications, there is an extensive shedding of presynaptic terminals from dendrites and somata of axotomized motoneurons (Figure 3). This loss was initially documented by qualitative elec-

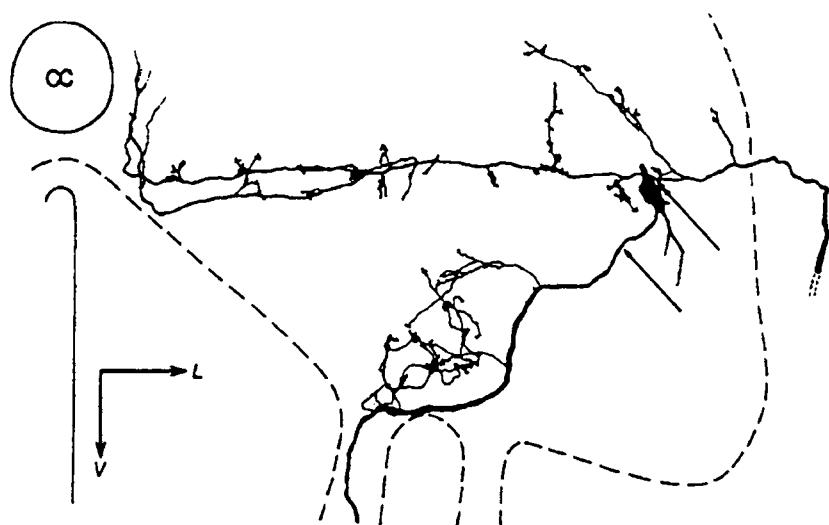


Figure 2. Example of a remarkable plastic response of axotomized motoneurons. The motoneuron has been injected with horseradish peroxidase, which has spread into its processes of which the figure shows the axonal tree. In addition to the main axon (**single arrow**) there is a newly formed axon (**double arrow**), originating from the motoneuron perikaryon. Reprinted with permission from *Nature* (Havton and Kellerth, 1987), copyright (1987) Macmillan Magazines Ltd.

tron microscopy (Blinzinger and Kreutzberg, 1968), but have since been repeatedly described in quantitative terms (for early studies see, e.g., Sumner and Sutherland, 1973; Chen, 1978). An even more detailed picture of these events has been obtained by examination of synaptic coverage of individual motoneurons, which have been filled with dyes that spread throughout the entire cell. Using this method it has been possible to show that a significant loss of synapses also occurs from motoneuron dendrites (Brännström and Kellerth, 1998). The vacated synaptic sites are rapidly covered by astroglial cells processes (Chen, 1978; Svensson and Aldskogius, 1993; see also Figure 3B), seemingly providing an increased "protection" or "isolation" of the neuron from surrounding influences.

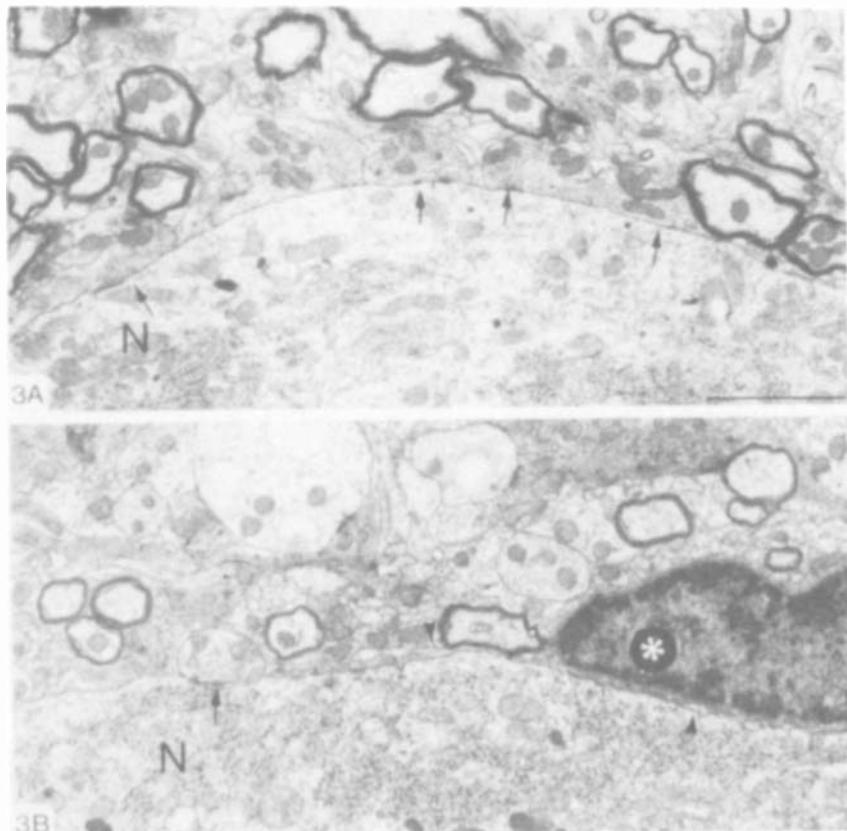


Figure 3. Electron micrographs demonstrating the effect on synaptic coverage of axotomizing motoneurons. (A) shows part of an intact motoneuron (N) with numerous synaptic terminals (**arrows**). (B) shows part of a seven day postaxotomy motoneuron (N). Only few synaptic terminals remain (**arrow**). A microglial cell (*****) is located close to the motoneuron, but separated from it by thin astroglial processes (**arrowhead**). Bar = 2 μ m. Reprinted with permission from Experimental Neurology (Svensson and Aldskogius, 1993), copyright, 1993, Academic Press).

C. Mechanisms Underlying Loss of Presynaptic Motoneuron Terminals

General Aspects on the Regulation of Motoneuron Responses to Axotomy

Current view emphasizes a multifactorial background for the reactions of motoneurons to axotomy (for review see Aldskogius and Svensson, 1993; Ambron and

Walters, 1996; see also Hayes et al., 1992). The early responses are probably initiated by the electric discharge set off by the injury ("the injury potential"), whereas later responses have a complex "molecular" basis: (1) the influx and subsequent somatopetal transport of molecules entering the injured axons ("positive signal"), (2) premature turnaround of ("abnormal") molecules normally transported anterogradely to the terminals to be modified prior to their return to the cell body, and (3) the depletion of retrogradely transported neurotrophic factors ("negative signal").

There is presently no hard evidence linking loss of presynaptic terminals to one or more of these mechanisms. It is perhaps significant that the downregulation of transmitter-associated molecules can be mimicked by blocking axonal transport in the intact motor nerve, and counteracted by local administration of brain-derived neurotrophic factor (BDNF), or neurotrophin (NT)-4/5 (Yan et al., 1994; Friedman et al., 1995; Tuszyński et al., 1996; Wang et al., 1997), or by access to degenerating peripheral nerve tissue (Rende et al., 1997). These findings indicate that features associated with maintenance of neural circuitry, perhaps including the normal presynaptic coverage, are critically dependent on the availability of target- and/or nerve-derived growth factors for the motoneurons. These mechanisms may cooperate with hormonal factors. Thus, administration of testosterone has been shown to significantly reduce loss of presynaptic terminals from axotomized motoneurons (Jones et al., 1997).

Perineuronal Glia May Participate in Removal of Presynaptic Terminals

Several previous studies have implicated perineuronal glial cells in the removal of presynaptic terminals from the surface of axotomized motoneurons. Already in the initial reports on the profound changes in the synaptic input to axotomized motoneurons it was noted that microglial cells were in strikingly close apposition to the terminals as well as to the corresponding site of the motoneuron soma (Blinzinger and Kreutzberg, 1968). This prompted the suggestion that microglia, a rapidly migrating cell with powerful phagocytic potential, are actively involved in synapse displacement.

Subsequent electron microscopic studies analyzing the neuron–glial structural relationships in detail came to the conclusion, however, that microglia were always excluded from the motoneuron cell membrane by sheetlike astrocytic processes, and would therefore probably not be in an efficient position for carrying out synapse displacement (Reisert et al., 1984; cf. Figure 3). Other studies already previously also emphasized the close interrelationship between reactive astrocytes and the axotomized motoneuron surface (Chen, 1978). The absence of any reports of actual phagocytosis of terminals by microglia may also be taken as evidence against a major role of these cells in synapse displacement. Further evidence in this direction are the results from experiments in which despite virtual eradication of microglial cells from the territory of axotomized motoneurons, displacement of

presynaptic terminals on axotomized motoneuron somata was not influenced (Svensson and Aldskogius, 1993).

Changes in Membrane Dimensions and/or Membrane Properties after Axotomy May Influence Synaptic Stability

An overwhelming proportion of presynaptic terminals on motoneurons is located on their dendrites. It is therefore highly significant that the dendritic tree of axotomized motoneurons undergoes a profound reduction in size (Bränström et al., 1992). The ensuing membrane instability in combination with a substantial “crowding” of presynaptic terminals may act as major forces in the shedding of synaptic terminals. We have previously suggested that changes in the presence, organization, and/or function of synapse anchoring proteins at the postsynaptic site are responsible for the displacement of presynaptic terminals on axotomized motoneurons. Previous studies have shown that postsynaptic receptor proteins and their mRNAs as well as ligand binding to these receptors are significantly down-regulated following motor axon injury (for review see Aldskogius and Svensson, 1993). Additional groups of molecules that are likely to be involved in regulating motoneuron synapse stability, but that have so far received little attention, are proteases (Turgeon and Houenaou, 1997), protease inhibitors (Osterwalder et al., 1996), and cell adhesion molecules (Schachner, 1997).

D. Functional Consequences of Loss of Presynaptic Motoneuron Terminals

Physiologic analyses of axotomized motoneurons have demonstrated a decrease in conduction velocity (Nishimura et al., 1992), an increased input resistance, a prolongation of the afterhyperpolarization (Gustafsson, 1979, Gustafsson and Pinter, 1984), an overall reduction in synaptic transmission (Mendell, 1988), and a particularly marked reduction in inhibitory input (Takata, 1981; Takata and Nagahama, 1984; Nishimura et al., 1992). There are also an increased influx of calcium, which may contribute to motoneuron degeneration (Laiwand et al., 1988; Tao and Aldskogius, 1998). The morphological changes reviewed above are often interpreted as processes serving to reduce “excitatory stress” of the axotomized motoneuron. In line with this notion in the finding of a downregulation of the expression of NMDA-receptor subunit protein mRNAs after axotomy (Piehl et al., 1995).

It is unclear, however, whether this mechanism helps motoneuron survival or restoration of the interrupted motor axon. It is possible that despite the apparent decrease in functional excitatory input, the axotomized motoneurons are more sensitive to the excitatory transmitter glutamate; e.g., blocking glutamate binding with MK801, an NMDA-receptor antagonist, significantly increases the survival rate of motoneurons following neonatal axotomy (Greensmith et al., 1994; Iwasaki et al., 1995).

From the foregoing discussion we hypothesize that the reduction in synaptic input to axotomized motoneurons is the result of inadequate trophic feedback from the target muscle. This situation suggests that the axotomized motoneurons "dedifferentiate" and may be prepared for a greater synaptic "flexibility" than in the intact nervous system. If so, is this useful for the functional adaptation of motor function after reinnervation of the denervated muscles?

A common complication following motor axon injury is that numerous regenerating axons will end up in the "wrong" muscle. The supraspinal regulation of coordinated muscle activity is based on a "body map" organization with motoneurons innervating different muscles located in separate "pools." This arrangement is typically deranged after regeneration of a damaged motor nerve. Numerous regenerating axons grow into the "wrong" branch and will therefore end up in an inappropriate muscle (for review see Aldskogius and Molander, 1990). The consequence will be poor coordination of activity of the reinnervated muscles, leading e.g., to so-called mass movements. A rearrangement of presynaptic terminals in line with the novel peripheral innervation pattern would remedy this lack of muscle coordination. However, to judge from the functional outcome, this plasticity appears to be very limited (see, e.g., Gruart et al., 1996). Thus, the potential for plasticity by the intrinsic connections of axotomized motoneurons appears to be insufficient to compensate for mismatches in motoneuron-muscle connectivity. These findings suggest that strategies to promote functional recovery after motoneuron injury should include attempts to maintain the presynaptic input.

III. REMOVAL OF PRIMARY SENSORY INPUT TO THE SPINAL CORD MAY OR MAY NOT INDUCE THE FORMATION OF NEW CONNECTIONS

A. Organization of the Sensory Input to the Spinal Cord

Sensory ganglion cells have a unique morphological organization, with their cell bodies located peripherally in sensory ganglia giving rise to dichotomizing processes, one branch projecting peripherally to innervate various peripheral target tissues, the other projecting centrally in the spinal cord or brainstem via spinal dorsal roots (cf. Figure 4).

The central terminations of primary sensory afferent processes are organized mainly according to two major principles in the spinal cord gray matter: (1) modality, i.e., in relation to sensory quality, and (2) somatotopy, i.e., reflecting specific body surface areas (Willis and Coggeshall, 1991). Nonmyelinated C-fibers, largely originating from small ganglion cells, convey impulses from nociceptors and thermoreceptors and terminate mainly in the dorsal part of the gray matter termed lamina II (Molander et al., 1984). Myelinated sensory fibers of larger calibers, and originating from large or medium-sized ganglion cells, mediate impulses from a wide range of non-nociceptive mechanical receptors and terminate more ventrally in lam-

iniae III–V or—with regard to sensory fibers from muscle spindles—even in the ventral horn. Although modalities are, in principle, segregated along the dorsoventral axis, the body surface is laid down mediolaterally with axis skin represented medially and the cutaneous innervation occupying the lateral part of the spinal cord dorsal horn.

B. The Response of Peripheral Sensory Nerve Injury Has Markedly Different Effects in the Spinal Cord Compared to Dorsal Root Injury

The sensory input to the spinal can be interrupted either by a lesion of the peripheral (peripheral nerve; Figure 4A) or of the central processes (dorsal root; Figure 4B) of sensory ganglion cells. After peripheral axotomy the central processes of the

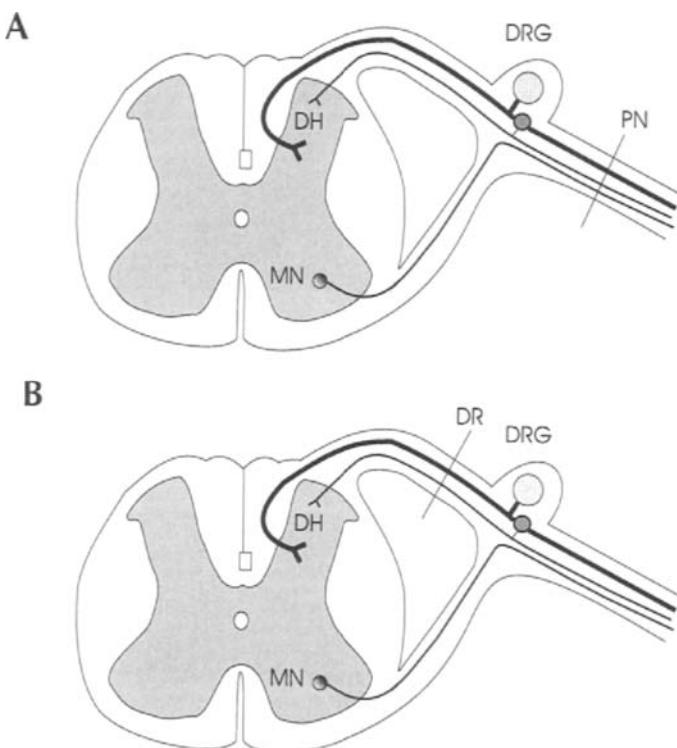


Figure 4. Dorsal root ganglion cells can be subdivided in a simplified manner in two major categories: small cells with nonmyelinated axons (darkly shadowed), which terminate in the superficial part of the dorsal horn, and large to medium-sized cells with myelinated axons (lightly shadowed), which enter the dorsal horn (DH) via the dorsal column and terminate more ventrally. The dorsal root ganglion cells can be axotomized in the peripheral nerve (A; PN) or in the dorsal root (B; DR). MN = Motoneuron.

injured sensory ganglion cells will be in continuity with the cell body permanently or at least for an extended period of time. These processes may, therefore, be modified by the cell body reaction. The regenerative response mounted by peripherally injured dorsal root ganglion cells will "spread" to the central processes (Richardson and Issa, 1984; Richardson and Verge, 1986). This is manifested, e.g., in an upregulation of the growth-associated protein GAP-43 (Woolf et al., 1990; Doubell et al., 1997), which has been strongly implicated in sprouting (Aigner et al., 1995). There is also a complex series of changes in the expression of neuropeptides and their receptors by peripherally axotomized sensory ganglion cells (Hökfelt et al., 1994). At the same time, degenerative features occur in their central processes, including a loss (Castro-Lopes et al., 1990) or atrophy (Coggeshall et al., 1997) of large numbers of terminals in the dorsal horn (cf. Figure 8). Thus, these responses, collectively often termed transganglionic changes (Aldskogius et al., 1985; 1992), display the typical contrasting pattern of regenerative and degenerative events (cf. section IIA).

In contrast, injury of the central processes effectively interrupts the connection between these processes and the ganglion cell body and the central processes will therefore undergo rapid and irreversible disintegration, leading to loss of stem axons as well as their associated primary afferent terminals (cf. Figure 7).

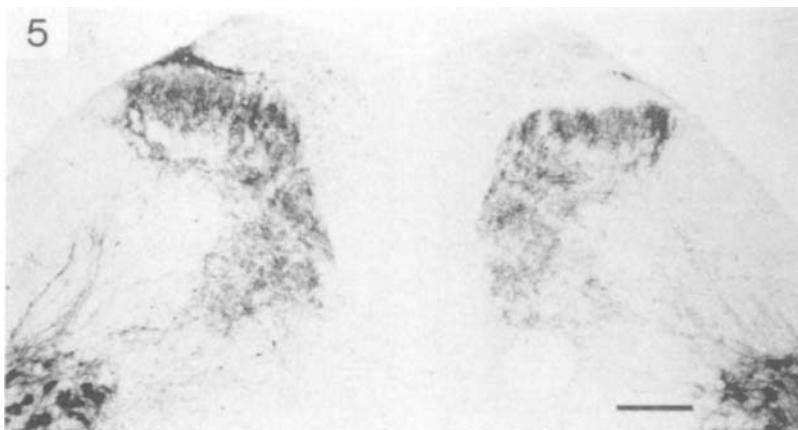


Figure 5. The distribution of primary sensory terminals in the spinal cord have been traced using "bulk" labeling from a peripheral nerve. The tracer cholera toxin B subunit (CTB), which labels myelinated afferents, has been injected into the intact (right) and injured (left) sciatic nerve (6 weeks postlesion survival time). Note the dorsal expansion by labeled fibers on the injured side. Reprinted with permission from the *Journal of Comparative Neurology* (Woolf et al., 1995), copyright, 1995, John Wiley & Sons.

Three principal aspects of plasticity of spinal cord primary sensory systems can be envisioned: (1) alterations in modality organization, (2) alterations in somatotopic organization, and/or (3) rearrangement of connections within the original modality or somatotopic territory. Given that these changes are presumed to involve the formation of new axons and/or axon terminals, these alterations would qualify for inclusion in the concept collateral sprouting or collateral expansion. If these newly formed collaterals make contact with postsynaptic neurons, the term *collateral reinnervation* would be appropriate.

C. Evaluating Anatomical Changes in Central Projections of Sensory Axons

The primary sensory projections to the spinal cord are typically visualized by using neuroanatomical tracers. After injection into a peripheral nerve or after application to its transected proximal stump these tracers are transported first retrogradely to the sensory ganglion cell bodies, and from there anterogradely into their terminals in the spinal cord, (so-called transganglionic transport; cf. Figure 5). The resolution of the tracing approach can be profoundly improved by injecting tracer into single functionally identified primary sensory axons and determine the precise distribution of these axons (cf. Figure 6). Finally, analyses of the distribution and labeling intensity of molecules expressed in the central terminals of primary sensory axons, such as the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) or the enzyme fluoride-resistant acid phosphatase (FRAP) have provided supplementary information.

D. Deafferentation Alone May Be Insufficient to Cause Expansion of Primary Sensory Afferents in the Spinal Cord

A commonly used experimental paradigm for analyzing plasticity in the spinal cord is the so-called "spared-root" preparation (Figure 7). Results from early studies exploring this paradigm suggested that neighboring intact dorsal root fibers extended into the denervated territory (Liu and Chambers, 1958). This interpretation has since been the subject of numerous studies, the majority of which have essentially confirmed the original findings (Hulsebosch and Coggeshall, 1981; Goldberger and Murray, 1982; McNeill and Hulsebosch, 1987; LaMotte et al., 1989; McNeill et al., 1990; Polistina et al., 1990; McNeill et al., 1991; Cameron et al., 1992; Florence et al., 1993; LaMotte and Kapadia, 1993), whereas other investigators have challenged the early findings and provided evidence that deafferentation alone results in no or minimal expansion by adjacent primary sensory neurons (Rodin et al., 1983; Molander et al., 1988; McMahon and Kett-White, 1991). This confusion presumably has several reasons, one of which could be injury-induced alterations in phenotypic expression or in ability to bind and transport exogenous tracers by the ganglion cells that are the subjects of study. Other

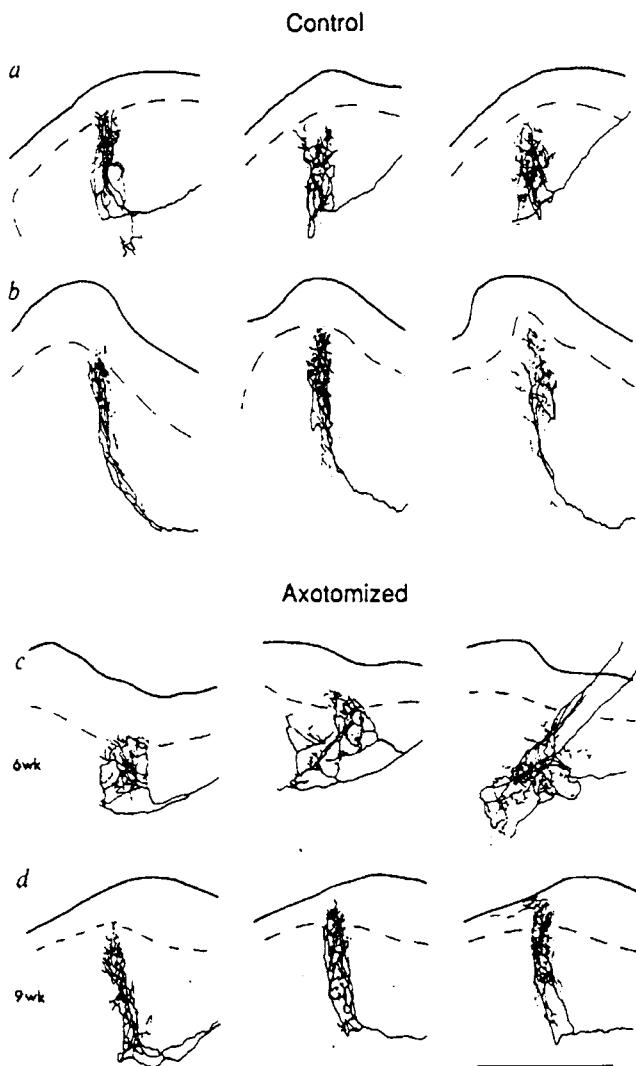


Figure 6. Camera lucida reconstructions of the terminal arbors in the spinal cord dorsal horn from two normal (**a, b**) and two peripherally axotomized (**c, d**) single myelinated sensory fibers of the sural nerve, a branch of the sciatic nerve containing almost exclusively cutaneous nerve fibers. The tracer horseradish peroxidase (HRP) was injected into the individual axons close to their entry in the spinal cord. The boundary between laminae II and III is indicated by the stippled lines. Terminal arbors of the intact sural nerve do not cross this boundary, whereas those belonging to axotomized primary afferents do. Bar = 250 μ m. Reprinted with permission from *Nature* (Woolf et al., 1992), copyright 1992, Macmillan Magazines Ltd.

methodological differences, including details of experimental approach and/or analytical techniques, are also likely to be involved. Only further studies will settle these controversies. At this point, it seems reasonable to make the conclusion that deafferentation alone is at best a weak stimulus for collateral sprouting.

A particularly illuminating approach developed in recent years has been to probe the sprouting response in the selected group of medium-sized to large-caliber A β primary afferents that project to laminae III–V. The central axons and terminals of this group of fibers can be selectively labeled after injection of the B-fragment of cholera toxin (CTB) into the peripheral nerve containing the peripheral processes of the corresponding sensory ganglion cells (cf. Figure 5). Using this approach it has been demonstrated that deafferentation by dorsal root injury does *not* prompt the neighboring primary sensory A β fibers to sprout outside their original territory (Mannion et al., 1998). The impact of this negative finding becomes particularly significant given that other peripheral sensory deafferentation injuries demonstrated strong evidence for sprouting by A β fibers (see section IIIE). The latter findings, therefore, serve as positive “controls.”

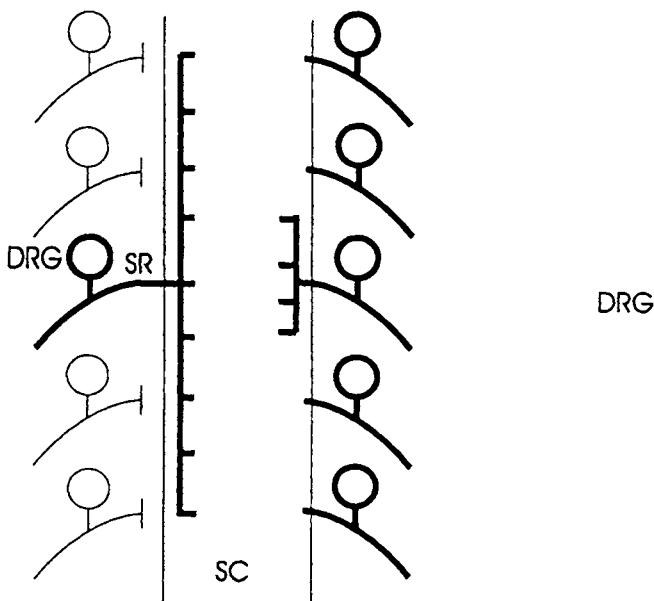


Figure 7. The principle of the “spared-root” model. A series of dorsal roots is cut on one side above and below an intact root (SR). According to some, but not other, studies (see text), there is an expansion of the termination area of the spared root compared to the contralateral uninjured side. DRG = dorsal root ganglion. SC = spinal cord.

However, even if there are substantial restrictions in the ability of primary sensory afferents to sprout after dorsal root injury, there are several observations compatible with modifications in intrinsic spinal cord systems as a result of the same injury (cf. Figure 6). The neuropeptide substance P (SP) is expressed by a subpopulation of sensory ganglion cells, but also by intrinsic spinal cord neurons. Following dorsal root injury, SP levels in the corresponding spinal cord segments drop, but subsequently return to virtually normal levels. This return is probably mediated by SP-expressing spinal cord neurons (Tessler et al., 1980, 1981, 1984; Wang et al., 1991; Zhang et al., 1993). One of the major descending supraspinal systems to the spinal cord gray matter is the serotonergic raphe spinal pathway. Some time after dorsal root injury the area in the spinal cord gray matter occupied by serotonergic fibers and terminals is markedly increased in the deafferented segments. (Polistina et al., 1990; Wang et al., 1991; Zhang et al., 1993; Kinkead et al., 1998). Finally, quantitative ultrastructural studies have shown that after an initial decline in synapse number, synaptic coverage in the spinal cord dorsal horn returns to normal levels (Murray and Goldberger, 1986; B. Zhang et al., 1995). These findings suggest that certain neuronal systems in the spinal cord have an advantage over the primary sensory neurons in increasing their projection after deafferentation by dorsal root injury.

E. Deafferentation by *Peripheral Nerve Injury* Is Sufficient to Produce Expansion of Primary Sensory Afferents

Injury to peripheral sensory nerve axons appears to induce more conducive conditions for sprouting in the spinal cord than complete deafferentation produced by dorsal rhizotomy. Peripheral sensory nerve injury interrupts the conduction of impulses from the peripheral target tissue to the spinal cord. However, although this implies a functional deafferentation vis-à-vis the peripheral target tissue, action potentials are still able to reach the spinal cord, either as a result of "spontaneous" or "nonphysiological" activity in the injured neurons. In the early period after injury, these action potentials may well be transmitted to postsynaptic neurons, perhaps preventing an early loss of primary sensory synaptic contacts in the spinal cord dorsal horn. Later on, there is a loss of primary sensory terminals (Castro-Lopes et al., 1990). At this stage, the affected synapses will therefore no longer be able to operate as sites for impulse transmission.

This sequence of transganglionic changes creates a condition which allows expansion of intact A β myelinated primary sensory afferents dorsally into laminae II, i.e., an area they do not normally occupy (Doubell et al., 1997). A variation of spinal cord deafferentation can be created by exposing a peripheral sensory nerve to the neurotoxin capsaicin, which causes selective degeneration of sensory C-fibers (Jancso and Lawson, 1990, Jancso 1992). This situation also results in expansion of intact A β -fibers into lamina II (Mannion et al.,

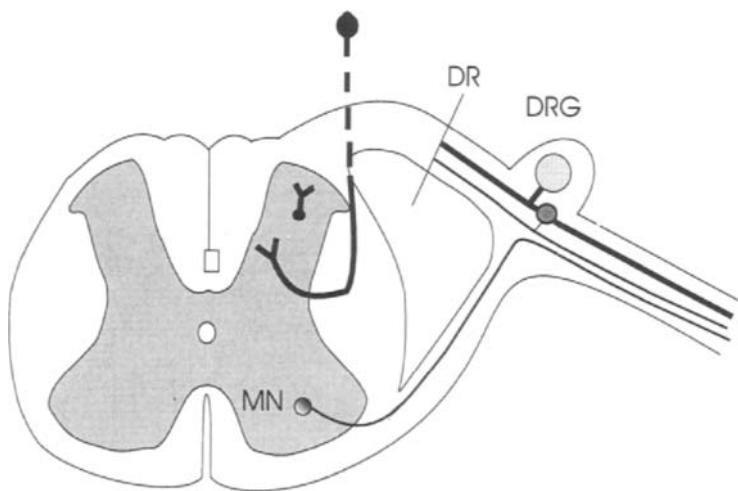


Figure 8. A lesion of the sensory processes in the dorsal root (DR) produces complete nerve fiber degeneration central to the injury, and induces sprouting by some local as well as some descending neuronal systems. DRG = Dorsal root ganglion. MN = Motoneuron.

1996). The results of these studies can therefore be interpreted as evidence for the view that deafferentation by peripheral nerve injury is a more efficient stimulus for primary afferent sprouting than dorsal root injury.

Peripheral Axotomy Enhances Central Expansion by Primary Sensory Afferents

Peripheral axotomy induces a regenerative mode throughout the injured sensory neurons (Richardson and Issa, 1984; Richardson and Verge, 1986; Woolf et al., 1990). The central processes of these neurons may therefore be “primed” for growth in this situation. Not surprisingly, the most extensive central expansion by primary sensory afferents into denervated territory appears to occur by afferents that have been injured themselves (Figures 5, 9). This expansion appears to occur among nonmyelinated (Molander et al., 1988) as well as myelinated (Woolf et al., 1992; Shortland and Woolf, 1993; Koerber et al., 1994; Woolf et al., 1995; see also Lekan et al., 1996) primary sensory afferents. This expansion can be at least transiently counteracted by the local administration of exogenous nerve growth factor (NGF; Bennett et al., 1996; Eriksson et al., 1997a). One of the mechanisms underlying this action is probably inhibition of the axotomy-induced central changes in the NGF-responsive C-fiber population in lamina II of the dorsal horn by substituting for the normal access of target-derived NGF.

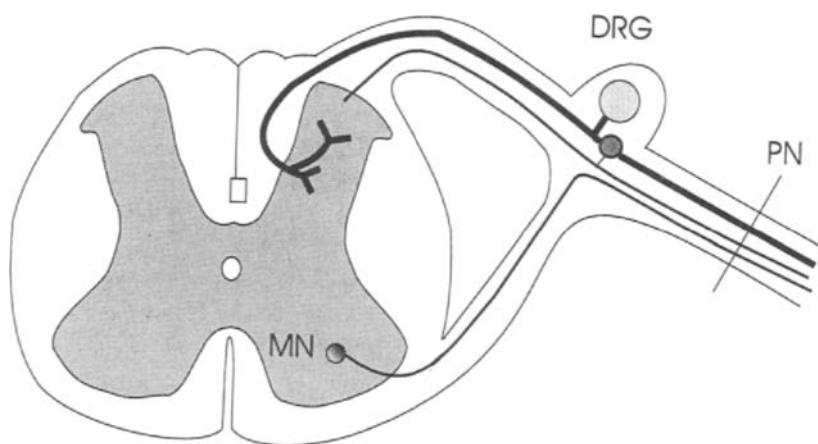


Figure 9. Diagram summarizing the main events after peripheral nerve injury (PN). Terminals from dorsal root ganglion (DRG) cells are lost in the superficial part of the spinal cord dorsal horn (thin line), and accompanied by an expansion of myelinated afferents into this area (bold lines). MN = Motoneuron.

E. Possible Factors Underlying Differences in Central Plasticity Following Deafferentation by Peripheral but Not Central Sensory Axon Injury

There is still considerable uncertainty about the details in the extent of collateral sprouting by primary sensory axons after different injuries outside the spinal cord. Nevertheless, existing information strongly indicate that it is highly significant for the anatomical outcome if spinal cord deafferentation occurs by injury to *peripheral* sensory processes or to the *central* processes. Revealing the mechanisms behind this difference is likely to help our understanding of how spinal cord plasticity is regulated. Presumably there are several cellular components involved in this regulation.

The Neuronal Response in the Spinal Cord Dorsal Horn

As emphasized above, following peripheral nerve injury the central processes are in continuity with their ganglion cell bodies for a considerable length of time, and are thereby able to influence the environment in the spinal cord dorsal horn in an “active” way. This may include the secretion of growth-promoting factors, such as brain-derived neurotrophic factor (BDNF), which is transported anterogradely to the spinal cord dorsal horn (Zhou and Rush, 1996). There is an upregulation of

the synthesis and its central transport after peripheral sensory nerve injury (Michael et al., 1997; Cho et al., 1998). This upregulation occurs in ganglion cells sensitive to nerve growth factor (NGF) (Verge et al., 1995), the majority of which projects to the superficial laminae of the spinal cord, i.e., the target area for much of the central sprouting taking place after peripheral nerve injury (Woolf et al., 1992; Shortland and Woolf, 1993). In this context the counteraction of primary afferent A β -fiber sprouting by exogenous NGF is obviously of interest. Moreover, studies *in vitro* have shown that the presence of neurotrophins produces a rapid, localized sprouting response from neurite shafts of sensory ganglion cells (Gallo and Letourneau, 1998). The recent findings that neurotrophins are involved in short-term postsynaptic signaling events in the central nervous system also adds to their potential influence on synapse reorganization (Lu and Figurov, 1997).

The Non-Neuronal Response in the Spinal Cord Dorsal Horn

There are marked differences in the glial cell reaction in the area affected by the peripheral nerve compared to dorsal root injury. The interaction between *astrocytes* and the postsynaptic neurons is presumably of crucial significance for the possibility of axonal extension and the formation of new synapses (for reviews see, e.g., Müller, 1992; Ridet et al., 1997). Astrocytes are presumably the main source of extracellular matrix molecules, which contribute to a nonpermissive environment for axon growth (Fawcett, 1997; Fitch and Silver, 1997). After dorsal root lesion, astrocytes proliferate and hypertrophy and appear to behave similar to “scar-forming” astrocytes (Liu et al., 1998; see also Aldskogius and Kozlova, 1998). The rapid disappearance of terminals after dorsal root injury may create a structural relationship between the vacated postsynaptic sites, which is unfavorable for the formation of new connections with neighboring primary sensory afferents. Peripheral nerve injury, on the other hand, although generating astrocytic hypertrophy (Gilmore et al., 1990; Hajós et al., 1990; Murray et al., 1990; Eriksson et al., 1997b), does not induce astrogliosis (Persson et al., 1995), and the products released from these cells may be quite different from the situation after dorsal rhizotomy.

Microglia are activated after peripheral sensory nerve injury as well as dorsal root injury (for review see Aldskogius and Kozlova, 1998), but the molecular expressions during this activation appears to be markedly different, e.g., after peripheral nerve injury these cells upregulate complement receptor 3 (CR3) expression as well as the synthesis and—presumably—release of complement components, including the terminal complex of complement (Liu et al., 1995), also called membrane attack complex (MAC; Walport, 1993). These components are, in fact, downregulated after dorsal root injury (Liu et al., 1998). This is in line with the interpretation that Wallerian degeneration in the CNS has a suppressive influence on the development of microglia to fully competent macrophages (Bell et al., 1994; Lawson et al., 1994).

Oligodendroglia and myelin-associated molecules, which appear to exert a significant inhibitory influence on injured axons in the CNS, also appear to counteract the potential for axonal expansion in the spinal cord (Schwegler et al., 1995; Schwab, 1996; Kapfhammer, 1997).

The Extracellular Environment in the Spinal Cord Dorsal Horn

Dorsal root lesion induces changes in the expression of cell adhesion molecules. The consequences of these expressions for axon growth in the adult spinal cord are unclear. Tenascin-C, which promotes axon growth under certain conditions (Faissner, 1997), is upregulated (Y. Zhang et al., 1995). There is an increased expression of the polysialylated isoform of the cell adhesion molecule N-CAM (Bonfanti et al., 1996), which promotes axonal extension and synaptogenesis during development (for review see Rutishauser and Landmesser, 1991). The axon guidance molecule, limbic system-associated membrane protein (LAMP), normally expressed by neurons in lamina II of the spinal cord, is first downregulated after dorsal root injury, but later upregulated on axons (presumably intrinsic ones) as well as postsynaptic neurons (Goldberger et al., 1993; Zhang et al., 1998).

How these and other molecules associated with axon growth and synapse formation are regulated after peripheral nerve injury is unknown. Another set of molecules of potential relevance in this context are the various guidance molecules, the coordinated expression of which has been shown to determine direction of axonal growth during development (Stoeckli, 1997).

Finally, proteases and extracellular calcium levels may be involved in controlling the expression and activation of molecules involved in sprouting. Activation of proteases, e.g. by microglial cell activity, may initiate a breakdown of axon stabilizing components, such as neurofilaments (Ziv and Spira, 1998). The membrane attack complex (MAC) of the complement system released by activated microglia binds to target cells, e.g., neurons, and is able to produce holes in the cell membrane through which water and ions, e.g., calcium, can enter (Walport, 1993). A limited amount of calcium influx stimulates cellular metabolism (Morgan, 1992), and could therefore promote sprouting and neurite extension (see below). Ongoing electrophysiological activity in peripherally injured primary sensory neurons may lead to calcium release in the spinal cord dorsal horn, which may in itself serve as a signal for sprouting (Amato et al., 1996; Kawano et al., 1997; Rajnicek and McCaig, 1997, Gitler and Spira, 1998), or may alter the normally relatively stable interrelationship between astrocytes and axon terminals (Ver nadakis, 1996).

After dorsal root injury the situation is obviously different. Huge amounts of extracellular calcium most probably enter the central processes of primary sensory axons because of inability of the disintegrating axons to regulate ionic fluxes across their membranes (cf. George et al., 1995). The subsequent fate of calcium "trapped" in the collapsing myelinated axons is completely unknown. It is possi-

ble, however, that this degeneration process does not provide the same possibility for a calcium-mediated "physiological" stimulation of axon or terminal sprouting.

F. Functional Consequences of Injury to Peripheral Sensory or Dorsal Root Axons

Sensory deafferentation of the spinal cord induces profound changes in connectivity in the spinal cord dorsal horn in a short-term as well as long-term perspective. These changes include expansions of receptive fields (Devor and Wall, 1978; Devor and Wall, 1981a, 1981b; Markus et al., 1984; Sugimoto et al., 1993; Koerber et al., 1994; Koerber and Mirnics, 1996; Wilson and Snow, 1987; Molander et al., 1998), changes in central excitability as well as changes in reflex activities (Wall and Devor, 1981; Woolf and Wall, 1982; Wall and Woolf, 1986). Although some of these changes may be explained by anatomical changes in connectivity, there is still a great deal of uncertainty about the significance of this process. It appears that alternative mechanisms such as disinhibition and sensitization from prolonged excitatory inputs play major roles in this situation. These aspects have been extensively reviewed elsewhere (Randic, 1996; Wilson and Kitchener, 1996; Baranauskas and Nistri, 1998). As pointed out above (Section I), the rearrangement of anatomical connections in the spinal cord dorsal horn following injury may be a significant factor underlying the development of neuropathic pain.

IV. CONCLUDING COMMENTS

As reviewed here, axon lesions outside the spinal cord commonly (or always?) create anatomical reorganization within a smaller or larger territory in the gray matter. Although motor axon injury appears to be inevitably associated with rearrangement of presynaptic terminals, sensory axon injury appears to have less predictable outcome, with the largest influences on the sensory systems occurring after peripheral sensory axotomy, rather than after the more extensive deafferentation induced by dorsal root injury. The anatomical reorganization is most probably accompanied by functional changes in the spinal cord circuitry, but these changes have been difficult to define, since synapse function can be altered after injury by several mechanisms not involving the formation of new connections (see section I).

Motoneurons and primary sensory neurons have marked anatomical and functional differences. Nevertheless, their response to peripheral axotomy shows principal similarities. Axotomized motoneurons retract part of their dendritic ramifications, and at least some of the peripherally injured sensory neurons withdraw their terminals in the spinal cord dorsal horn. Although conclusive evidence is lacking, available information indicate that both of these processes may be regulated by interruption of peripheral target contact. It is postulated that these

withdrawal processes in combination with the axotomy-induced non-neuronal responses and changes in extracellular matrix components form the key to understanding the fundamental differences in the anatomical and functional dynamics of spinal cord circuitry after peripheral nerve injury *vs.* lesions of central pathways.

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CERULOPLASMIN

STRUCTURE AND FUNCTION OF AN ESSENTIAL FERROXIDASE

Samuel David and Bharatkumar N. Patel

I.	Introduction	212
II.	Amino Acid Sequence and Protein Structure	213
	A. Primary Structure	213
	B. Secondary and Tertiary Structure	214
	C. Copper Centres	216
	D. Iron-binding	217
III.	Comparisons with Other Copper-Containing Proteins	219
	A. Comparisons with Azurin and Plastocyanin	219
	B. Comparisons with Ascorbate Oxidase and Laccase	220
	C. Comparisons with Coagulation Factor V and VIII	220
	D. Comparison with Yeast Fet3	221
IV.	Other Forms of Ceruloplasmin	222
	A. Membrane-Anchored Form	222
	B. Truncated Form	225
V.	Mutations in the Ceruloplasmin Gene	226
VI.	Role of Ceruloplasmin	227

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A.	Iron-induced Free Radical Formation	227
B.	Lack of Ceruloplasmin Leads to Neurodegeneration	229
C.	Iron-Mediated Neuronal Damage in Other Neurological Disorders	229
D.	Copper-Iron Connection in Other Diseases	230
E.	Antioxidant Effects of Ceruloplasmin	232
VII.	Concluding Remarks	233
	Acknowledgments	233
	References	233

I. INTRODUCTION

Ceruloplasmin was first identified in 1948 by Holmberg and Laurell as a plasma protein and given this name because of its sky-blue color (Holmberg and Laurell, 1948). Serum ceruloplasmin is secreted by the liver (Kingston et al., 1977), and is very abundant being present at levels of 300 to 450 µg/ml. Only a few other proteins such as albumin and transferrin are found at higher concentrations in serum. This glycoprotein of approximately 135 kDa carries 90 to 95% of the copper in serum. Ceruloplasmin has a long evolutionary history, and is found in mammals, birds, and reptiles (Holmberg and Laurell, 1948; Calabrese et al., 1988; Musci et al., 1993). In addition, a variety of oxidases in prokaryotes and eukaryotes have been shown to have certain structural similarities to ceruloplasmin.

Several functions have been attributed to ceruloplasmin based on both *in vitro* and *in vivo* data; including roles in copper transport, antioxidant defense, and iron metabolism. However, recent findings in patients with an inherited deficiency of ceruloplasmin (aceruloplasminemia), suggest that its major role *in vivo* is in iron metabolism and preventing free radical formation. The absence of ceruloplasmin leads to a striking degree of iron deposition in various tissues, and to severe neuropathological changes. These findings suggest that the primary function of ceruloplasmin is to oxidize the ferrous form of iron [Fe(II)] to the ferric form [Fe(III)] as proposed by Frieden and colleagues more than three decades ago (Osaki et al., 1966). Furthermore, recent studies have shed exceptional light on the structure of ceruloplasmin, leading to insights into the function of this molecule.

In this chapter we review the structure-function relationship of ceruloplasmin, compare its structure with those of other blue copper oxidases, discuss the phenotype resulting from mutations in the ceruloplasmin gene, and examine the recent evidence for different forms of ceruloplasmin in mammals. Particular attention will be paid in the latter part of this review to the important role of ceruloplasmin in mobilizing iron out of the brain and its potential role in neurodegenerative disease.

II. AMINO ACID SEQUENCE AND PROTEIN STRUCTURE

A. Primary Structure

The first complete sequence of human ceruloplasmin based on amino acid sequencing of the three major proteolytic fragments of 67, 50, and 19 kDa was reported by Putnam and colleagues (Takahashi et al., 1983). This analysis revealed that human serum ceruloplasmin was composed of a single polypeptide chain of 1046 amino acids and contained four potential sites for asparagine-linked glycosylation. The complete cDNA sequence of human ceruloplasmin was later determined by several groups, confirming the amino acid sequence data and further revealing the presence of a 19 amino acid N-terminal leader peptide (Koschinsky et al., 1986; Yang et al., 1986; Mercer and Grimes, 1986). The sequence of the signal peptide is consistent with it being a secreted protein. The complete cDNA sequence of rat ceruloplasmin was subsequently published by Fleming and Gitlin (1990), and more recently Gitlin's laboratory also published the cDNA sequence

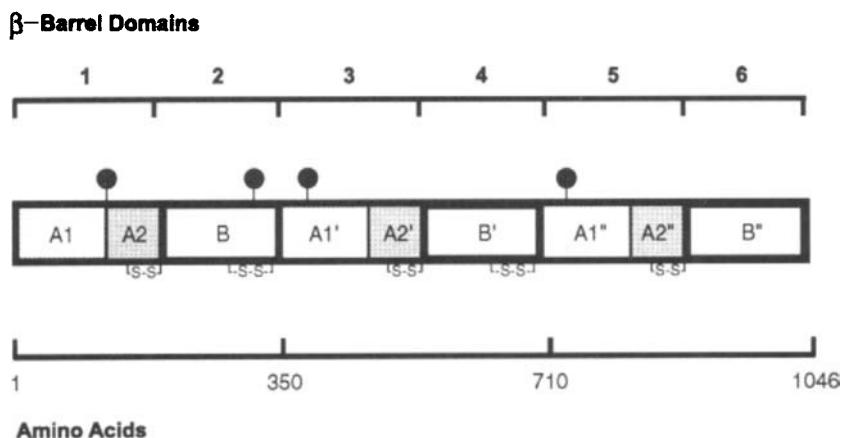


Figure 1. Schematic drawing of the structure of human ceruloplasmin illustrating the internal triplication. Each of the triplicated segments contains an A and B domain. The A domains can in turn be subdivided into A1 and A2 subdomains. The six β-barrel domains that correspond to the A and B domains are also indicated above. Thus domains 1, 3, and 5 are homologous to each other, as are domains 2, 4, and 6. Locations of potential glycosylation sites (•) and disulphide bonds (S-S) are also indicated. Note that the five disulphide bonds are located in domains 1 to 5. The amino acid ruler is shown below the Figure.

of mouse ceruloplasmin (Klomp et al., 1996). Comparisons of cDNAs from the three species reveals a remarkable sequence identity of greater than 90%.

Even prior to the complete amino acid sequence determination of ceruloplasmin, it was apparent that ceruloplasmin had an internal triplicated structure (Dwullet and Putnam, 1981; Takahashi et al., 1983; Ortell et al., 1984). It was found to be composed of three homologous segments of approximately 350 amino acids each (Figure 1) (Takahashi et al., 1983). Each of these segments are approximately 40% identical in sequence when compared pairwise and all three segments share an identity of approximately 30%. As expected, these homologies of the segments at the amino acid level also extend to the nucleotide level. In addition, detailed analysis of different proteolytic fragments of ceruloplasmin led Putnam and colleagues (Takahashi et al., 1983; Ortell et al., 1984) to propose that each of the three homologous segments in ceruloplasmin was composed of two or three distinct domains based on proteolysis studies and sequence comparisons (Figure 1). Each of these domains is most similar to the corresponding domain in the other segments. These results correspond well with the domain structure as revealed by earlier studies from several laboratories based on the chemical and enzymatic proteolysis of ceruloplasmin (Rydén, 1972; Kingston et al., 1977; Moshkov et al., 1979). From these studies emerged a six-domain model of ceruloplasmin that has been substantiated by electron microscopy studies (Samsonidze et al., 1979; Prozorovski et al., 1982), spectroscopic and calorimetric analyses (e.g., Bonaccorsi di Patti et al., 1990), and most recently by X-ray crystallography (Zaitseva et al., 1996; Lindley et al., 1997).

B. Secondary and Tertiary Structure

The six domains of ceruloplasmin are arranged in a triangular fashion (Figure 2) (Zaitseva et al., 1996). Each domain consists of a β -barrel composed of eight anti-parallel β strands that show similarity in their arrangement to that seen in other blue copper oxidases such as plastocyanin, azurin, and ascorbate oxidase (Messerschmidt and Huber, 1990; Zaitseva et al., 1996). A more detailed comparison of ceruloplasmin to these and other copper containing proteins is provided below. An extensive loop is present between β -strands 1 and 2 of each domain, and, since all six β -barrels point in the same direction, the six loops form a canopy on the "top" part of the molecule. A strong distribution of negative charge between these loops serves to attract positively charged ions such as Fe(II) to the copper centers (see below) and, together with the steric effects due to the loops, may account for the substrate specificity of ceruloplasmin. The three-dimensional configuration of the molecule is maintained in part by five disulphide bridges located in domains 1 to 5 (see Figures 1 and 2) (Takahashi et al., 1983; Messerschmidt and Huber, 1990; Zaitseva et al., 1996). These disulphide bonds are positioned along the "bottom" aspect of the molecule and connect the last β -strand (i.e., strand 8) of each domain to either strand 7 (in domains 1, 3, and 5) or strand 2b (in domains 2 and 4).

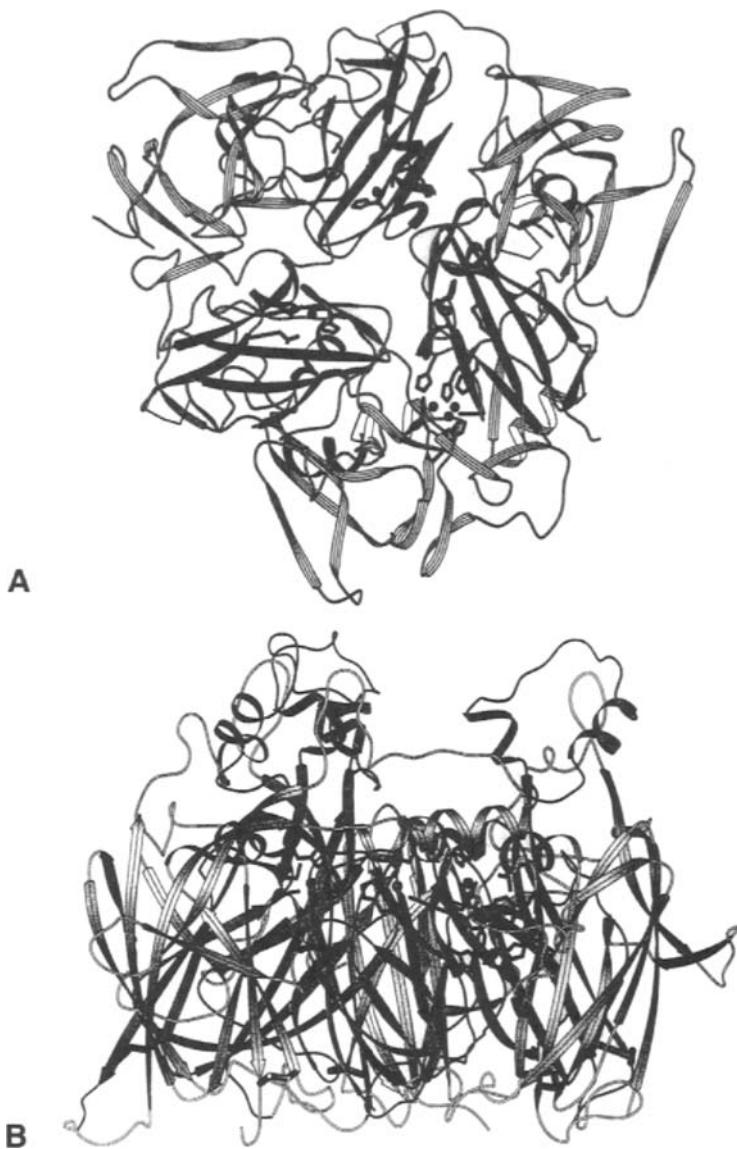


Figure 2. A view of the human ceruloplasmin molecule along the pseudo-threefold axis (**A**) and perpendicular to this axis (**B**). Note the trinuclear copper cluster in the bottom part of (**A**). The extended loops between the first and second strands of each of the β -barrels, which form a canopy over the "top" of the molecule is seen in figure B. (Reproduced from Zaitseva et al., 1996.)

(Zaitseva et al., 1996). The incorporation of copper into apoceruloplasmin also contributes to its tertiary structure, as removal of the copper results in the molecule acquiring a molten globule conformation (De Filippis et al., 1996).

C. Copper Centers

Early chemical and spectroscopic analyses indicated that ceruloplasmin contains six to seven copper atoms per molecule (Magdoff-Fairchild et al., 1969; Rydén, 1984; Calabrese et al., 1988, 1989). Analysis of the putative copper ligands based on comparisons with other copper-containing proteins, and direct structural analysis suggest the presence of three mononuclear copper binding sites in domains 2, 4, and 6 and a trinuclear copper center located at the interface

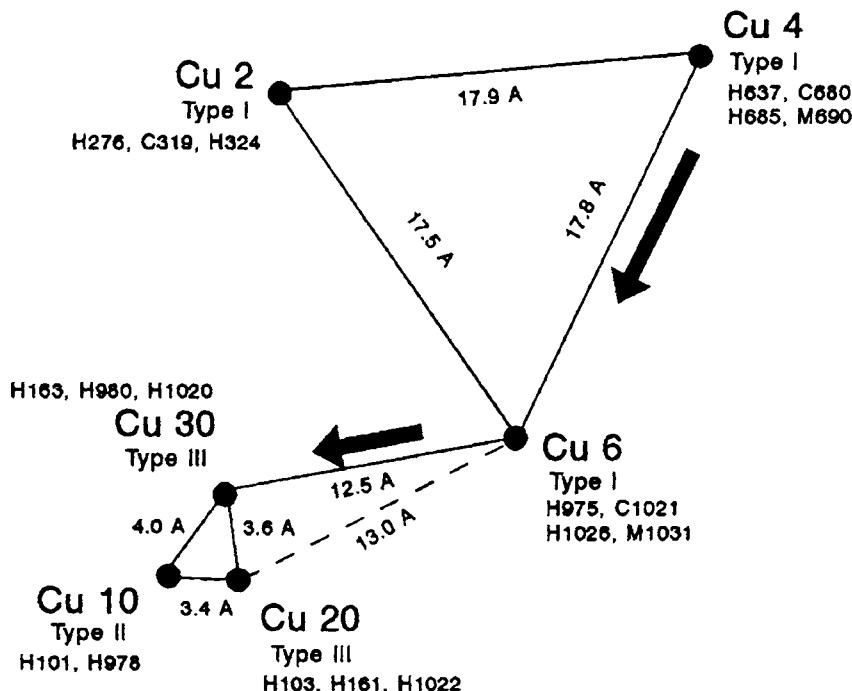


Figure 3. Schematic showing the positions of the various coppers in human ceruloplasmin. Cu 2, Cu 4, and Cu 6 represent mononuclear copper atoms in domains 2, 4, and 6, respectively. Cu 10, Cu 20, and Cu 30 represent coppers in the trinuclear copper center. The arrows represent the electron transfer pathway. The intercopper distances are in angstroms. (Figure modified from Zaitseva et al., 1996).

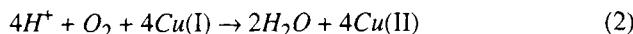
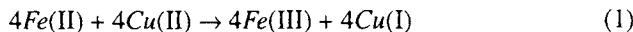
between domains 1 and 6 (Figure 2) (Takahashi et al., 1983; Ortel et al., 1984; Calabrese et al., 1989; Messerschmidt and Huber, 1990; Zaitseva et al., 1996; Lindley et al., 1997). These six coppers comprise the three mononuclear type I copper atoms, and one type II and two type III coppers, forming the trinuclear cluster. The mononuclear copper atoms serve to translocate electrons from Fe(II) iron to the trinuclear center, resulting in the reduction of dioxygen to two molecules of water (Calabrese et al., 1989; Zaitseva et al., 1996). It is thought that in the trinuclear center, one oxygen atom bridges the two type III coppers and another oxygen atom is attached to the remaining type II copper (Figure 3). A possible electron transfer pathway was recently proposed by Zaitseva et al. (1996) and is shown in Figure 3. Based on this model, the primary function of the single copper atoms in domains 2 and 4 might be to funnel electrons to the mononuclear copper in domain 6. The transfer of electrons from the mononuclear copper in domain 6 to the trinuclear cluster would, therefore, be of prime importance to the ferroxidase activity.

Histidine residues serve as important ligands for the coppers (Figure 3) (Ortel et al., 1984; Messerschmidt and Huber, 1990; Zaitseva et al., 1996). The three copper atoms in the trinuclear center at the interface of domains 1 and 6 are bounded by four pairs of histidines. Two of these pairs are derived from domain 1 (H101, H103; H161, H163), and two pairs from domain 6 (H978, H980; H1020, H1022). The cysteine that separates the last pair (C1021) acts as a ligand for the mononuclear copper in domain 6, thus linking this mononuclear copper site with the trinuclear copper center. At each of the mononuclear copper sites (in domains 2, 4, and 6), in addition to one pair of histidine residues, the copper is liganded by a cysteine and a methionine (except in domain 2 where the methionine is lacking) (Ortel et al., 1984; Messerschmidt and Huber, 1990; Zaitseva et al., 1996). The replacement of the methionine with a leucine in the domain 2 copper site can be expected to result in a high redox potential as is seen with laccase (Messerschmidt and Huber, 1990).

In addition to these integral copper sites, two labile copper binding-sites have been identified in domains 4 and 6 and are 9 to 10 Å distant from the nonlabile sites (Lindley et al., 1997). These labile binding sites are thought to be occupied by copper based on X-ray fluorescence analysis and show 50% occupancy. Two additional copper-binding sites have also been seen under extreme experimental conditions (Lindley et al., 1997). It has been proposed that the labile binding sites could be used for copper transport by ceruloplasmin as copper would be easily released from these sites (Lindley et al., 1997).

D. Iron Binding

As is the case for ascorbate oxidase and laccase, ceruloplasmin oxidizes its substrate [Fe(II)] with the concomitant reduction of molecular oxygen. Molecular oxygen binds to the trinuclear cluster and is reduced to two molecules of water by the transfer of four electrons from the substrate:



X-ray diffraction studies suggest that plausible sites for the binding of ferrous iron are the two labile copper-binding sites in domains 4 and 6 (Lindley et al., 1997). These domains bind copper weakly and it appears that binding Fe(II) to both sites involves displacement of the labile coppers. The Fe(II) is oxidized at the labile binding sites to Fe(III) and then shifted to a nearby "holding site" composed of negatively charged side chains (Lindley et al., 1997). The electron is then transferred from the nearby mononuclear coppers to the trinuclear copper cluster, where it partially reduces molecular oxygen. The oxidation of four Fe(II) atoms leads to the complete reduction of molecular oxygen. Ceruloplasmin has a high affinity (low K_m value of 3.9 μM) for molecular oxygen and is about 90% saturated at physiological O_2 concentration in venous blood of about 30 μM . This helps ensure that ceruloplasmin completely reduces molecular oxygen to water and does not generate toxic oxygen radicals (Hassett et al., 1998).

Because both domains 4 and 6 contain these labile binding sites and holding sites, the possibility exists that ceruloplasmin might be able to bind and oxidize two Fe(II) ions. The oxidation of Fe(II) at the mononuclear copper site in domain 6 may proceed faster than at the mononuclear copper in domain 4, as the electron has a shorter path to travel from the former copper to the trinuclear cluster (Lindley et al., 1997). This type 1 copper in domain 6 might correspond to the "fast" type 1 copper that is quickly reoxidized by molecular oxygen (Carrico et al., 1971; De Ley and Osaki, 1975; Sakurai and Nakahara, 1986; Messerschmidt and Huber, 1990) and therefore might be the most relevant type 1 site. This might be functionally significant, as the rate-limiting step in the ferroxidase activity of ceruloplasmin is likely to be the electron transfer from the reduced type 1 copper to the trinuclear center (Hassett et al., 1998). The type 1 copper in domain 2 does not appear to participate in the oxidation of Fe(II), as it is permanently reduced and redox-inactive (Machonkin et al., 1998). The inability of this copper to be oxidized under physiological conditions appears to be due to its high reduction potential (~1.0 V) because of the substitution of the ligand methionine with a leucine (Machonkin et al., 1998; Lindley et al., 1997). In addition, this copper lacks a nearby Fe(III) holding site, further suggesting that it does not participate in the ferroxidase activity (Lindley et al., 1997). These data support earlier findings showing two different standard redox potentials for ceruloplasmin (Deinum and Vännngard, 1973).

It is not yet known whether the Fe(III) at the holding sites is available for direct transfer to apotransferrin, or whether the Fe(III), which is much less soluble than Fe(II), is first chelated by small chelating molecules prior to transfer to apotransferrin. The possibility that Fe(III) might be first transferred to another protein is suggested by studies showing that ceruloplasmin can directly associate

with ferritin and load iron to it (Reilly et al., 1998; Juan and Aust, 1998). Whether direct transfer of Fe(III) from ceruloplasmin to apotransferrin occurs is not yet known.

III. COMPARISONS WITH OTHER COPPER-CONTAINING PROTEINS

Ceruloplasmin shares homologies to a number of copper-containing proteins found in animals, plants, and fungi. Among these are the cupredoxins azurin and plastocyanin, the oxidases ascorbate oxidase, laccase, and cytochrome oxidase, and other serum proteins such as coagulation factors V and VIII. Recently, a protein that mediates high-affinity iron uptake in the yeast, *Saccharomyces cerevisiae*, called Fet3, was shown to be a copper-binding ferroxidase with homology to ceruloplasmin (Askwith et al., 1994; De Silva et al., 1995; Yuan et al., 1995; De Silva et al., 1997).

A. Comparisons with Azurin and Plastocyanin

Although ceruloplasmin is a large multi-copper oxidase containing at least three different spectroscopically distinguishable types of copper, it nonetheless shares common features with the bacterial electron transfer protein azurin (Messer-schmidt and Huber, 1990; Adman, 1985) and the plant electron transfer protein plastocyanin (Colman et al.; 1977). These cupredoxins are small proteins of approximately 10 to 14 kDa consisting of a single domain in the form of a β -barrel (Adman, 1991). They have a single type 1 (blue) copper, characterized by an intense absorption near 600 nm, and an electron paramagnetic resonance (EPR) spectrum of $A < 95 \times 10^{-4} \text{ cm}^{-1}$ (Adman, 1991). In both azurin and plastocyanin, the type 1 copper is bound by a trigonal bipyramidal geometry of ligands consisting of a methionine sulfur and a peptide carbonyl oxygen on either side of a triangular plane formed by two histidine nitrogens and a cysteine sulfur. The β -barrel structure of azurin is thought to facilitate electron transfer as the β -sheet structure is more effective at mediating long-range couplings than other structures such as the α -helix. This is in part due to the nearly linear coupling pathways along the polypeptide backbone in the β -sheet spanning 3.4 Å per residue compared to only 1.5 Å per residue for the coiled α -helix (Beratan et al., 1991; reviewed by Gray and Winkler, 1996).

Ceruloplasmin is composed of six domains, each consisting of a β -barrel similar to that found in the small cupredoxins. The three mononuclear coppers in ceruloplasmin are coordinated in a similar manner to the copper in azurin and plastocyanin. The coppers in domains 4 and 6 have the typical type 1 copper ligands with two histidines, a cysteine, and a methionine. The remaining mononuclear copper in domain 2 is coordinated in a similar manner, but lacks the methionine ligand.

B. Comparisons with Ascorbate Oxidase and Laccase

Although there is an unmistakable resemblance of ceruloplasmin to the single-copper proteins azurin and plastocyanin, ceruloplasmin is more similar to the multicopper oxidases ascorbate oxidase and laccase. Ascorbate oxidase is a plant protein whose role is not fully known, but it may be involved in plant growth as it is most abundant in the cell wall (Ohkawa et al., 1989). This protein usually exists as a dimer of two 68 kDa subunits and is highly specific for its substrate, ascorbate. Laccase is a 64 kDa protein found in tree sap and in fungi, and oxidizes aromatic amines and phenolic compounds and may be involved in the breakdown of lignin (Germann and Lerch, 1986). Like ceruloplasmin, both of these proteins reduce dioxygen in the process of oxidizing their substrates. Although ascorbate oxidase, laccase, and ceruloplasmin have different substrates, the strategies that the enzymes use to oxidize their disparate substrates are strikingly similar. Laccase contains four copper atoms: one type I, one type II, and two type III coppers (Germann et al., 1988). Ascorbate oxidase has a similar complement per subunit of each of the three types of copper as seen in laccase (Ohkawa et al., 1989). Both laccase and the ascorbate oxidase monomer consist of three domains, each of which is a β -barrel structure similar to the individual domains of ceruloplasmin. The type I copper in ascorbate oxidase is located in domain 3 and the remaining three coppers form a trinuclear cluster between domains 1 and 3. This arrangement is nearly identical to that in laccase and very similar to that in ceruloplasmin. Domains 1 and 3 of ascorbate oxidase correspond to domains 1 and 6 of ceruloplasmin (Messerschmidt and Huber, 1990). The trinuclear cluster in all three are formed by one type II and two type III coppers.

The homology of ceruloplasmin to other multicopper oxidases, such as ascorbate oxidase and laccase, led to the proposal that these proteins may have evolved from a two-domain ancestral protein that was itself likely derived via gene fusion or gene duplication of an azurin-like gene (Dwulet and Putnam, 1981; Rydén, 1988). Ascorbate oxidase and laccase would then have acquired a third genetic element giving rise to their three domain structure. This basic two-domain structure is triplicated in ceruloplasmin. The various possible evolutionary pathways leading to these copper-binding oxidases have been discussed previously (Ortel et al., 1984; Messerschmidt and Huber, 1990; Zaitseva et al., 1996).

C. Comparisons with Coagulation Factors V and VIII

Although ceruloplasmin has homologies to the multi copper oxidases mentioned above, its greatest homologies are to the plasma coagulation factors V and VIII. The complete cDNA sequence for both factor V (Jenny et al., 1987) and factor VIII (Vehar et al., 1984; Toole et al., 1984) are available. Amino acid sequence alignments of factor V, factor VIII, and ceruloplasmin indicate the presence of highly conserved domains between these proteins. All three molecules show

evidence of internal triplication (Vehar et al., 1984; Toole et al., 1984; Jenny et al., 1987). Factors V and VIII have a similar domain structure and these molecules share a 40% amino acid sequence identity in domains A and C. Ceruloplasmin shares a 30 to 35% sequence identity with the three A domains of both factors V and VIII, which are arranged in a triangular fashion similar to that in ceruloplasmin (Pan et al., 1995; Villoutreix and Dahlback, 1998). This homology of ceruloplasmin to the coagulation factors in the A domains is only slightly lower than the homology between the different A domains of ceruloplasmin itself (30% - 40%). The homologies between these three proteins extend as well to the secondary and tertiary structures. As in ceruloplasmin, the A domains form a β -barrel structure (Pan et al., 1995; Pemberton et al., 1997). Although neither factor V nor factor VIII demonstrates oxidase activity, both contain a single type II copper atom per molecule (Mann et al., 1984; Bihoreau et al., 1994) which is located at the interface between domains A1 and A3 (Pan et al., 1995; Villoutreix and Dahlback, 1998). This copper atom contributes to the conformation of the proteins and, in the case of factor VIII, there is evidence that the presence of this single type II copper mediates intersubunit interactions and increases the catalytic activity of the protein (Bihoreau et al., 1994; Pan et al., 1995; Tagliavacca et al., 1997; Pemberton et al., 1997; Sudhakar and Fay, 1998). These striking similarities suggest that ceruloplasmin, factor V, and factor VIII are derived from an ancestral gene that had already undergone internal triplication (Dwulet and Putnam, 1981; Takahashi et al., 1983; Ortel et al., 1984). The strong homologies between the A domains of the three proteins suggest a requirement for conservation within this domain. The other domains in factor V and factor VIII that do not share homologies with ceruloplasmin suggests that each segment has mutated independently in a divergent manner (Church et al., 1984).

D. Comparison with Yeast Fet3

Fet3p, a ferroxidase identified in the yeast *S. cerevisiae*, is a protein that most resembles ceruloplasmin with respect to function although ceruloplasmin demonstrates greater structural homologies with the proteins mentioned above. Fet3p was identified as a protein involved in the high affinity uptake of iron in *S. cerevisiae* (Askwith et al., 1994; Dancis et al., 1994; De Silva et al., 1995; Yuan et al., 1995; De Silva et al., 1997). Mutations in the *FET3* gene abolish the ability of yeast to grow in medium containing low amounts of iron (Askwith et al., 1994). The sequence of Fet3p revealed that it was homologous to ceruloplasmin as well as to the other multicopper oxidases laccase and ascorbate oxidase. Further studies demonstrated that Fet3p contained copper and was a cell surface ferroxidase that had a transmembrane and a short cytoplasmic domain (De Silva et al., 1995). In addition, comparison of the rate of Fe(II) oxidation to O₂ consumption gave a stoichiometry of approximately 4:1, the same as that for ceruloplasmin (Osaki, 1966) and consistent with the reduction of molecular oxygen to water. Thus, it appears

that Fet3p is structurally and functionally similar to ceruloplasmin. Alignment of the sequence of Fet3p with ceruloplasmin, laccase, and ascorbate oxidase revealed that Fet3p has two multicopper oxidase motifs (De Silva et al., 1995). The second of these motifs, amino acids 483–494 in Fet3p, contains the conserved sequence HCHXXXH that in ceruloplasmin is found near the C-terminal and that contributes ligands for the mononuclear copper in domain 6, as well as the type III coppers in the trinuclear copper cluster. EPR spectrum analysis of Fet3p and analysis of the copper content revealed that Fet3p likely has one each of the three types of copper atoms (Hassett et al., 1998). A comparison of the rates of Fe(II) oxidation revealed that ceruloplasmin was more effective as a ferroxidase than Fet3p, having a 50-fold greater rate of Fe(II) turnover (Hassett et al., 1998). The lower catalytic activity of Fet3p might be due to the presence of a coordinating leucine (as suggested by sequence alignment) for the type I copper instead of the methionine present in ceruloplasmin, leading potentially to a higher reduction potential and consequently slower electron transfer from the type I copper to the type II and type III coppers at the O₂-binding site. Detailed structural analysis of Fet3p, including the nature of its copper-binding sites, substrate binding, and three-dimensional structure are needed for a more complete comparison to ceruloplasmin and the other multicopper oxidases.

IV. OTHER FORMS OF CERULOPLASMIN

A. Membrane-Anchored Form

Until recently, ceruloplasmin was considered to be exclusively a soluble protein secreted by the liver into the plasma, and by the choroid plexus into the cerebrospinal fluid (CSF). Serum ceruloplasmin does not cross the blood–brain barrier, and its level in the CSF is extremely low (Del Principe et al., 1989). Our recent work has led to the discovery of a novel membrane-bound form of ceruloplasmin in rats that is localized preferentially on the surface of a population of non-neuronal cells (astrocytes) in cultures of the neonatal rat central nervous system (CNS) (Patel and David, 1997) (Figure 4). Similar cell surface expression of ceruloplasmin has also been detected in leptomeningeal cells (which cover the surface of the brain), and some fibroblasts. The cell surface localization of ceruloplasmin on astrocytes and leptomeningeal cells appears to be unique, as hepatocytes and cells of the choroid plexus, both of which are known to produce ceruloplasmin, do not express this molecule on the cell surface (Figure 4). Evidence for a cell surface form of ceruloplasmin was obtained from immunofluorescence labeling by incubating live CNS cell cultures with either a monoclonal antibody (1A1) generated in our laboratory or a polyclonal anti-ceruloplasmin antiserum. Although in our earlier reports we were unaware of the identity of the cell surface antigen recognized by our monoclonal antibody (Mittal and David, 1994a, 1994b), our later studies

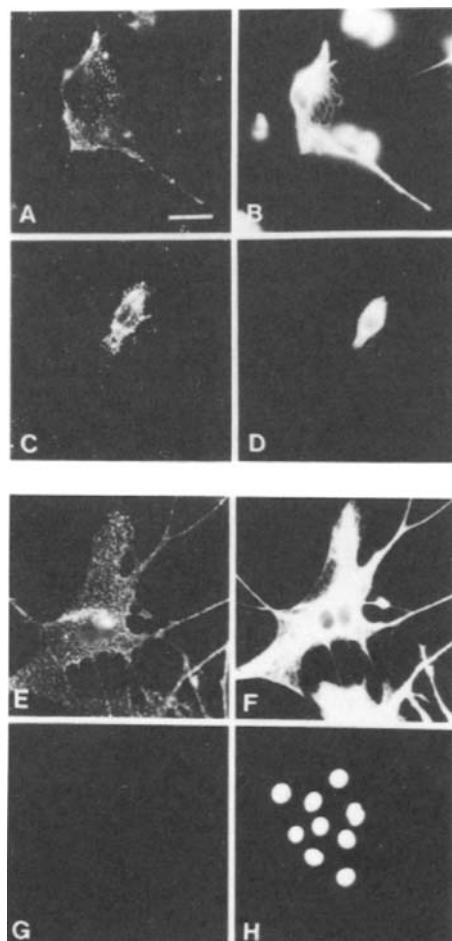


Figure 4. Immunofluorescence labeling showing cell surface localization of ceruloplasmin on the surface of neonatal rat astrocytes stained with either a polyclonal anticeruloplasmin antibody (**A**) or monoclonal anticeruloplasmin antibody (**E**). Similar cell surface staining is also seen on rat C6 glioma cells (**C**) but not on cells of the choroid plexus (**G**). Antiglial fibrillary acid protein labeling of these astrocytes is shown in **B**, **D**, and **F**. Nuclear yellow staining of choroid cells is shown in **H**. Bar = 30 μ m. (Reproduced from Patel and David, 1997.)

showed it to be ceruloplasmin (Patel and David, 1997). These earlier studies in which we carried out radioiodination of cell surface proteins on cultured astrocytes followed by immunoprecipitation also confirmed that this molecule is associated with the astrocytic plasma membrane (Mittal and David, 1994a). We and

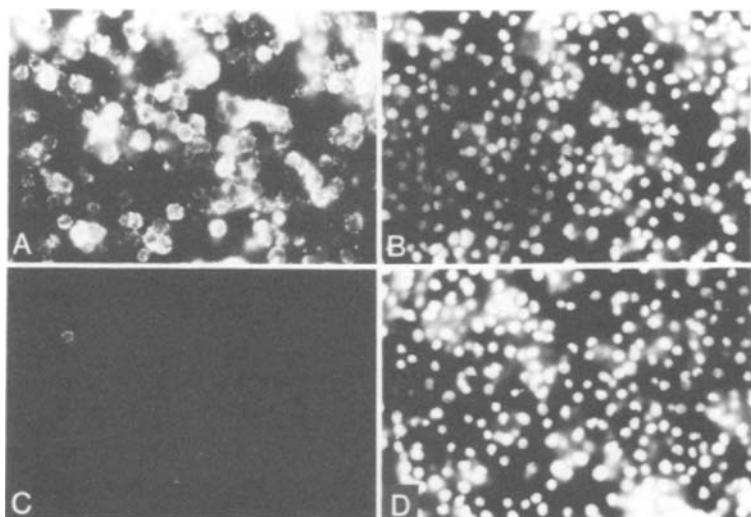


Figure 5. PI-PLC treatment of cell suspensions of C6 glioma cells removes cell surface labeling with anticeruloplasmin antibody (**C**). Nuclear yellow labeling of the same field is shown in (**D**). Control cells stained identically but without PI-PLC treatment show robust staining with anticeruloplasmin antibody (**A**). Nuclear yellow staining of corresponding field (**B**). (Reproduced from Patel and David, 1997.)

others have also shown by metabolically labeling astrocyte cultures with ^{35}S -methionine that astrocytes synthesize ceruloplasmin (Zahs et al., 1993; Mittal and David, 1994a; Klomp et al., 1996). Our initial monoclonal antibody blocking experiments indicated that the membrane-bound form of ceruloplasmin might mediate weak adhesive interactions *in vitro* between neurons and astrocytes (Mittal and David, 1994a, 1994b). These interactions may be mediated via a pentapeptide (YIGSK in humans) that is similar to the YIGSR cell adhesion recognition sequence found in the extracellular matrix molecule laminin (Graf et al., 1987). Whether such adhesive interactions occur *in vivo* is not yet known.

We have also shown that the surface localization of ceruloplasmin on astrocytes and C6 glioma cells can be removed by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC), which specifically cleaves glycosylphosphatidylinositol (GPI) anchors from GPI-linked proteins (Patel and David, 1997) (Figure 5). Additional evidence for GPI anchoring of ceruloplasmin was obtained by immunoaffinity-purifying ceruloplasmin cleaved from the surface of C6

glioma cells by PI-PLC and Western blotting the immunoaffinity purified ceruloplasmin with a polyclonal antibody that specifically recognizes GPI anchors. This antibody reacted strongly with membrane-bound ceruloplasmin purified from C6 glioma cells, but not with similarly purified ceruloplasmin from rat serum (Patel and David, 1997). Furthermore, our studies also demonstrated that GPI-anchored ceruloplasmin possesses oxidase activity, indicating that it also functions as a ferroxidase. Peptide-mapping studies indicated further that the membrane-bound and secreted forms of this molecule differ mainly in the GPI-anchoring C-terminal region. Our results have been confirmed by the work of Salzer et al. (1998) who also demonstrated that the GPI-anchored form of ceruloplasmin is the major GPI-anchored protein on the surface of astrocytes and is also present on the surface of Schwann cells.

Several earlier studies have reported the presence of ceruloplasmin mRNA in the brain by Northern blotting (Thomas et al., 1989; Gaitskhoki et al., 1990; Shvartsman et al., 1990; Yang et al., 1990; Klomp and Gitlin, 1996; Klomp et al., 1996) and RNAase protection assays (Klomp and Gitlin, 1996). Ceruloplasmin mRNA has been localized *in vivo* to certain astrocytes in the retina and brain by *in situ* hybridization (Klomp et al. 1996; Klomp and Gitlin, 1996; Levin and Geszvain, 1998). Increased expression of ceruloplasmin has been detected in retinal ganglion cells and glial cells in the retina after optic nerve crush injury (Levin and Geszvain, 1998). We have also observed widespread distribution of ceruloplasmin in the adult mammalian brain (Doroudchi and David, unpublished observations). Our recent studies indicate that the GPI-anchored form of ceruloplasmin is the major form in the brain (Patel et al., in press). The two forms of ceruloplasmin are derived from a single gene because only one chromosomal locus for ceruloplasmin has been identified (Royle et al., 1987).

B. Truncated Form

A 125 kDa truncated form of ceruloplasmin has been identified in the liver and bile, and is thought to be the major biliary isoform (Davis et al., 1996). Earlier work also showed a lower molecular weight copper-binding protein in bile that cross-reacted with anticeruloplasmin antibodies (Iyengar et al., 1988). It has been proposed that the 125 kDa form of ceruloplasmin may serve as a copper carrier for the excretion of copper via the bile (Davis et al., 1996; Chowrimootoo et al., 1997). Studies by Davis et al., (1996) indicate that the 125 kDa ceruloplasmin is not derived from deglycosylation of the full-length 132 kDa form. It may be the product of a different mRNA transcript. If this truncation involves the C-terminal end of the molecule, it would result in the loss of the trinuclear copper center and thus loss of ferroxidase activity (see below). A role for copper excretion/transport could therefore be a likely function for such an isoform.

Table 1. Known Mutations in the Ceruloplasmin Gene

Mutation	Consequence	References
5 bp insertion at nucleotide 1287	Frameshift producing truncated polypeptide of 445 amino acids	Miyajima et al., 1987 Harris et al., 1995
G to A point mutation at nucleotide 2630	Nonsense mutation changing Trp ⁸⁵⁸ to a stop codon-truncated polypeptide of 857 amino acids	Daimon et al., 1995a, 1995b Takahashi et al., 1996
Adenine insertion at nucleotide 607	Frameshift producing truncated polypeptide of 187 amino acids	Okamoto et al., 1996
Deletion of nucleotide 2389 (G)	Frameshift producing truncated polypeptide of 788 amino acids	Harris et al., 1996
5 bp deletion (nt 3019–3023) in mRNA	Defective splicing producing truncated polypeptide of 990 amino acids	Yoshida et al., 1995
A to G point mutation at splice acceptor site of intron 6	Defective splicing producing truncated polypeptide of 387 amino acids	Yazaki et al., 1998

V. MUTATIONS IN THE CERULOPLASMIN GENE

The human ceruloplasmin gene is located on chromosome 3q21–25 (Yang et al., 1986; Royle et al., 1987) and serum ceruloplasmin is encoded by 19 exons spanning greater than 50 kb of genomic DNA (Daimon et al., 1995b). At the time this chapter was written, six different mutations in the human ceruloplasmin gene have been identified. The mutations are recessive and homozygous patients with this condition aptly named aceruloplasminemia develop motor deficits, retinal degeneration, and dementia (for review, see Gitlin, 1998; also see below). Six different mutations have been identified in seven different families (Table 1). All of these mutations produce truncated polypeptides that are expected to lack ferroxidase activity. Three of the six mutations (Miyajima et al., 1987; Harris et al., 1995; Okamoto et al., 1996; Yazaki et al., 1998) produce severely truncated polypeptides that would lack any of the tertiary structure of ceruloplasmin. The more limited truncations (Yoshida et al., 1995; Daimon et al., 1995a; Takahashi et al., 1996; Harris et al., 1996) might still maintain most of the tertiary structural features of wild-type ceruloplasmin, but are unlikely to retain ferroxidase activity, as they would at least lack the C-terminal 75 amino acids that provide crucial ligands for the mononuclear copper in domain 6 as well as for the coppers in the trinuclear cluster. However, even in the least truncated mutations (Yoshida et al., 1995; Daimon et al., 1995a; Takahashi et al., 1996), ceruloplasmin is either at least 100-fold lower in patients compared to control subjects (Yoshida et al., 1995) or undetect-

able (Takahashi et al., 1996). This suggests that the mutant ceruloplasmin does not undergo appropriate cellular processing and might be prematurely degraded.

VI. ROLE OF CERULOPLASMIN

The structure of ceruloplasmin, the similarities of its copper centers to those of several other copper oxidases, and the striking findings of excessive iron deposition in particular in the liver and brain of people with aceruloplasminemia point strongly to an important role in mobilizing iron out of tissues via its ferroxidase activity. The presence of labile copper binding sites suggests that it may also function to some extent as a copper transporter as originally proposed (Harris, 1993). However, the ferroxidase function of this molecule appears to be its primary role. Ceruloplasmin is therefore involved primarily in maintaining iron homeostasis and preventing iron-mediated free radical injury.

A. Iron-Induced Free Radical Formation

Iron is the central component of a number of metalloenzymes, such as aconitase, and the cytochromes of the electron transport chain. On the other hand, free iron can generate highly toxic free radicals because it is a redox-active transition metal. Ferrous iron can react with hydrogen peroxide to produce ferric iron leading to the formation of hydroxyl radicals (Fenton reaction) (Figure 6). Also, via the Haber–Weiss reaction, iron can react with molecular oxygen to yield a variety of reactive oxygen species, such as superoxide anions, hydrogen peroxide, and hydroxyl radicals (Figure 6). Several enzymes and substrates exist that can neutralize or prevent free radical formation. These include among others, superoxide

Fenton Reaction



Haber-Weiss Reaction

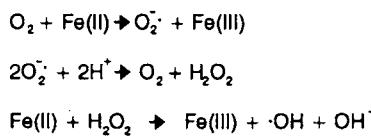


Figure 6. Fenton and Haber–Weiss reactions.

dismutase, glutathione peroxidase, glutathione, catalase, ascorbic acid, and ceruloplasmin. Ceruloplasmin plays an important role in preventing the formation of free radicals by controlling the levels of highly toxic iron within cells.

A number of enzymes, binding proteins, and transporters have been identified that are involved in mobilizing, transporting and sequestering iron (De Silva et al., 1996; Jefferies et al., 1996; Kaplan and O'Halloran, 1996). In mammals, transferrin, a protein that tightly binds ferric iron appears to be the major iron carrier in plasma, transporting iron from sites of storage, such as the liver to tissues utilizing iron. The iron-loaded transferrin binds to a high-affinity receptor on the cell surface and is subsequently internalized via clathrin-coated vesicles. The bound iron is eventually released following acidification of these vesicles (Jefferies et al., 1996). The oxidation of ferrous iron to the ferric form is necessary for iron incorporation into transferrin, as transferrin binds largely the ferric form. Ceruloplasmin appears to play a key role in the oxidation of ferrous iron and hence its release from cells and loading onto apotransferrin (Young et al., 1997). In the absence of ceruloplasmin, iron fails to be mobilized out of tissues and accumulates in cells as is seen in aceruloplasminemia. This interpretation is also supported by studies of patients and mice with hereditary atransferrinemia. In this condition, a lack of transferrin results in a failure of iron to be mobilized out of sites of storage, such as the liver, to tissues utilizing iron. This results in severe iron deposition in the liver and other organs, as well as anemia caused by the lack of iron available to erythroid cells (Bernstein, 1987; Hamill et al., 1991).

Although receptor-mediated uptake of iron bound to transferrin is clearly an important mechanism of iron mobilization for numerous tissues and organs, certain cell types have been found to possess a transferrin-independent mechanism of iron uptake. This mechanism of iron uptake has been identified in several cell types (Sturrock et al., 1990) and it might play a particularly important role in cells that do not express the transferrin receptor. It has been suggested that this transferrin-independent system might also be important for iron transport into the liver because in atransferrinemia dietary iron accumulates in this organ despite the lack of transferrin-mediated iron uptake (Dickinson et al., 1996). Although the identity of the iron transporter is still not known, iron uptake by this system appears to require the activity of a cell surface ferrireductase (Jordan and Kaplan, 1994; reviewed by De Silva et al., 1996), suggesting that Fe(III) iron must first be reduced to Fe(II) prior to uptake. Given this requirement for reduction of iron, ceruloplasmin with its oxidase activity might function to inhibit iron uptake by this transferrin-independent uptake system by reducing the level of Fe(II) available for transport. Indeed, this possibility is supported by the recent identification of an iron uptake system by cells that is stimulated by hydroxyl radicals generated by the Fe(II)-catalyzed Fenton reaction (Richardson and Ponka, 1995). Ceruloplasmin was able to completely inhibit iron uptake by this system, most likely by limiting the amount of Fe(II) iron available to catalyze the Fenton reaction (Richardson and Ponka, 1995).

Although the major physiological role of ceruloplasmin is to mobilize iron out of tissues, and thus prevent iron accumulation, recent work suggests that under conditions of severe iron deficiency, ceruloplasmin may increase the uptake of iron by certain cell types (Mukhopadhyay et al., 1998; Attieh et al., 1999). Even though the mechanism of iron uptake by this system is not known, it appears to involve a putative metal transporter that is specific for trivalent cations, including Fe(III), that is activated in iron-deficient cells (Attieh et al., 1999).

B. Lack of Ceruloplasmin Leads to Neurodegeneration

The strongest evidence that ceruloplasmin plays a role in preventing the accumulation of intracellular iron comes from patients with aceruloplasminemia. This is a rare autosomal recessive disorder in which mutations in the ceruloplasmin gene leads to almost undetectable levels of serum ceruloplasmin (Miyajima et al., 1987; Logan et al., 1994; Harris et al., 1995; Morita et al., 1995; Yoshida et al., 1995; Harris et al., 1996; Okamoto et al., 1996; Takahashi et al., 1996; Miyajima et al., 1998). These patients develop motor incoordination and other neurological symptoms between the ages of 40 to 50 years. Liver biopsies reveal coarse granular deposits of iron in the cytoplasm of hepatocytes and Kupffer's cells (Morita et al., 1995). These cytoplasmic iron deposits were seen within secondary lysosome-like structures as well as larger nonmembrane bound inclusions. At autopsy, iron deposition was seen in a number of organs including the liver, pancreas, kidney, heart, spleen, thyroid gland, and CNS. The most severely affected organs were the liver, pancreas, and CNS. Iron accumulation in the CNS varied in different regions and was detected in a number of areas such as the caudate nucleus, putamen, globus pallidus, thalamus, dentate nucleus of the cerebellum, frontal cortex, red nucleus, substantia nigra, and retina. Both glia and neurons were found to possess iron deposits. Another striking feature of the CNS pathology is the neuronal degeneration that is seen in many of these regions (Morita et al., 1995; Yoshida et al., 1995). In some areas, such as the caudate and putamen, the severe degeneration led to cavitations (Morita et al., 1995). The neurodegeneration appears to be a consequence of oxidative stress induced by iron deposition as discussed above. In support of this, Miyajima et al., (1998) have reported increases in the level of iron and of lipid peroxidation in the CSF of individuals with aceruloplasminemia.

C. Iron-Mediated Neuronal Damage in Other Neurological Disorders

Oxidative stress has been proposed to play a role in the pathogenesis of a number of neurodegenerative diseases (Olanow and Arendash, 1994). Besides aceruloplasminemia, iron deposition has been observed in the CNS regions that are affected in several neurodegenerative diseases, such as, amyotrophic lateral sclerosis (Oba et al., 1993; Yasui et al., 1993; Ince et al., 1994; Kasarskis et al., 1995);

Hallervorden-Spatz disease (Halliday, 1995), Parkinson's disease (Hirsch et al., 1991; Sofic, 1991; Yasui et al., 1993), and in the cerebral cortex and amyloid plaques in Alzheimer's disease (Connor et al., 1992; Good, 1992). Whether a lack of ceruloplasmin localized to certain regions of the CNS contributes to the iron deposition seen in these conditions is yet to be established.

There are reports that in Alzheimer's disease there is a reduction in ceruloplasmin in the cerebral cortex (Connor et al., 1993), as well as a marked reduction in ceruloplasmin oxidase activity in the plasma (Snaedal et al., 1998). However, other reports have shown increases in ceruloplasmin in the brain in Alzheimer's disease and this has been interpreted as possibly reflecting increased oxidative stress or an acute phase response (Loeffler et al., 1996). In Parkinson's disease, the auto-oxidation of dopamine not only produces toxic semiquinone radicals (Schipper et al., 1991), the hydrogen peroxide produced by this reaction can react with ferrous iron to produce highly toxic hydroxyl radicals, which can cause lipid peroxidation and cell death. Molecular oxygen can also react with ferrous iron to generate hydroxyl radicals. Furthermore, saturation of melanin (which is found in the substantia nigra pars compacta, the brainstem region affected in Parkinson's disease) with large amounts of iron results in the formation of hydroxyl radicals (Kienzl et al., 1995). It is therefore possible that a lack of ceruloplasmin could contribute to iron-mediated free radical injury in this disease, but this has yet to be established.

In addition to the iron deposition caused by aceruloplasminemia, earlier studies demonstrated that rapidly growing animals made copper deficient developed anemia associated with severely diminished levels of serum ceruloplasmin and plasma iron, accompanied by accumulation of iron in tissues (Gubler et al., 1956; Lee et al., 1968). Interestingly, administration of ceruloplasmin to such animals resulted in mobilization of iron from the liver and its loading onto transferrin. These results further point to the important role ceruloplasmin plays in the mobilization and egress of iron from the liver. It likely also plays a similar role in the egress of iron from other tissues and organs, including the brain.

D. Copper-Iron Connection in Other Diseases

The copper-iron connection has been most clearly demonstrated in humans with Wilson's disease (for review, see Brewer and Yuzbasiyan-Gurkan, 1992). Wilson's disease is an autosomal recessive disorder of copper transport with symptoms arising in late childhood due to copper toxicity. These patients have marked copper accumulation in a number of organs, including the brain and liver, with associated hepatic disease and neurological symptoms of motor impairment as well as psychiatric disorders. Neuronal degeneration occurs in various parts of the brain, including the cortex, basal ganglia, brainstem, and thalamus. A reduction in the level of plasma ceruloplasmin in patients with Wilson's disease was discovered as early as 1952 (Bearn and Kunkel, 1952; Scheinberg and Gitlin, 1952).

However, no significant disturbance of iron metabolism has been observed in Wilson's disease, suggesting that the low level of ceruloplasmin that is present in Wilson's disease is nonetheless sufficient to permit normal iron metabolism. The reason for the reduction of ceruloplasmin and lack of its oxidase activity in Wilson's disease was identified with the cloning of the gene responsible for the disease. A number of laboratories identified mutations in a copper-transporting P-type ATPase that is expressed in the liver and kidney (reviewed by Chelly and Monaco, 1993). The deficiency in ceruloplasmin oxidase activity results from the lack of incorporation of copper into ceruloplasmin. In the absence of copper the molecule likely acquires a conformation that makes it more susceptible to proteolytic degradation thus accounting for the lower serum levels. This has been most extensively investigated in the Long-Evans cinnamon coat (LEC) rat model of Wilson's disease, which has a mutation in the rat homologue of the Wilson's disease gene (*WND*). In the rat model, synthesis of apoceruloplasmin is normal, with normal levels in plasma (Yamada et al., 1993). However, most of the ceruloplasmin in the circulation is in the apo- form and contains no detectable copper, and therefore accounting for the lack of ceruloplasmin oxidase activity in the serum. This observation is consistent with the localization of the *WND* gene product to the Golgi apparatus (Payne et al., 1998) where it would be involved in copper incorporation into copper-containing proteins such as ceruloplasmin. Indeed, recent studies have demonstrated that retrovirus-mediated expression of the wild-type *WND* gene product leads to restoration of holoceruloplasmin synthesis and ceruloplasmin oxidase activity in the LEC rat (Terada et al., 1998).

Disruption of copper homeostasis is also a hallmark of Menkes disease, which is also caused by mutations in a copper-transporting P-type ATPase that shares 60% identity with the Wilson's disease ATPase (reviewed by Hamer, 1993). Ceruloplasmin levels and oxidase activity are also severely reduced in Menkes disease. Unlike the Wilson's disease protein, the P-type ATPase affected in Menkes disease is expressed in all cell types except the liver (Paynter et al., 1994; Kuo et al., 1997) and is localized to the endoplasmic reticulum, trans-Golgi apparatus, and vesicular compartment (Ackland et al., 1997; La Fontaine et al., 1998). It also functions to convey copper to the secretory pathway (Yamaguchi et al., 1996; Payne and Gitlin, 1998). Because the Menkes gene product is not expressed in the liver but is expressed in the brain and other tissues, the expression of ceruloplasmin in the brain would be expected to be affected, especially since serum ceruloplasmin does not cross the blood-brain barrier. In contrast, in Wilson's disease, ceruloplasmin expression in the brain is unlikely to be affected, as the Wilson's gene is expressed primarily in the liver and kidney. The fact that serum ceruloplasmin levels are reduced in Menkes disease in which the defective P-type ATPase is not expressed in the liver, suggests that other factors also regulate ceruloplasmin gene expression in the liver in this disease. These findings warrant a closer examination of the contribution of iron to the pathological changes seen in the CNS in Menkes disease.

E. Antioxidant Effects of Ceruloplasmin

Ceruloplasmin is thought to be an important antioxidant in serum (Gutteridge and Quinlan, 1993). It has been shown to be effective in inhibiting lipid peroxidation stimulated by iron and copper (Gutteridge, 1983; Yamashoji and Kajimoto, 1983). Ceruloplasmin inhibits Fe(II) and Cu(II)-catalyzed lipid peroxidation by about 70 to 85%. Ceruloplasmin has also been shown to inhibit superoxide and ferritin-dependent lipid peroxidation of phospholipid liposomes (Samokyszyn et al., 1989). It can also block superoxide-mediated reductive mobilization of iron from ferritin. Ceruloplasmin may reincorporate iron into ferritin, thus reducing the amount of toxic ferrous iron (De Silva and Aust, 1993; Reilly et al., 1998; Juan and Aust, 1998). Other studies have also shown that ceruloplasmin can block free radical-induced carbonyl formation in cellular proteins in endothelial cells *in vitro* (Krsek-Staples and Webster, 1993). Ceruloplasmin combined with desferroxamine was also able to prevent ischemia-reperfusion damage in kidney transplantation *in vivo* (Baron et al., 1994). It also had a protective effect against free radical damage to perfused rat heart in an *ex vivo* model (Chahine et al., 1991). In this model ceruloplasmin was more effective in preserving cardiac function as compared with equimolar concentrations of superoxide dismutase. It has also been proposed to serve antioxidant functions in rheumatoid arthritis (Gutteridge, 1986), aging (Prothro, 1989), and immunity (Yang et al., 1996). Ceruloplasmin has also recently been shown to be capable of oxidizing and detoxifying the catecholamine neurotoxin 6-hydroxydopamine (Medda et al., 1996). Taken together, these findings suggest that ceruloplasmin likely plays an important role in protecting a variety of tissues from free radical injury.

VII. CONCLUDING REMARKS

Although ceruloplasmin is a very abundant serum protein, its three-dimensional structure was only recently obtained. The elucidation of its tertiary structure, its copper-binding centers, as well as the striking phenotype of patients with aceruloplasminemia resulting from mutations in the ceruloplasmin gene, has provided a clearer understanding of the role of this glycoprotein in iron homeostasis. The recent discovery of a novel GPI-anchored form of ceruloplasmin in the brain raises interesting questions as to the need for such a modification in the brain. Another important question concerns the relative levels of this membrane-anchored form and the secreted form of ceruloplasmin in the brain. Given the frequent observation of iron accumulation in a variety of neurodegenerative diseases, it is of vital importance to establish the role ceruloplasmin plays in these diseases. Because there is only a single gene locus for ceruloplasmin, work needs to be done to characterize the tissue and cell-type specific promoters and enhancers that regulate the

expression of the different forms of ceruloplasmin in different regions of the central nervous system.

In addition, a more detailed understanding of the function of ceruloplasmin and its role in iron metabolism also requires identification of other proteins that help maintain iron homeostasis. The recent discoveries of the gene mutated in the iron overload disease hereditary hemochromatosis and the gene mutated in mice with microcytic anemia (*mk* mice) are beginning to provide a clearer view of iron metabolism. The gene mutated in hereditary hemochromatosis is called *HFE* and encodes a type I transmembrane glycoprotein that is homologous to class I major histocompatibility complex proteins (Feder et al., 1996). *HFE* protein binds the transferrin receptor and reduces its affinity for iron-loaded transferrin, thereby reducing the rate of iron entry into cells by receptor-mediated endocytosis (Lebrón et al., 1998). The mutation in the *mk* mice is in a membrane-spanning iron transport protein called *Nramp2* that transports Fe(II) iron into the cell by proton-coupled active transport. *Nramp2* is ubiquitously expressed with relatively high levels found in the duodenum and the brain (Gruenheid et al., 1995; Gunshin et al., 1997). A clearer understanding of the precise role of ceruloplasmin in the movement of iron in and out of cells will have to await the identification and characterization of other proteins involved in iron transport.

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EXPRESSION OF A NOVEL NUCLEAR PROTEIN IS CORRELATED WITH BRAIN DEVELOPMENT

Arumugham Raghunathan and Mohan C. Vemuri

I.	An Overview of Transcriptional Factors/DNA Binding Proteins in the Nervous System	240
II.	Structural Designs of the Transcription Factors Involved in CNS Development	240
	A. Homeobox (Helix-Turn-Helix)	240
	B. Helix-Loop-Helix	241
	C. Leucine Zippers	242
	D. Zinc Fingers Proteins	242
	E. HMG Proteins	243
III.	A Novel Nuclear Brain-Specific DNA-Binding Protein.	244
	Acknowledgments	258
	References	258

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I. AN OVERVIEW OF TRANSCRIPTIONAL FACTORS/DNA BINDING PROTEINS IN THE NERVOUS SYSTEM

The central nervous system (CNS) comprises an enormous array of neuronal phenotypes that originate and develop from the neural tube. Such a diverse cellular phenotypic development involves both positive and negative transcriptional regulation. Following cell genesis, neurons transmit the information by their electrophysiological potential and by activating second messenger signal cascades, which in turn recruit and activate constitutive transcription factors (CTFs), to produce inducible transcription factors (ITFs). These factors orchestrate transcriptional regulation in tune with the neuronal responses (Armstrong and Montminy, 1993).

Over the last 10 years, several transcription factors involved in the early development of the CNS, particularly in neural induction, maturation, and specification stages of development have been identified (Scotting and Rex, 1996). These transcription factors share specific structural designs common to several eukaryotic DNA binding proteins (DBPs) and contribute to CNS development through their precise timing of their expression.

II. STRUCTURAL DESIGNS OF THE TRANSCRIPTION FACTORS INVOLVED IN CNS DEVELOPMENT

Most of these transcription factors share a structural design in their DNA binding motif and the amino acid sequences in these motifs in general parallel with the structure of eukaryotic DBPs. So far, at least about eight different structural variants in the transcription factors involved in CNS development have been identified and the search is still far from complete. The variants include the following.

A. Homeobox (Helix-Turn-Helix)

These were first identified in bacteria as regulatory proteins and their three-dimensional structure revealed that they bind to DNA as dimers and have three α -helical regions separated by short turns in their structure. This arrangement was termed the *helix-turn-helix* motif. It was shown that amino acid substitutions in the α -helices nearest to the carboxyl end of each subunit of the dimer disturb the binding in the major groove of the DNA. These findings together with X-ray studies showed clearly that a protein helix in each of the dimers occupies the major groove in two successive turns of the DNA helix. When the nucleotide sequences of many homeotic genes were studied, it was seen that a region encoding about 60 amino acids was remarkably conserved in all these genes. This region, called the *homeobox*, is conserved in genes from *Drosophila* to mammals.

Several homeobox containing transcription factors involved in CNS development have been identified. Well-recognized members include *Hox*, *Pou*, *XPOU2*, *Otx*, *Emx*, and *Dlx*. *Hox* genes are expressed in many parts of the CNS except in the forebrain and midbrain (Guazzi et al., 1998). *Otx2* is another factor belonging to the homeodomain, which shows regional restricted expression in the forebrain. This was isolated in mice and humans as a counterpart of the *orthodenticle* gene in *Drosophila* involved in the development of the head in flies (Simeone et al., 1993). POU proteins are strongly involved in the development of the nervous system (Pevny and Lovell-Badge, 1997). POU factors contact target DNA in two adjacent major grooves with their homeodomain and a juxtaposed POU-specific domain (Klemm et al., 1994). POU domain was originally identified in the mammalian proteins Pit-1, Oct-1, and Oct-2. There are about 40 POU proteins identified in animals from nematodes to mammals. These proteins are grouped into six subclasses, with members of class III and class IV being expressed predominantly in the developing and adult nervous system (Herr et al., 1988). XPOU2 is one such case that is involved in the early stages of development contributing to neural induction (Witta et al., 1995). *Emx* and *Dlx* are transcription factors with homeodomains that are expressed in the early stages of anterioposterior specification in CNS development (Rubenstein et al., 1994).

B. Helix-Loop-Helix

A group of proteins that bind to a consensus sequence CANNTG (also known as the E box), share a common motif that is proposed to result in two amphipathic helices interrupted by a loop, called the helix-loop-helix (HLH) motif. HLH domains mediate dimerization and a basic region located near the N-terminal of the HLH domain is responsible for DNA binding. HLH proteins are ubiquitous and tissue specific. The interplay among the HLH proteins is particularly interesting, as it helps to understand the regulation of tissue specific gene expression (Kadesch, 1993).

Several members of basic HLH transcription factors are expressed in restricted domains of the developing nervous system (Guillemot, 1995). Good examples of HLH factors include MASH, HES1, and NeuroD. Neurogenins, which are expressed during the differentiation of autonomic and olfactory sensory neurons, are implicated in neuronal determination factors, as overexpression of neurogenins induces ectopic neurogenesis (Ma et al., 1996). Mash (Guillemot et al., 1993), HES1 (Ishibashi et al., 1994), and Neuro D (Lee et al., 1995) are expressed in a distinct pattern, which suggests that they might be responsible for the diversity of neuronal cell types (Ma et al., 1997). However, the mechanisms regulating the expression of these transcription factors remain still to be determined.

C. Leucine Zippers

The first and most extensively studied protein of this large group is CCAAT/enhancer binding protein beta (C/EBP β), which is a transcriptional regulator of the basic leucine zipper family. A helical domain with leucines projecting uniformly on one side is a common feature in the members of this family. In the proposed DNA binding motif of C/EBP, which is a dimer, the structural models describe the protein dimerization domain as a region with a repeat of five leucines (L1 to L5) forming leucine zipper and an adjacent DNA-binding domain containing clusters of basic amino acids. The leucine zipper is highly conserved in several other transcription factors, identified in many non-nuclear proteins of neural as well as non-neural tissues (O'Neil et al., 1990). The helical region is amphipathic and the array of leucines on the other side is hydrophobic. During activation, the surfaces of protein coils bearing the leucine get interlocked in a "zip up" manner.

C/EBP β is widely expressed in the CNS of adult mice prominently in cells of the hippocampus, dentate gyrus, cerebellar Purkinje cells, and granule cells. It has been shown that the C/EBP protein and gene are direct downstream targets of the nerve growth factor (NGF) receptor in neurotrophin signaling in the brain (Sternberg and Johnson, 1998). Other leucine zipper protein factors *c-fos* and *jun* are involved in the immediate early development of nervous system. The *jun* and *fos* function as transcriptional regulators in the form of homo- or heterocomplexes that bind to DNA. *Fos* functions specifically as a heterodimeric complex, whereas *jun* functions both as homo- and heterodimeric complexes (Okuno et al., 1991). The basic regions of *fos* and *jun* occupy the major groove on different strands of the DNA helix and project out from the side of the DNA helix opposite to the leucine zipper, thus allowing transcriptional activation through DNA bending (Kerppola and Curran, 1997). The *c-fos* protein is an immediate early gene product and has recently been implicated in controlling the neural responses of maternal behavior in rats (Numan et al., 1998).

D. Zinc Finger Proteins

Zinc fingers are DNA-binding structural motifs present in several eukaryotic protein nucleic acid interactions. The first positive acting regulatory protein to be well characterized was transcription factor IIIA (TF_{III}A) required for RNA pol III transcription of the 5S-rRNA genes. The protein has nine repeated domains that contain cysteines and histidines spaced at regular intervals. The purified protein has zinc associated with it for its activity. The TF_{III}A structure contains DNA-binding motif with coordinated binding of Zn atoms through properly spaced cysteines and/or histidines to impart tetrahedral symmetry to the coordinate complex. Residues between the coordinate amino acids loop out in a fingerlike projection. In the zinc finger folded structure of the protein, the repeated domains form loops in such a way that a zinc ion is bound between a pair of

cysteines and a pair of histidines. A phenylalanine or tyrosine residue and a leucine residue occur at a nearly constant position in the loops, which are required for binding to DNA.

The Egr proteins, *Egr-1* (also known as NGF1-A, krox-24, zif268 and TIS8), *Egr-2* (also known as *krox-20*), *Egr-3*, and *Egr-4* (NGFI-C and pAT133) are closely related members of a subclass of zinc finger motif containing proteins. These are immediate early gene-encoded transcription factors, rapidly induced in developing and adult rat brain and controlled in a discrete manner. The Egr proteins belong to the Cys2His2 class, share 90% conserved residues in the zinc finger domain and recognize the same consensus DNA binding motif.

Approximately 300 to 500 genes encoding zinc fingers of the Cys2His2 class have been estimated in mammalian genome (Becker et al., 1995). A recent excellent review of Egr transcription factors in the nervous system, physiological regulation by Egr proteins, expression and target genes provides a complete picture of the inducible transcription factor subfamily of zinc finger proteins (Beckman and Wilce, 1997). There is an ever-increasing literature with the addition of new members in this category such as rapsyn, a 43-kDa receptor-associated protein; the zinc finger domain of this protein is known to provide a binding site for acetylcholine receptor clustering (Bezakova and Bloch, 1998). Similarly, Bassoon, a novel zinc finger CAG/glutamine repeat protein, is localized in presynaptic nerve terminals, constituting as a key candidate in the neurotransmitter release (tomDieck et al., 1998). Opl is still another most recent candidate identified as a zinc finger protein that regulates neural determination and patterning in *Xenopus* contributing in the activation of midbrain, dorsal neural, and neural crest fate in CNS development (Kuo et al., 1998).

E. HMG Proteins

These proteins are so referred due to their capability for high mobility on sodium dodecyl sulfate (SDS)-polyacrylamide gels; these group of proteins have a very acidic C-terminus. Of the total amino acids, 64% are acidic, including polyglutamic/aspartic acid residues. The remaining residues in the acidic C-terminus tail are serine (25%), asparagine (4%), and glycine (7%). The acidic C-terminal tail is necessary for efficient DNA binding. Although all HMG proteins share these common characteristics, they still have been categorized based on their size, sequence similarity, and DNA-binding properties of HMG-1/2, HMG 14/17, and HMG I/Y. Of these, HMG 1/2 is highly conserved. The C-terminus has been shown to contain two domains, A and B, with 30 and 50% β -helix, respectively. Domains A and B are homologous, processing a conserved sequence box of 75 residues with aromatic and basic amino acids termed the HMG box (Jantzen et al., 1990, Baxevanis and Landsman, 1995). HMG 1/2 can bind to single- and double-stranded DNA, with a preference to the single strand DNA (Einck and Bustin, 1985).

Several HMG proteins have been implicated in the regulation of development in CNS. The Sox group is particularly famous in the development of glial cells in CNS. Sox proteins bind to DNA mainly through minor groove contacts, with a domain that was originally identified in HMG-1/2. Contrary to the HMG box, the so called Sry-box (sox) mediates sequence specific DNA binding (Laudet et al., 1993). The sox proteins have been grouped into six different groups and they have been expressed in developing and adult nervous system, involving both early and later phases of developmental expression (Pevny and Lovel-Badge, 1997).

Besides these major groups of DBPs involved in the regulation of CNS development, few other minor categories of transcriptional factors have been identified. They include (1) *winged helix motif* in the HNF3 β transcription factor, which is expressed in the early stages of neural induction with dorso ventral specification in chicks (Kelly and Melton, 1995; Ruiz et al., 1995), (2) *paired box motif* in the pax transcription factor expressed in the early development of CNS with dorsoventral specification in the neural tube (Gruss and Walther, 1992), and (3) *LIM class homeobox motif* in the LIM/Islet transcription factor, which is involved in both early and late stages of CNS development in head and motor column specification (Tsuchida et al., 1994; Shawlot and Behringer, 1995). The work on these motifs has just been started and has to be developed over time.

In addition to acting independently, several of these transcription factors are known for their functional and cooperative interaction among one another. Such functional interactions exist between POU and Sox groups. Similarly, AP-1 families of leucine zippers like *fos* and *jun* have distinct functions in CNS. C-*Jun* triggers the differentiation of photoreceptors, whereas *c-fos* expression is related to development of apoptosis. The studies on interactions between these transcriptional factors in CNS are increasing and attracting the attention of neuroscientists as they basically explain and contribute to the understanding of neuronal plasticity at molecular and genetic level.

We describe a single-strand DNA binding protein that seems to be involved in the developmental regulation of rat brain. By behavior it appears to be a protein related to the HMG group of proteins, although its molecular size does not allow it to fall under that category. The detailed isolation and characteristics of the protein are described in the following section.

III. A NOVEL NUCLEAR BRAIN-SPECIFIC DNA-BINDING PROTEIN

Brain-specific gene expression and gene regulation in different subsets of cells need a crucial involvement of nonhistone chromosomal (NHC) proteins. NHC proteins are assumed to play a crucial role during development and differentiation of nervous system. In brain, particularly in neurons, NHC proteins exceed their ratio to either histones or DNA compared with other tissues (Wu et al., 1973). It is

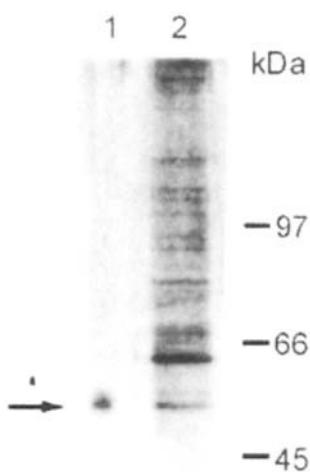
known that about 5000 to 10,000 new mRNAs appear during the course of postnatal development of rodent brain, inclusive of brain-specific mRNAs, whereas the number of proteins studied and characterized according to the present literature is an underestimate considering the various functions of the brain. A considerable number of these proteins could be DNA-binding regulatory proteins. A better understanding of the structural and functional complexity of brain could be achieved by an insight into the properties and functions of DNA-binding proteins specific to the brain.

Evidence is accruing supporting NHC protein involvement in the development of CNS and brain-specific functions (Utset et al., 1987). Expression of various homeobox-containing genes and proto-oncogene encoding DNA-binding proteins in CNS constitutes regional and cell type specific functions in brain at different stages of development (Ruppert and Wille, 1987). Similarly POU-domain genes are believed to encode transcriptional regulatory proteins in rat brain (He et al., 1989). Although homeobox gene products or POU gene products are expressed throughout CNS developmental stages, it is reported that NHC proteins may occur only in postmitotic brain cell types and at specific developmental stages (Heizmann et al., 1980). Once the specific target function is carried out, the NHC protein might not be synthesized in the cell through out the life span of the animal. As such, many of these developmental and stage-specific NHC proteins exert their actions through transcriptional regulation by binding to DNA in a sequence-specific or nonspecific manner. There have been very few studies of NHC proteins in mammalian CNS. Hence identification and characterization of proteins from brain binding to DNA is crucial in order to understand brain-specific gene expression and function. This study reports the isolation and intensive characterization of a single-strand DNA binding (ssDBP) nonhistone nuclear protein from the brain.

Wistar strain albino rats were killed by decapitation, brains were rapidly removed, and a 10% (w/v) homogenate was prepared in ice-cold TEMN buffer (10 mM Tris-HCl pH 7.4, 1mM EDTA, 6 mM β -mercaptoethanol and 50 mM NaCl) containing 1 mM PMSF in a Dounce homogenizer using pestle B. The homogenate was centrifuged at $750 \times g$ for 10 min at 4°C. The pellet was resuspended in TEMN buffer and nuclear extracts were prepared using Dounce homogenizer with pestle A followed by ammonium sulphate precipitation. The 60% ammonium sulphate pellet was collected by centrifuging at $10,000 \times g$ at 4°C for 10 min, resuspended in the TEMN buffer and dialyzed overnight against four changes of the TEMN buffer. DNA in the extract was precipitated with 10% polyethylene glycol. The DNA-free nuclear protein extract was dialyzed against the TEMN buffer and used for chromatography.

In order to identify the proteins capable of binding to single-strand DNA, ssDNA-cellulose affinity chromatography was employed. DNA-cellulose chromatography was performed as described by Alberts and Herrick (1978). The DNA-free nuclear protein (50 mg) was loaded onto a single-strand (ss) DNA-cellulose column (10×1.5 cm) preequilibrated with TEMN buffer. Proteins bound

A



B

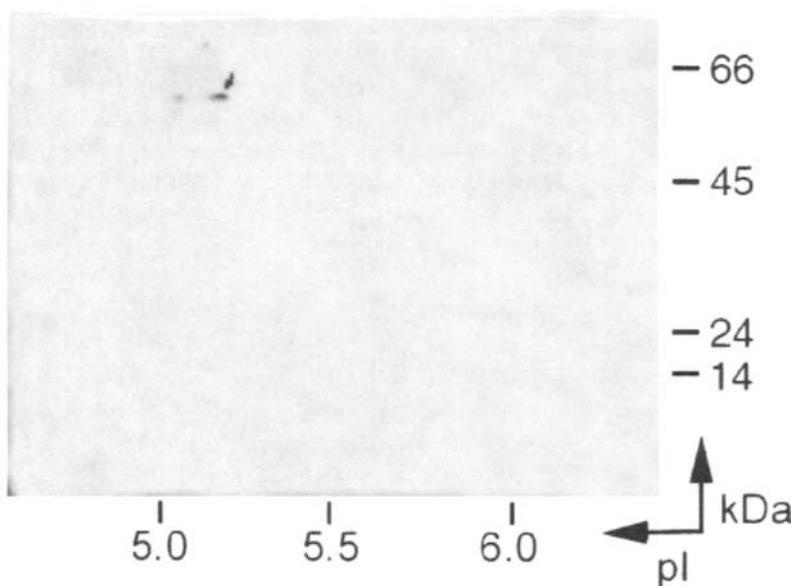


Figure 1. (A) SDS-PAGE of total nuclear proteins and ssDBP. **Lane 1:** ssDBP eluted at 0.2 M NaCl; **Lane 2:** total nuclear proteins. (B) Two-dimensional electrophoresis of 56 kDa protein. IEF was done in tubes (3 mm ID) using 2% ampholine (1.6% 5 to 7 pH and 0.4% 3 to 10 pH) and second dimension in a 10% polyacrylamide resolving gel with a 4.75% stacking gel:

to ssDNA-cellulose were eluted stepwise with the TEMN buffer containing 0.1, 0.2, 0.5, 1.0, and 2.0 M NaCl. Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. One-milliliter fractions were collected and analyzed for proteins. Several minor proteins were eluted with 0.1 M NaCl, but they were ignored, as proteins bound to DNA cellulose below 150 mM salt concentration were not of significant binding type. The O.D. 280 peak fractions of 0.2 M NaCl contained a prominent single-protein band with a molecular mass of 56,000. To determine the purity, molecular weight, and *pI* of the ssDBP, the 0.2 M fraction from the DNA-affinity column was subjected to 2DE analysis. SDS-PAGE was performed essentially as done by Laemmli (1970) with modifications as suggested by Thomas and Kornberg (1975). Two-dimensional electrophoresis was done as described by O'Farrel (1975). Silver staining of proteins in gels was done as described by Blum et al., (1987). The 56-kDa protein band was resolved at a *pI* of 5.1 to 5.2 and showed no molecular heterogeneity in SDS-PAGE gels (Figure 1).

On a preliminary search on a protein database network, employing the molecular mass and isoelectric point, no such protein with DNA-binding activity was found from the existing database. However, a literature search revealed the presence of a 56-kDa protein in the subplate of cerebral cortex involved in developmental regulation (Naegele et al., 1991). But, on further examination, it was found to be different except for the similarity in molecular mass. Because no other protein was identified, it is presumed that 56 ssDBP from the brain is a novel protein, and hence further work was carried out with this protein, referred hereafter as 56 ssDBP.

The extent of binding of 56 ssDBP to ssDNA from rat brain was accomplished by a dot-blot technique involving adsorption of the protein on a Genescreen-plus membrane and the latter was probed with non-radioactively labelled ssDNA (Genius System, Boehringer-Mannheim). After incubation, DNA-protein complexes were alone retained on Genescreen-plus and the DNA was stained with Nitroblue tetrazolium (NBT) to visualize DNA-protein complexes. The data obtained in the present study showed an increased affinity of the ssDBP to the DNA with increasing concentrations of protein (Figure 2).

The DNA-binding protein dot-blot assay indicated that 56 ssDBP preferentially binds to ssDNA from rat brain. In addition, gel retardation experiments were carried out in this study (Garner and Revzin, 1981) to qualitatively demonstrate DNA-ssDBP interaction. The DNA in the gel was visualized by fluorescence after immersing the gel in a solution containing ethidium bromide. In principle, in a mixture of DNA-protein solution on a gel, the unbound DNA separates from bound DNA-protein complex, and hence the DNA-protein complex has a diminished electrophoretic mobility; the retarded DNA in the DNA-protein complex can be visualized as a fluorescent complex after ethidium bromide treatment. In our study, a fixed amount of pBR322 DNA (1.2 kb fragment) was allowed to bind to different amounts of 56 ssDBP, and the mix-

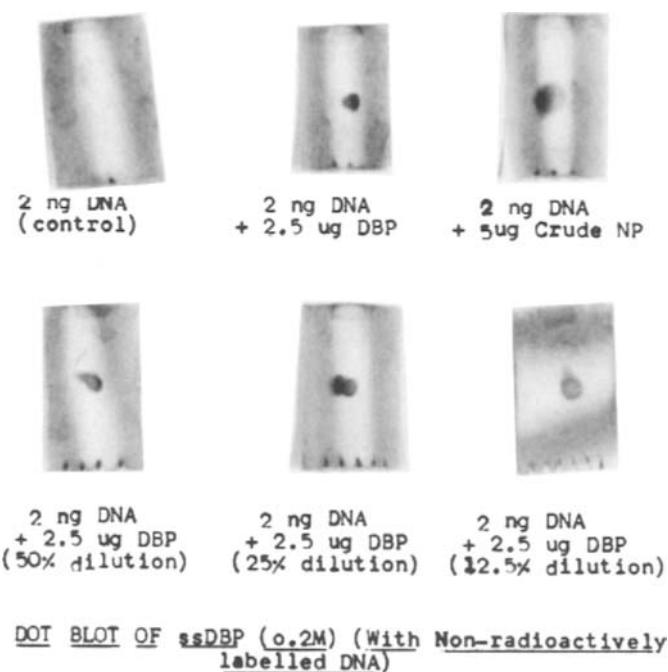


Figure 2. Dot-blot analysis of ssDBP using non-radioactively labeled DNA. 2.5 µg 56 kDa protein at different dilutions (100%, 50%, 25%, and 12.5%) and crude nuclear proteins (NP) were blotted onto a Gene-Screen plus membrane and later hybridized with 2 ng of non-radioactively labeled DNA and the complexes was stained with NBT.

ture of DNA-protein complex was subjected to electrophoresis on 5% polyacrylamide gel. The data suggested a clear binding of 56 ssDBP to pBR322 DNA with increasing protein levels (1, 2, 4, 6, 8, and 10 µg). This data complements dot-blot assays confirming the ssDNA binding nature of 56 ssDBP (Figure 3).

Absorption spectrum of the protein (10 µg) was studied using a Shumadzu UV/VS spectrophotometer between 200 and 400 nm. Fourth-derivative spectra of the purified protein (in 10 mM Tris-HCl pH 7.4, 20 mM NaCl) was recorded in a Shimadzu UV/Vis spectrophotometer at 25°C. Emission spectra (excitation at 268 nm) and excitation spectra (emission at 340 nm) of the protein was recorded at 25°C using the F-3010 Hitachi fluorescence spectrophotometer. The spectral characteristics of the 56 ssDNA binding protein showed a maximum absorption at 268 nm and the absorbance showed no shift in the

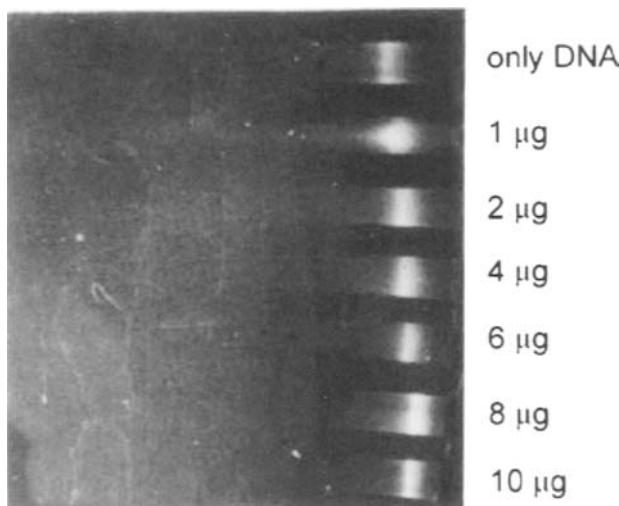


Figure 3. Gel retardation assay. pBR322 DNA (2 μ g) was incubated with 10, 8, 6, 4, 2, and 1 μ g of 56 kDa (ssDBP) in a binding mixture and electrophoresed in a 5% polyacrylamide gel. Only pBR322 DNA was coelectrophoresed in the last lane.

presence of DNA. The fluorescence emission spectrum of the 56 ssDBP showed a maximum emission at 340 nm, which suggests the presence of tryptophan in the protein, but in a buried condition (Figure 4).

Proteins with exposed tryptophan in the conformational structure show a maximum emission at around 358 nm. The excitation spectra for 56 ssDBP showed a broad maximum excitation at 270 nm. Titration of a fixed amount of protein with increasing amounts of ssDNA revealed an initial steep increase in fluorescence followed by a shallow increase at high concentrations of ssDNA. The fourth-derivative spectrum of 56 ssDBP showed a peak at 300 nm. The fourth-derivative peaks of tryptophan are chiefly in the minimum of largest wave length 280 to 300 nm. The peak observed in the present study at 300 nm suggests that tryptophan is in a buried conformation (Figure 5). The data are in agreement with the fluorescence studies.

Circular dichroism (CD) spectra of the protein was recorded in a Jasco-20 scanning automatic recording spectrophotometer. All spectral data were represented as mean residue ellipticity or molar ellipticity $[\theta]$. Circular dichroism is defined as $A_L - A_R$, with a unit of $\text{cm}^2\text{mole}^{-1}$. The CD spectrum studies on the interaction of the 56 ssDBP with DNA suggest that the protein has no influence on the conformation of ct DNA, as no change is observed in the wavelength from 240 to 300 nm (Figure 6). This rules out the possible involvement

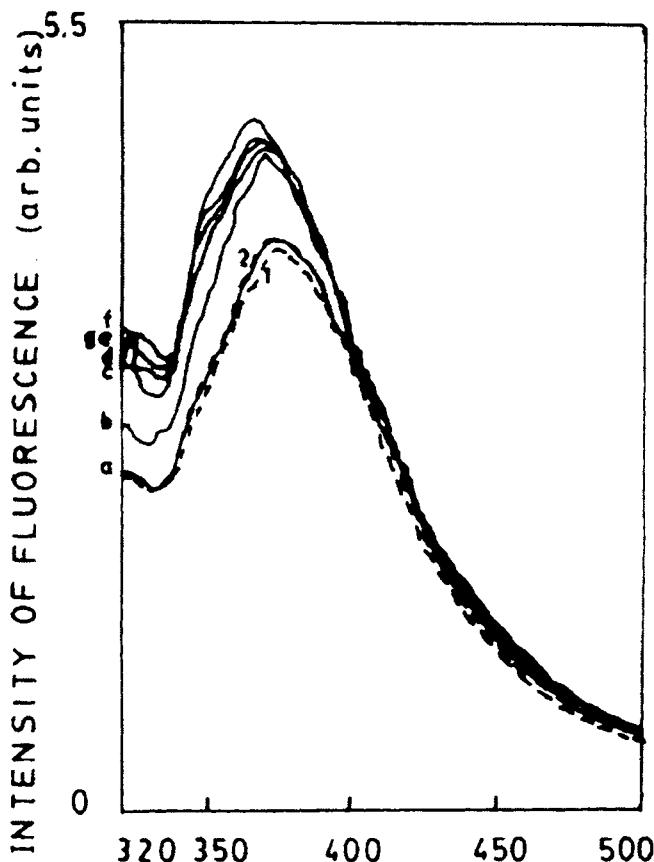


Figure 4. Intrinsic fluorescence spectrum of 56 ssDBP. 30 µg protein was taken in 1 mM Tris-HCl pH 7.4, 50 mM NaCl. Spectrum was recorded in the absence of and increased amounts of calf thymus ssDNA. Fluorescence spectrum of (a) 56 ssDBP alone, 30 µg; b-(g) 30 µg of 56 ssDBP plus 0.3, 0.6, 0.9, 1.5, 3.0 and 6.0 µg of ssDNA only buffer with 3.0 µg (2) buffer with 6.0 µg of ssDNA as control.

of the ssDBP in structural regulation of DNA/chromatin. However, stoichiometric and titration studies are required further to confirm this result.

The protein (50 µg) was subjected to acid hydrolysis using 6N HCl at 110°C for 22 hours to determine the amino acid composition. The hydrolysates were analyzed in a Pharmacia LKB alpha plus automatic amino acid analyzer. Protection was done for cysteine, methionine, and tyrosine using appropriate protecting reagents. The amino acid composition of the 56-kDa protein has been determined as shown in Table 1. The protein is relatively rich in glycine,

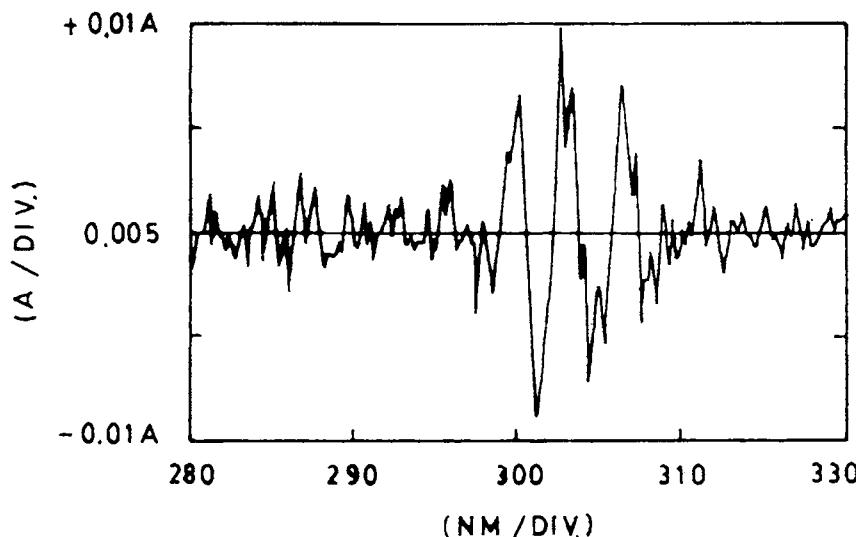


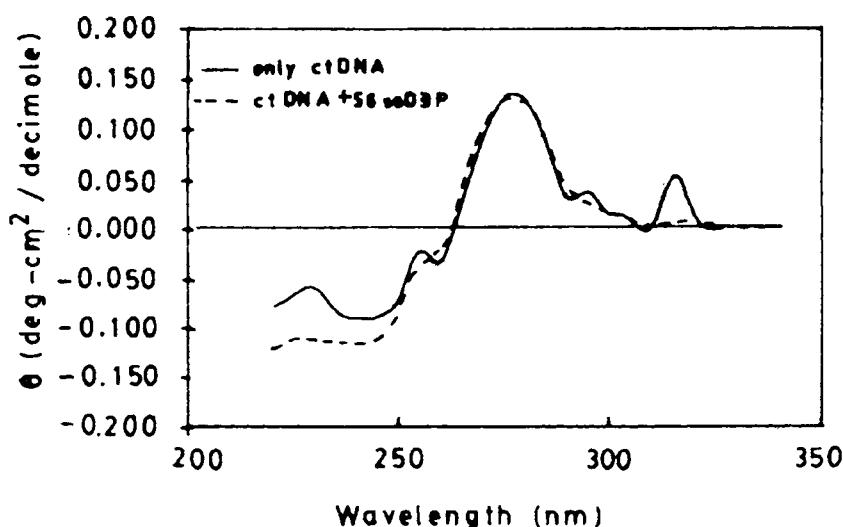
Figure 5. Fourth derivative spectra of 56 kDa protein. The protein was taken in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl and the derivative was recorded using a Shimadzu UV/VIS spectrophotometer.

serine, and glutamic acid. The mole percent of the acidic amino acid is 22.41%, whereas the basic amino acids is 7.28%. Cysteine, methionine, proline, and tyrosine were not detected, whereas arginine and histidine were found in trace amounts. Fluorescence emission spectra indicated the presence of tryptophan; however, in the amino acid analysis, it could not be identified as acid hydrolysis of the protein results in the breakage of the indole ring of tryptophan.

Thermal denaturation profiles of DNA in the absence and presence of this protein was obtained by heating the DNA in 1 ml of 10 mM Tris-HCl pH 7.2 and 25 mM NaCl. The rate of heating was 2°C per minute. An increase in absorbance at 260 nm was measured in a programmed UV/VIS spectrophotometer attached with a thermoprogrammer. The melting curve was simultaneously recorded. These studies were carried out in 10 mM Tris-HCl (pH 7.5) containing 25 mM NaCl. This buffer was used, as phosphate or cacodylate were reported to interact directly with arginine residues through ionic and hydrogen binding and reduce protein-DNA interaction. Under the present experimental conditions the T_m of calf thymus DNA was found to be 79°C (Figure 7). Adding 56 ssDBP had no significant effect on the melting of DNA. DNA/protein ratios of 0.5, 1.0, 1.5, and 2.0 also showed similar melting

Table 1. Amino Acid Analysis of 56 ssDBP

Amino Acid	Composition (mol%)
Aspartic acid	6.715
Threonine	4.103
Serine	17.025
Glutamic acid	15.687
Glycine	27.654
Alanine	9.757
Valine	3.492
Isoleucine	1.406
Leucine	3.292
Phenylalanine	2.634
Histidine	0.951
Lysine	6.754
Arginine	0.527
Total	100.0

**Figure 6.** Circular dichroism studies of calf thymus DNA in the presence (---) and absence (--) of 56 kDa protein. The spectra were recorded in 1 mM Tris-HCl, pH 7.4, and 20 mM NaCl using a 0.1-cm path length cuvet.

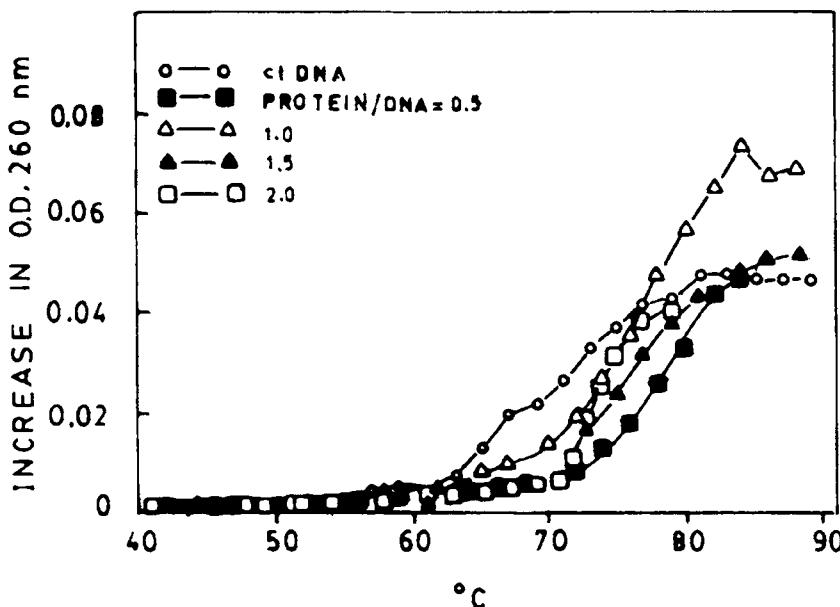


Figure 7. Effect of 56 kDa protein on the melting of calf thymus DNA. Melting of DNA was performed in buffers containing 1 mM Tris-HCl, pH 7.4, 1 mM EDTA and 25 mM NaCl in the absence of 56 kDa protein, and in the presence of different ratio of protein/DNA content.

features suggesting that the 56 ssDBP has no effect on the melting property of DNA.

Functional studies of 56 ssDBP using polyclonal antibodies showed interesting aspects of the protein. In order to identify the possible distribution of 56 ssDBP, the nuclear proteins from different tissues (heart, liver, kidney, lung, muscle, and brain) of rat were separated on 15% SDS-PAGE gels, transferred on to nitrocellulose membranes (Towbin et al., 1979) and probed with polyclonal sera raised against 56 ssDBP. The Western blots showed the presence of this protein only in neural tissue, but not in other tissues, suggesting that ssDBP is specific to central nervous system (Figure 8).

Since the ssDBP was specific to the brain, the distribution of ssDBP in brain regions, brainstem, midbrain, cerebellum, and cerebral cortex was examined employing Western blots. All the four regions of brain showed the presence of 56 ssDBP. However, the brainstem had a relatively higher amount of ssDBP (approximately three-fold) than the mid brain followed by the cerebellum and cerebral cortex (Figure 9). The fact that it is a ssDNA-binding protein, its selective expression in brain and relatively high levels in the brainstem

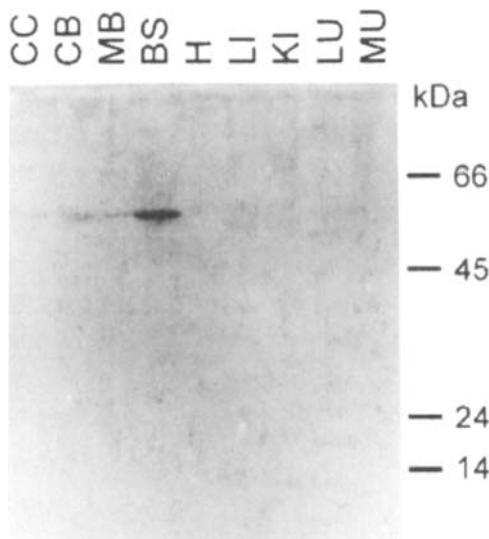


Figure 8. Western analysis to study tissue-dependent expression of the 56 kDa. Nuclear proteins were extracted from, H (heart), Li (liver), Ki (kidney), Lu (lung); and Mu (muscle) of 60-day old rat. The proteins were separated on a 12% SDS-PAGE and transferred onto a nitrocellulose membrane, treated with primary antibody and stained.

suggests that it may have some crucial role in the brainstem besides brain-specific gene expression.

Changes in ssDBP during cerebral development were examined by preparing nuclear extracts from the brains of rats of different ages from 1 day to 90 days. The analysis (Figure 10) showed that the protein starts appearing around 30 days of age, and its concentration increases with age up to 90 days.

In the early days of development i.e., before 30 days, the protein might be either absent or below the level of detection by the present method. However, the fact that the protein appears after a specific stage of development suggests its regulatory role in brain-specific functions.

An attempt was made to screen for the presence of 56 ssDBP in selective cell lines of neural as well as non-neural cell origin. C6 glioma (39th passage; rat glial brain tumor induced by N-nitroso-methylurea), Neuro-2A, Hep G2 (human hepatoma), K-562 (human erythroleukemia), and KG-1 (acute myelogenous leukemia) were obtained from NFATCC, Pune, INDIA, and grown in 25 cm² T-flasks under appropriate conditions. All the cell lines were maintained in logarithmic growth in a CO₂ incubator. Nuclei were isolated from these cell lines and processed for the presence of 56 ssDBP. Immunoblot studies with

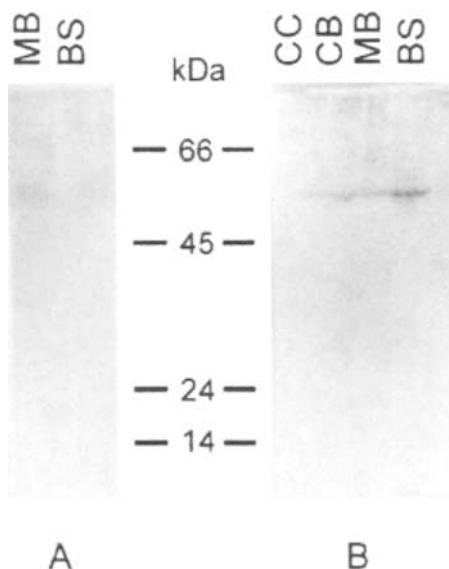


Figure 9. Western analysis to study regional distribution of 56 kDa in brain. Nuclear proteins were extracted from CC (cerebral cortex), CB (cerebellum), MB (midbrain), BS (brainstem) of 60-day old rat brain. (A) Two lanes were treated with pre-immune sera to check nonspecific cross reactivity (B). Treated with antisera to 56 ssDBP.

nuclear proteins showed the presence of 56 ssDBP only in Neuro-2A cells and C6 glioma (Figure 11), suggesting that the protein might be specific only to CNS. The protein was absent in cells such as KG-1 and K562 (leukemia) and Hep G2 (hepatoma) suggesting that the protein might not be related to DNA replication events.

The brain is a complex organ with cellular, regional, and functional heterogeneity (Lajtha, 1982; Korr, 1986; Tobin and Khrestchatsky, 1989). The cellular diversity in the CNS is further remarkable for the variety of cell types, which differ distinctly by size, shape, pattern or connections, and chemical composition. The diverse functions and complexity of the brain are partly attributed to the flexibility of gene regulation in brain cells, which is speculated to be effectively carried out by DNA-protein interactions. Analysis of nuclear protein interactions with the genome helps to explain cell-type-specific gene expression.

Several studies have reported DBPs from other tissues, but very few reports are available about the brain. Some include a 35-kDa protein in "mitotically arrested neurons" (Kuenzle, 1984); 24 and 30 kDa proteins from rat brain having preferential affinity for single-strand DNA (Falaschi et al., 1984); pax3, a novel murine DBP expressed during early neurogenesis (Goulding et al., 1991); Zif 268; and

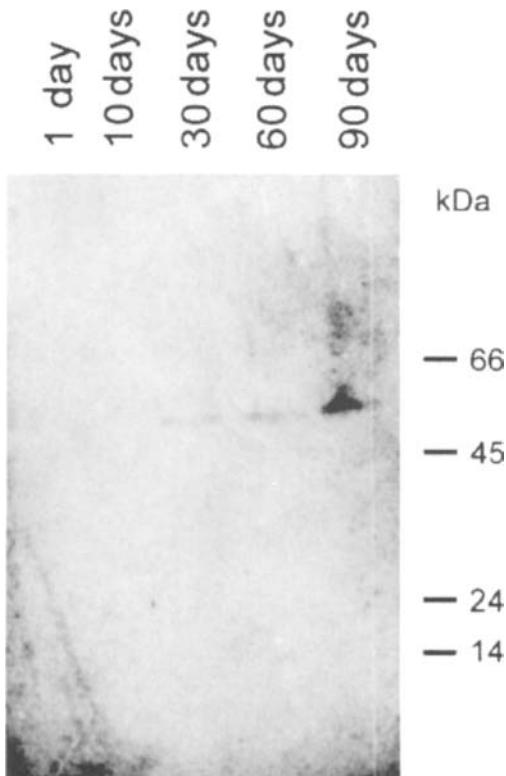


Figure 10. Western analysis to study developmental expression of the 56-kDa protein. Immunoreactive profiles of nuclear proteins from 1-, 10-, 30-, 60-, and 90-days old rat brain.

Krox 20 transcriptional regulatory factors that contain zinc finger DNA-binding domain, which are rapidly regulated in rat brain by neuronal stimulation (Bhat et al., 1992). Astrocytes and glioblastoma cells express novel octamer DBPs, designated as N-Oct proteins, which are distinct from the ubiquitous Oct-1 and B-cell type Oct-2 proteins and are considered to be transcriptional activators for genes specifically expressed in cells of neural origin (Schreiber et al., 1990). Our study accomplishes the isolation and characterization of a single-strand DNA-binding protein (56 ssDBP), a novel nuclear protein specifically restricted to the brain and turns out to be associated with brain development.

Ultraviolet and CD spectral analyses imply that the interaction of 56 ssDBP with DNA might not alter the conformation of DNA. The ability of 56 ssDBP to reduce the melting temperature of double-strand DNA by selectively binding to transient single-strand DNA and preventing it from renaturation was assessed by

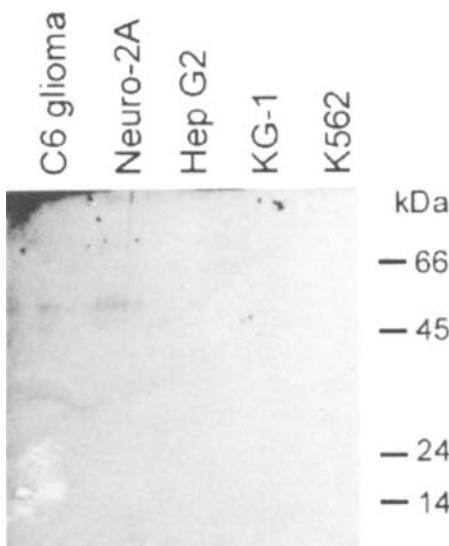


Figure 11. Immunoreactive profiles of 56 ssDBP in cancer cell lines. Nuclear proteins were extracted from C6 glioma, Neuro-2A, Hep-G2, KG-1, and K562 cell lines, and probed for immunoreactivity with antisera to 56 ssDBP.

studying the thermal denaturation profiles of ctDNA in the presence and absence of 56 ssDBP under varying protein/DNA ratios. The T_m of ctDNA was not altered by the protein, which indicated that the DNA-ssDBP interaction does not involve a change in the denaturation profiles of DNA or in the melting kinetics. However, at a high protein concentration a slight decrease in T_m was noticed. This could be due to the presence of both double-strand and single-strand DNA. Usually under these conditions the double-strand DNA has a higher negative charge density, and therefore the peptide-binding constant to double-strand DNA is higher than that of single-strand DNA. This argument holds true for proteins like histones. But in this study, the 56ssDBP has more affinity for single-strand DNA than for double-strand DNA. However, under conditions of high protein concentration chances exist for the protein to bind to DNA and cause a minor alteration in the T_m , as noticed in the present study.

The fluorescence titration of ssDBP with single-strand DNA showed slightly increased levels of fluorescence. This suggests that the ssDBP indeed binds to single-strand DNA, but with a very moderate affinity, a feature characteristic of regulatory proteins eluted at low ionic strength buffers (such as 200 to 300 nm) from DNA affinity columns. Immunological studies using the antibodies to 56 ssDBP by Western blots showed interesting observations. In terms of distribution, the

protein was found to be tissue specific-highly specific to the brain; other tissues do not show even weak immunoreactivity, a feature suggesting a selective enrichment and presence of this protein only in the brain. Even within the brain, regional heterogeneity was observed in the distribution of this protein being more abundant in the brainstem and less in the cerebral cortex. The study indicates a very subtle and clear profile of the protein distribution in a gradual increasing pattern from the deep most regions of the brain to outermost brain regions, such as the brainstem, midbrain, cerebellum, and cerebral cortex. Further during development, the protein starts appearing in the brain around the age of 30 days.

Of the cell lines studied—C6, neuro-2A, Hep G2, KG-1, and K562—the protein was present (as assessed by Western blots) only in Neuro2A and C6-glioma cells indicating the distribution of the protein in both neurons and astrocytes in a nonselective manner. The biological significance of the occurrence of 56 ssDBP in brain is speculative because of the complexity of brain. The protein is less abundant and, based on its isolation, it can be categorized into a rare DNA-binding regulatory protein selectively present in the brain and therefore might be involved in brain-specific gene expression. Identification, isolation, and characterization of 56 ssDBP selectively from different brain regions offers a novel opportunity for further examination of brain-specific gene expression. The selectivity of tissue-specific gene expression depends primarily on the transcription factors present in a given cell type and their recognition of specific sequences of promoters and enhancers of a particular gene to be transcribed. The modular arrangement of DNA through promoter and enhancer elements, and the interplay of specific nuclear factors at these DNA modules, result in tissue-/cell-specific gene regulation. The 56 ssDBP isolated in the present study needs to be tested for such selective binding to specific sequences of DNA in order to identify its regulatory role with specific promoter and/or enhancers contributing to brain-specific gene expression.

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STRUCTURE–FUNCTION RELATIONSHIPS OF THE NUCLEAR ENVELOPE

Christopher Maske and David J. Vaux

I.	Introduction	262
II.	The Problem of Open Mitosis	262
	A. Nuclear Envelope Breakdown	264
	B. The Form of the NE during Mitosis	265
III.	The Membranes of the Nuclear Envelope	268
	A. Compartments of the Nuclear Envelope	268
	B. Proteins of the Inner Nuclear Membrane	271
IV.	Associations between Nuclear Envelope Membranes and Underlying Structures	275
	A. Nuclear Lamina	275
	B. The Role of Lamins in Differentiation	277
	C. Functions of the Lamina	278
V.	Transport across the Nuclear Envelope	279
VI.	Specializations of the Nuclear Envelope	281

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A.	Structures within the Nuclear Envelope	281
B.	Modifications of the Morphology of the Nuclear Envelope	284
C.	Disease-Related Changes in the Nuclear Envelope	287
VII.	Phospholipid and Signaling in the Nuclear Envelope	288
VIII.	Calcium Signaling and the Nuclear Envelope	290
IX.	Conclusion	293
	References	294

I. INTRODUCTION

The nuclear envelope (NE) is a double-membrane structure that encloses the chromatin and forms a physical and functional barrier between the nucleoplasm and cytoplasm (reviewed in Gerace, 1988; Gerace and Foisner, 1994; Gant and Wilson, 1997). The outer nuclear membrane (ONM) faces to the cytoplasm and is continuous with the rough and smooth endoplasmic reticulum (ER) that emanates from its surface. The inner nuclear membrane (INM) invests the chromatin, contains a characteristic protein composition, and is lined by a filamentous meshwork called the nuclear lamina. Between the two membranes is a lumen that is continuous with ER lumen. The outer and inner membranes are in continuity with each other at the nuclear pore complexes and form the pore membrane domain. The NE may also project into the nucleus as a double-membrane invagination from the nuclear periphery as an apparent specialization.

The NE is emerging as a highly specialized and dynamic structure, with important contributions to the proper functioning of the interphase cell. In part, this is due to the unique position that the NE occupies between the genetic material of the nucleoplasm and the differentiated environment of the cytoplasm. Although the major bidirectional communication between the nucleus and the cytoplasm occurs via the nuclear pore complexes, it is becoming apparent that the NE itself can play a significant role in signaling between these two compartments. Emerging themes based on new biochemical information about components of the NE, new insights into morphological specializations of the NE and their possible functions, together with an examination of the role of the NE itself in signaling via calcium or phosphatidylinositol, form the basis of this review.

II. THE PROBLEM OF OPEN MITOSIS

All cells have to resolve a fundamental problem associated with the need to both use and replicate their genetic material. The processes of transcription and of replication require temporal and spatial organisation in any cell type. In a prokaryotic system, the genetic material is in the form of a single closed circular DNA chromosome. Although the prokaryotic genome is not segregated into

chromosomes, the single circle of genomic DNA is often supplemented by autonomously replicating small circular plasmids, some of which bear genes important for the life cycle of the bacterium. The hallmark of the prokaryote is a genome that is not separated from the cytoplasm by a membrane envelope; replication, transcription, and translation all occur in a single compartment. Despite the apparent simplicity of this arrangement, it is clear that the machinery for replication and transcription is not randomly dispersed throughout the cytoplasmic space, but is instead concentrated into a small area of the cell. Furthermore, this area is morphologically distinct and almost always attached to the inner face of the bounding cytoplasmic membrane. As we will see in this review, a tripartite association between chromatin, the protein machinery responsible for maintenance and use of the genome, and cellular membranes is a feature common to both prokaryotes and eukaryotes.

Until very recently, the process of cell division in bacteria could be regarded as fundamentally different to the process in a eukaryotic cell (Jacob et al., 1963). However, recent results suggest that the obvious differences in scale, complexity, timing, and membrane and cytoskeletal behavior have obscured fundamental similarities in the strategy used to ensure that the two daughter cells receive a single complete set of genetic material (Sharpe and Errington, 1996; Sharpe et al., 1998). Studies in bacterial species capable of the specialized replication and segregation steps required for spore formation have been particularly revealing (Glaser, et al., 1997). Division in *Bacillus subtilis* requires the participation of a cytoskeletal structure formed by a protein related to the tubulin that forms the spindle apparatus in eukaryotic mitosis, and a site-specific association of the daughter chromosomes with this primitive "spindle apparatus."

One problem not faced by prokaryotes is how to segregate replicated chromosomes into daughter cells when there is a nuclear envelope to enclose the chromosomes during interphase. Some lower eukaryotes, among them *Saccharomyces* species, solve the problem by assembling the apparatus for mitotic segregation within the nuclear envelope, so that the envelope remains intact. This requires a controlled fission event to separate the envelope into two closed structures around the daughter nuclei, but prevents mixing of nucleoplasm and cytoplasm.

Higher eukaryotes assemble a large, complex apparatus in the cytoplasm for segregation of chromosomes and require the release of the chromosomes from the confines of the interphase nucleus in order for the mitotic spindle apparatus to carry out its function. An advantage of this system is that the definition of the axis of separation may be used by other components of the cell that also require specific segregation into daughter cells. For example, both the Golgi apparatus and ER fragment during mitosis in order that these single-copy organelles can be partitioned between the two daughter cells (Porter and Machado 1960; Lucocq and Warren 1987). Although a stochastic segregation may account for inheritance of endoplasmic reticulum fragments, this does not appear to be the mechanism for Golgi partitioning. Following a green fluorescent protein (GFP)-tagged Golgi

resident protein through mitosis in living cells revealed that Golgi fragmentation preceded an alignment and separation of Golgi fragments by the microtubules of the mitotic spindle (Shima, et al., 1998). Thus, the presence of a cytoplasmic machinery permits a controlled division of many cellular structures, and not just the genome. However, this "open mitosis" has a significant metabolic cost. The complete loss of integrity of the nuclear envelope as the nucleus is disassembled during pro-metaphase to release the condensed chromosomes means that any nucleoplasmic–cytoplasmic concentration gradients established during interphase by selective transport at the nuclear pore complexes are lost at once.

The loss of nuclear envelope integrity at mitosis can also be exploited by higher eukaryotes to count replication in the S phase and synchronize the DNA replication cycle with that of cell division. This is achieved by the incorporation of a soluble cytoplasmic protein called licensing factor into the re-forming nucleus during late anaphase (Blow and Laskey, 1988; Laskey, et al., 1996). Because this protein lacks any internalization signal for import into an intact nucleus, this is the only time in the cell division cycle that the protein can gain access to the nucleus. This protein is essential for DNA replication, but can be used only once; thus a single S phase with a complete replication of the nuclear DNA is possible, but no further replication can take place until the licensing factor has been replenished by another cell division.

A. Nuclear Envelope Breakdown

Mitosis requires major structural changes in the nucleus that will allow for reliable separation of chromosomes to the two daughter nuclei. Chromosome condensation and spindle pole formation are therefore accompanied by depolymerization of the nuclear lamina, vesiculation of the nuclear envelope, and solubilization of the nuclear pore complexes—structural changes required for an open mitosis in higher eukaryotes. These events are the result of a complex interplay between components of the nuclear envelope and the chromatin, which at the conclusion of mitosis must result in a replication competent nucleus (Gant and Wilson, 1997).

The NE breaks down at mitosis into vesicles. This can be demonstrated by transmission electron microscopy (TEM) of mitotic cells (Yang, et al., 1997a) and by fractionating mitotic extracts (Newport, 1987) and is therefore not subject to debate, although the extent of the vesiculation in different systems may vary. There is, however, some controversy over the form of the NE during mitosis (Gerace and Foisner, 1994). One school proposes that the membrane of the NE is dedicated to this structure and maintains its identity during mitosis. The breakdown of the envelope in this instance is achieved by vesiculation into small vesicles and redistribution to the daughter chromosomes at opposite poles of the cell (reviewed in Gant and Wilson, 1997). A second mechanism proposes that the NE loses its identity during mitosis and gets incorporated into the bulk ER. The distribution of membrane is in the form of vesicles and larger cis-

ternal structures, which are a feature of the ER during mitosis. Reconstitution of the NE is achieved when the protein component that defines it is redistributed to the inner membrane by diffusion (Gerace and Foisner, 1994). There is evidence to support both mechanisms.

The disassembly of the nuclear envelope is not a cataclysmic event, but appears to occur in a stepwise fashion starting with depolymerization of the lamina following mitotic phosphorylation and subsequent gradual deformation and disintegration of the membrane. Microtubules appear to have a role in this process (Georgatos, et al., 1997). Furthermore, disassembly requires functional nuclear pores for the import into the nucleus of mitotic kinases and an intact nuclear lamina prior to disassembly (Collas, 1998).

Envelope breakdown into NE-derived vesicles can theoretically occur in different ways. The vesiculation may be domain specific; that is, the integrity of the inner and outer nuclear membranes may be maintained in separate vesicles. This would imply a separate budding event from the two membranes and the resulting vesicles would have a protein content representative of any enrichment in the respective membranes from which they were derived—be this inner, outer, or pore membrane. Alternatively, vesicles may contain membrane derived from both the inner and outer membrane and therefore the protein components of both. This implies an internal fusion event between inner and outer membranes at the time of vesiculation to produce so-called mixed vesicles. In both cases the nuclear envelope identity is maintained and produces a population of vesicles that are functionally and immunocytochemically different from the ER.

B. The Form of the NE during Mitosis

What then is the evidence for nuclear envelope-derived vesicles during mitosis? The most frequently used system in support of mitotic vesiculation makes use of *Xenopus* oocytes (Newport, 1987). Preparation of mitotic extracts from oocytes and incubation with demembranated sperm chromatin produces a replication and transport competent nucleus on a time scale of 5 to 60 minutes, suggesting a high level of fidelity with actual events (Drummond, et al., 1999). Analysis of the extract reveals vesicles that can be divided into two populations according to functional criteria (early chromatin binding versus late fusogenic), ultrastructural features (rough ribosome containing versus smooth vesicles), and biochemical characteristics (specific protein enrichment for example) (Vigers and Lohka, 1991; Drummond et al., 1999). The ability to study *in vitro* reconstituted nuclei using *Xenopus* has allowed a great insight into the process of nuclear disassembly and reassembly.

A population of vesicles exists that is characterized by its ability to bind chromatin, which is later subject to fusion with a second group of vesicles that bind to them. It is possible to summarize the characteristics of the chromatin binding population from some of these studies. The early chromatin binding vesicles are

trypsin (Wilson and Newport, 1988; Newport and Dunphy, 1992) and phosphorylation (Vigers and Lohka, 1992) sensitive, suggesting that a receptor may be involved. Binding of vesicles does not require prior lamin assembly around the chromatin, but subsequent NE growth and nuclear function, that is replication, is dependent on lamina assembly (Newport et al., 1990; Goldberg et al., 1995). Binding is not energy dependent and N-ethylmaleimide (NEM) does not affect binding capability (Newport and Dunphy, 1992). Vesicles bind to chromatin, as is shown in all of these studies, but may also bind to lambda DNA, suggesting a lack of dependence on actual sequence (Newport, 1987). In addition, the chromatin binding vesicles are studded with ribosomes which indicates that they contain membrane of ONM/ER origin. They do, however, also contain markers for the INM (Wilson and Newport, 1988; Wiese et al., 1997). It is therefore unlikely they have formed by domain-specific breakdown of the NE. There are, however, studies that argue against this. Hela cells (Chaudhary and Courvalin, 1993), lymphocytes and rat liver nuclei (Buendia and Courvalin, 1997) have been shown to have a domain-specific disassembly and reassembly, with vesicles containing INM markers associating with chromatin first at reassembly.

Analysis of the *in vitro* nuclear reformation using the *Xenopus* cell-free system by field emission in lens-scanning electron microscopy (FEISEM) has demonstrated intermediates of both the NPC and the NE (Wiese et al., 1997; Gant et al., 1998). Once again two populations of vesicles are demonstrated—early binding rough vesicles and late fusogenic smooth vesicles. Biochemical evidence also showed that the chromatin-binding vesicles contained biochemical markers of all three membrane domains, indicating that domain-specific vesicles did not exist. Furthermore, smoothing the envelope into a mature structure required functional nuclear pore complexes. Lamin accumulation at the inner membrane surface took place only once nuclear pores were assembled, suggesting that lamin association with chromatin is not the major route for vesicle recruitment.

Drosophila and sea urchin embryos have been employed with very similar results, namely that two populations of vesicles exist and that the binding of vesicles is trypsin dependent, whereas fusion is energy dependent (Collas, Courvalin et al., 1996; Collas and Poccia 1996; Ulitzur, Harel et al., 1997). The correlation between the results in these experimental systems gives merit to the notion of NE-derived vesicles during mitosis. However, when considering whether the same applies to other cells and tissues, it may be prudent to ask whether these similarities are the result of using cell-free extracts, or are a property of embryonic tissues themselves.

In summary, investigators have shown that vesiculation of the nuclear envelope occurs at the onset of mitosis and that the nuclear envelope maintains some kind of functional identity during mitosis because of the presence of a distinguishable population of vesicles. However, there is disagreement as to whether these vesicles are derived from specific domains or whether they are mixed. Despite this

evidence there is still a debate about the mechanism of vesiculation and whether it occurs in all cell types.

There is growing evidence to suggest that the NE does not maintain its identity during mitosis and becomes incorporated into the bulk ER in mammalian cultured cells. Mitotic phosphorylation, therefore, causes depolymerization of the nuclear lamina, a loss of interaction between the inner membrane proteins (IMPs) and their lamin and chromatin binding sites, and hence a loss of the anchoring role of this binding (Foisner and Gerace, 1993). It is generally accepted that IMPs are anchored in this domain by binding to the underlying lamina and chromatin. A loss of this binding would bring on the reverse of this localization and allow the inner membrane protein to diffuse freely throughout the bulk ER. A key to the maintenance of asymmetry, therefore, is the timing of lamina depolymerization relative to envelope vesiculation. Asymmetry can not be maintained in the face of a depolymerized, phosphorylated lamina (and phosphorylated IMPs). It has been shown that the NE persists after the lamina has depolymerized (Georgatos et al., 1997).

This has been powerfully demonstrated as being the case using a live cell system and a GFP construct of an IMP that retains the domains for inner membrane localization. The onset of mitosis causes a rapid loss of asymmetry and diffusion of the chimeric protein into the ER. Significantly, there were no areas of accumulation during mitosis, suggesting the absence of focal enrichment of the marker. Reaccumulation of the inner membrane marker at the close of mitosis was rapid and was achieved by anchorage at the sites of chromatin binding by membrane and the depletion of marker from the ER mitotic store (Ellenberg et al., 1997).

Other inner membrane proteins have been shown to behave in a similar fashion, including lamina-associated polypeptides (LAP1, LAP2) (Yang et al., 1997a), and emerin (Manilal et al., 1998). Indeed EM images of mitotic cells show that vesicles do exist in these cells, but that these vesicles contain ER and inner membrane markers together with no apparent enrichment of either. The vesicles were of different sizes—of the order of 50 to 500 nm—with a population of smaller tubular structures also present. Given that the NE represents only 5% of the ER membrane in the average cultured cell, a specific NE compartment during mitosis should reveal itself with immunolabeling at the EM level. Further studies with EM are necessary to demonstrate whether recruitment of membrane to the chromatin following mitosis is in the form of larger cisternae rather than smaller vesicles.

Within the framework of the above models, it is possible to describe the cycle of events taking place in the NE during mitosis. The beginning of mitosis sees the phosphorylation of lamins and IMPs during prometaphase, which depolymerizes the lamina and alters the affinity of the IMPs for their binding substrates. The inner membrane proteins therefore diffuse out of the INM compartment into the bulk ER making the nuclear envelope essentially indistinguishable from ER. The NE fragments along with the rest of the intracellular membrane structures, including the ER and Golgi but not the mitochondria, into tubular-cisternal membranes and

vesicles. These membrane structures may be subject to microtubule-dependent partitioning to the daughter cells. This has certainly been shown for Golgi membrane, and membrane structures have been noted to be associated with the mitotic spindle (Waterman-Storer et al., 1993). Chromosome segregation occurs during anaphase followed in late anaphase by the recruitment of membrane to the chromatin surface. This is brought about by phosphatases that restore the affinity of the inner membrane proteins for chromatin. Targeting membrane to the chromatin is therefore through binding mediated by the IMPs and possibly also by lamin B, which remains associated with membrane during mitosis.

Following targeting of the membrane, in the form of cisternae and smaller vesicle structures, envelope growth is brought about by the energy-dependent fusion of vesicles. Further envelope growth and lamina formation are dependent on functional nuclear pores, which allow for import of nucleophilic proteins including the lamin subunits. Nuclear pore reassembly in the *Xenopus* reconstituted nuclei occurs once fusion has taken place. In these nuclei further smoothing of the envelope is dependent on nuclear pore function. The G1 nucleus is established once chromatin has decondensed and the gradients between nucleoplasm and cytosol have reestablished themselves; the nuclear envelope continues to grow until S phase and the onset of replication—the final test for a successful mitosis.

III. THE MEMBRANES OF THE NUCLEAR ENVELOPE

A. Compartments of the Nuclear Envelope

The ONM contains ribosomes and is indistinguishable from the bulk ER membranes (Puddington et al., 1985; Pathak et al., 1986; Yang et al., 1997a). Membrane proteins synthesized in the ER and outer membrane are free to diffuse to the INM and such proteins are present at a density equal to that in the ER (Torrisi et al., 1987). Furthermore, the diffusion speed of membrane proteins in the peripheral ER and ONM have been compared and found to be the same (Ellenberg et al., 1997).

Membrane proteins in the ER and ONM gain access to the INM by diffusion through the pore membrane domain that invests the nuclear pore complex (NPC). This is in contrast to soluble nucleophilic proteins that are transported across the NPC channel as a result of their nuclear localization sequence. A proportion of these membrane proteins are dedicated inner membrane proteins (IMPs) and will become retained by their binding interactions at the inner membrane surface. Those proteins not destined for the INM are subject to the same diffusional motion back into the ER (Soullam and Worman, 1995; Gorlich 1998). There exists a 10-nm channel lateral to the pore complex and separating this structure from the pore membrane which may allow for protein traffic through this region (Hinshaw et al., 1992). Proteins with cytoplasmic/nucleoplasmic domains of 70 kDa or greater do

not gain access to the inner membrane even with the appropriate localization sequences (Soullam and Worman, 1995). It has not been established if the tertiary structure of a protein contributes to their ability to translocate to the INM, but data suggest that the constraint is purely size limiting (Soullam and Worman, 1995). The orientation of proteins with respect to their luminal and cytoplasmic/nucleoplasmic domains is not altered during the transfer from outer to inner membrane.

The pore membrane is in itself a specialized area of the nuclear membrane. Two proteins that are targeted to this region have been characterized. Gp210 is a 210 kDa high-mannose glycoprotein that is targeted exclusively to the pore membrane of higher eukaryotes (Wozniak et al., 1989). It has interactions with proteins of the nuclear pore complex, which likely accounts for its localization to this area. The molecule consists of a large 200 kDa N-terminal domain, which is orientated to the NE lumen; a single transmembrane domain; and a short cytoplasmic C terminal domain (Greber et al., 1990). The membrane-spanning domain and cytoplasmic tail are shown to target the protein to this specialized area of the NE (Wozniak and Blobel, 1992). Gp210 may be involved in anchoring the NPC in the membrane. The small C-terminal domain is consistent with transport of inner membrane proteins through this region. NPC function, both passive and active transport, is disrupted by binding of an antibody to the luminal N-terminal domain, suggesting that this portion of the molecule is important for function and implicating gp210 as a cofactor in nucleocytoplasmic transport (Gerace and Foisner, 1994).

POM121 is a 121-kDa membrane-bound protein with a large number of O-linked glycosylation sites. It is a predominantly cytoplasmic protein and contains many potential phosphorylation sites. A function for POM121 has not been elucidated (Hallberg et al., 1993; Gerace and Foisner, 1994). POM121 has a single hydrophobic-predicted membrane-spanning domain that may be large enough to traverse the membrane twice in quick succession. The majority of the molecule is adjacent to the nuclear pore and contains pentameric sequence repeats found in nucleoporins that may function to bind and stabilise the pore complex. The large cytoplasmic domain is likely to prohibit access to the inner membrane of the nucleus (Hallberg et al., 1993).

Between the double membrane of the NE is a lumen, continuous with that of the ER. There is clearly a free flow of contents of the ER to the NE lumen and the two would therefore be expected to have very similar soluble content. There are small differences associated with the membrane proteins. The inner membrane aspect of the lumen presents the luminal domains of an enriched population of IMPs. Although there is currently no study on the binding properties of these luminal domains, they do conceivably offer an opportunity for retention of luminal proteins drifting in from the ER lumen. It is also possible that IMPs that are as yet uncharacterized exist as enzymes and are targeted to the inner membrane by interaction with the lamina. The lamin B receptor (LBR) may be a candidate for enzymatic activity at the INM surface and is discussed below.

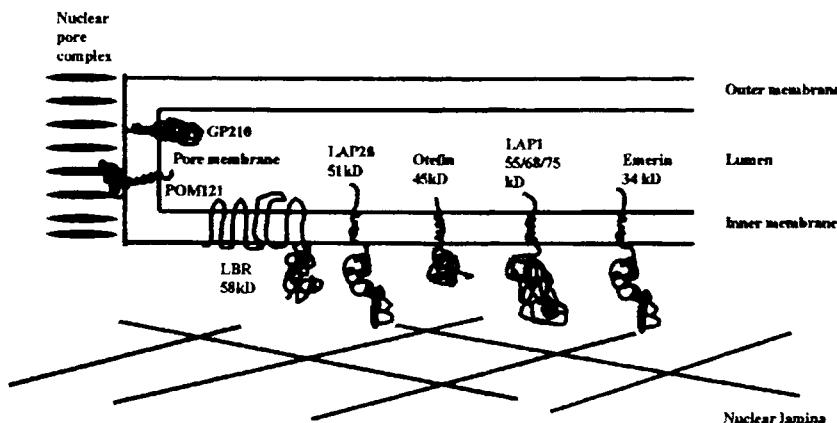


Figure 1. Proteins of the inner nuclear membrane and pore membrane. The schematic diagram depicts the known proteins of the inner nuclear membrane and pore membrane domains. The molecular weights of these proteins are included. The relationship of the different nuclear membrane domains is shown relative to the nuclear pore complex and nuclear lamina. The inner membrane proteins are anchored in this region by binding to the underlying nuclear lamina. LBR-lamin B receptor; LAP2-lamina associated polypeptide 2; LAP1 – lamina associated polypeptide 1.

In contrast to the outer membrane, which is indistinguishable from the ER, the interphase INM demonstrates a high degree of specialization. There have been five proteins characterized that are specifically targeted to the INM, including the LBR, which is described in detail below. LAP 1, LAP 2, emerin, and otefin (*Drosophila*) are well-described IMPs. What are the features of these proteins that result in their accumulation at the INM? The known INM proteins all contain at least one putative membrane-spanning domain and, with the exception of emerin, have demonstrated binding to lamins and chromatin. The nucleoplasmic domains, and, in some cases, the transmembrane region, are the sequence areas responsible for retention at the inner membrane. Interactions with the lamina are likely to play a major role in anchoring membrane proteins in the INM. In the case of LAP2 the retention signal is specifically the lamin-binding domain of the nucleoplasmic region (Furukawa and Kondo, 1998). It has been shown that LAP1 exists as an equilibrium between the INM and the ER, though greatly concentrated in the INM (Powell and Burke, 1990). Having defined the environment of the nuclear envelope it is now possible to discuss the membrane proteins that occupy its inner membrane in more detail (Figure 1).

B. Proteins of the Inner Nuclear Membrane

Lamin B Receptor

The LBR, also known as p58, is one of the most studied of the IMPs. LBR is found in a conserved form in the higher eukaryotes and in the sea urchin (Ye and Worman, 1994; Collas, et al., 1996). The human form is a 58-kDa, 615 amino acid integral membrane protein with eight putative membrane-spanning regions (Worman et al., 1990; Ye and Worman, 1994). The chicken (Worman et al., 1990) and human forms of LBR are 68% homologous and the protein is expressed in a host of cell lines (Schuler et al., 1994; Ye and Worman, 1994). The N-terminal domain of 208 amino acids contains phosphorylation sites and DNA-binding sequence motifs (Ye and Worman, 1994). Together with immunocytochemical data this has localized this domain to the nucleoplasmic side of the membrane (Smith and Blobel, 1993; Soullam and Worman, 1993). The C-terminal domain comprises eight membrane-spanning regions and has homology to enzymes in the sterol synthetic pathway of yeast (Schuler et al., 1994; Ye and Worman, 1994).

The N-terminal domain of LBR has been shown biochemically to bind DNA and lamin B (Ye and Worman, 1994). The DNA-binding domain corresponds to a basic serine/arginine-rich stretch in the amino terminal (residues 71 to 100)(Ye and Worman, 1994). Lambda DNA binds to LBR through this region, suggesting that the interaction is nonspecific. Indeed, no specific DNA sequence elements have been identified that bind to LBR. Precipitation of cell extracts with LBR, however, produces a number of proteins including lamins A and B, an LBR-specific kinase, p34 (which has homology to a splicing factor associated protein p32), and an 18-kDa protein found in avian erythrocyte NE. These proteins form what is termed the LBR complex and their precipitation indicates that a large number of interactions involving LBR are present at the inner membrane surface (Simos and Georgatos, 1992; Simos and Georgatos, 1994; Simos et al., 1996).

LBR is targeted to the INM of interphase cells through its nucleoplasmic N-terminal domain and to the first membrane-spanning domain of the C-terminal domain adjacent to the nucleoplasmic tail (Smith and Blobel, 1993; Soullam and Worman, 1993; Soullam and Worman, 1995). There is a greatly decreased diffusional mobility of the protein once it has localized to the INM (Ellenberg et al., 1997) and bound to the nuclear lamina. These two regions of the protein can target LBR to the inner membrane independently of each other and can also localize an unrelated polypeptide to this same compartment. Furthermore, the N-terminal domain contains a degenerate bipartite nuclear localization sequence, which causes it to accumulate in the nucleoplasm if expressed without a transmembrane domain. This may be the remnant of a gene fusion—an enzyme involved in sterol synthesis and a soluble nuclear protein.

The role of the first transmembrane domain in anchoring of the LBR molecule at the INM is unclear. It has been speculated that it interacts with other helical

membrane-spanning regions or dimerizes with another LBR molecule (Smith and Blobel, 1993). These interactions would not account for the greatly decreased diffusional mobility (Ellenberg et al., 1997) but may create a size exclusion for transport back across the pore membrane. It is also possible that the farnesyl modification of lamin B, which localizes it to the membrane, interacts with the first transmembrane segment of LBR (Georgatos et al., 1994; Smith and Blobel, 1994).

It is possible that interactions between the IMPs and chromatin not only localize these proteins to the inner membrane but also contribute to the higher-order organization of interphase chromosomes (Marshall et al., 1996; Lamond and Earnshaw, 1998). Not only has LBR been shown experimentally to be important for chromatin binding at the nuclear periphery (Pyrpasopoulou et al., 1996), but the interaction may be mediated by heterochromatin proteins, a family of chromatin-associated proteins that are involved in position effect variegation in *Drosophila* (Ye and Worman, 1996). LBR precipitates HP1 from HeLa cell extracts, suggesting that the interaction occurs *in vivo* as well as *in vitro*. In support of this, heterochromatin also has an association with the NE during interphase (Mathog et al., 1984). Using hydrophobic cluster analysis, Ye and colleagues (Ye et al., 1997) further showed that LBR contains two predicted globular domains in its nucleoplasmic part. The first globular domain corresponding to amino acids 1 to 60 was deduced to be important for lamin B binding. The second globular domain nearer the membrane-spanning region (aa 116 to 210) binds to HP1. Interestingly, this region contains a phosphorylation site for cdc2 kinase, phosphorylation of which would significantly change the conformation of this domain (Ye et al., 1997). This provides an insight into how LBR may interact with chromatin in a cell cycle-dependent manner. The region of LBR needed for *in vitro* DNA binding (aa 71 to 100) did not show any predicted secondary structure, but has features consistent with DNA-binding proteins. This region may be important for association with chromatin at mitosis when nucleoplasmic proteins are not necessarily present.

LBR contains in its primary sequence consensus sites for both cdc2 kinase and protein kinase A, both of which are phosphorylated by the respective kinases (Appelbaum et al., 1990; Courvalin, Segil et al. 1992; Simos and Georgatos 1992). In addition, the molecule is phosphorylated at arginine-serine motifs in the N-terminal domain by another kinase called LBR kinase, which belongs to a class of arginine-serine (RS) kinases (Nikolakaki et al., 1996). These sites are separate from the cdc2 and protein kinase A sites and affect the association of a protein, p34, with LBR. LBR precipitates a number of proteins of which p34 is one. It is homologous to a human nuclear protein, p32, which interacts with Splicing Factor 2 (Nikolakaki et al., 1996). Although not directly associated with splicing factors, LBR may interact with mRNA as well as with chromatin through these linker molecules. It will be interesting to know whether such an interaction takes place only in particular domains of the nucleus. Intranuclear invaginations of the nuclear envelope have a long intranuclear course and have been shown to associate

nucleoli (Fricker et al., 1997). These structures would be likely areas for such an mRNA interaction to occur.

Human LBR is 42% homologous to the yeast sterol C-14 reductase, which is an enzyme in the cholesterol biosynthetic pathway (Ye and Worman, 1994; Holmer et al., 1998). It has recently been shown that the hydrophobic domain, the region of LBR which is homologous to the yeast enzyme, has catalytic properties itself (Silve et al., 1998). Specifically, the multiple membrane-spanning C-terminal domain was able to restore ergosterol synthesis, the reaction product for the enzyme, in a mutant yeast lacking the sterol C-14 reductase enzyme. Despite a 36% homology to C-24 reductase, LBR did not show any activity of this kind in mutant yeasts (Silve et al., 1998). It is postulated that LBR may inactivate sterol hormones at the nuclear surface (Silve et al., 1998). Two novel LBR-like proteins have been cloned and found to have similar homologies to plant and yeast sterol reductase genes (Holmer et al., 1998). LBR is alone among all of these proteins in having an N-terminal cytoplasmic/nucleoplasmic domain. The two other LBR-like proteins, along with other members of the cholesterol synthetic pathway HMGCo-A reductase and squalene synthase, localize to the endoplasmic reticulum (Holmer et al., 1998). The pathophysiological significance of this enzyme activity needs to be established.

Lamina-Associated Polypeptides

Lamina-associated polypeptide 2 (LAP2) was cloned in the rat (Furukawa et al., 1995) and has subsequently been shown to be a member of a larger family of proteins. There are three isoforms of LAP2, termed α (75 kDa), β (51 kDa), and γ (39 kDa), which are alternatively spliced forms of the same gene product (Harris et al., 1994, 1995). The β and γ isoforms contain single transmembrane domains, whereas the larger LAP2 α is a soluble protein. The isoform that corresponds to the original cloned in rat is the β isoform. It has been shown to associate with chromatin and lamin B *in vitro*, both of which are reversed by phosphorylation (Foisner and Gerace, 1993), and is targeted to the NE during interphase (Furukawa et al., 1995, 1998). The LAP2 β nucleoplasmic domain, much larger than the luminal domain, is 410 amino acids long and contains two distinct binding domains for chromatin and lamina, respectively (Furukawa et al., 1998). The lamin-binding domain is necessary and sufficient for localization of the protein to the INM. The chromatin-binding domain is not in itself able to direct LAP2 β to the INM, but may be important in recruiting membrane to chromosomes at the conclusion of mitosis.

Studies using the binding domains of LAP2 β as competitive inhibitors have demonstrated that binding of LAP2 β to the lamina following mitosis is important for nuclear growth during G1 (Yang et al., 1997b). Progression into the S phase was inhibited in this study possibly as a result of a checkpoint involving nuclear size, but not as a direct inhibitory effect of LAP2 β on replication. Furthermore,

inhibition of chromatin-binding to LAP2 β (by its chromatin binding domain) blocks lamin accumulation suggesting that binding of chromatin by LAP2, and other inner membrane proteins, provides the necessary environment for lamins to polymerise (Gant et al., 1999).

In contrast LAP2 α is localised to the nuclear interior during interphase and at the spindle poles during mitosis. It is therefore not an IMP, but may be a component of the nucleoskeleton. Of note is that the sequence common to LAP2 β and LAP2 α is also the domain with which LAP2 β binds to chromosomes (Furukawa et al., 1998). Not surprisingly LAP2 α has been shown to associate with chromosomes during telophase (Dechat, Gotzmann et al. 1998).

LAP1 also has three isoforms, denoted LAP1A, B, and C, which arise from differential splicing of the same gene transcript (Senior and Gerace, 1988; Martin et al., 1995). LAP1 has been shown to bind DNA and lamin *in vitro* (Foisner and Gerace, 1993). LAP1C has been cloned and characterized and is a 506 amino acid type II membrane protein with potential phosphorylation sites on its amino terminal nucleoplasmic domain (Martin et al., 1995). There is differential expression of these isoforms—LAP1A and B are expressed only in differentiated cells, whereas LAP1C is constitutively expressed in all cells (Martin et al., 1995). This mirrors the expression of the lamins. LAP1C has been shown to bind less strongly to lamins, implicating LAP1A and B in forming a more established nuclear lamina in differentiated cells. LAP1C immunocytochemistry reveals a punctate staining pattern at the nuclear periphery and not the uniform pattern seen in other INM proteins. This is thought to be the result of complex formation—LAP1C complexes under physiological conditions with lamin B and protein kinase A (Maisond et al., 1997). This complex formation may explain why certain vesicles during mitosis contain INM components and others not, and why some associate with chromatin following mitosis (Maisond et al., 1997). Furthermore, LAP1C colocalizes with lamin B to the mitotic spindle, suggesting that lamins may associate with microtubule-associated proteins during segregation of chromosomes and by complexing with LAP1C bring about the ordered sorting of vesicles at mitosis (Maisond et al., 1997).

Other Inner Membrane Proteins

Emerin is a 254 amino acid, 31 kDa inner membrane protein with 40% homology to LAP2. It has one hydrophobic membrane-spanning region and is expressed in most tissues (Manilal et al., 1996). Its location in the cell is the inner membrane. During mitosis emerin diffuses into the ER in much the same way that LBR does (Manilal et al., 1998). Emerin is the only inner membrane protein that has been linked to a human disease phenotype, namely Emery–Dreifuss muscular dystrophy (Bione et al., 1994).

Otefin is a 45-kDa *Drosophila* INM protein that has a large nucleoplasmic amino terminal domain and a transmembrane domain at the carboxy terminus

(Padan et al., 1990; Ashery-Padan, Weiss et al., 1997). It has no homologies and is the only inner membrane protein to be characterized in *Drosophila*, the others mentioned in this list being absent. Otefin interacts with lamins through its nucleoplasmic domain (Goldberg et al., 1998) but is more sensitive to extraction from the NE than other inner membrane proteins (Ashery-Padan, Ulitzur et al., 1997). Furthermore, it is localized to the nucleus if the putative transmembrane region is deleted suggesting that it has functional NLS sequences. Constructs involving a large nucleoplasmic β galactosidase domain of 170-kDa were still targeted to the inner membrane contrary to previous evidence that a size limit exists. It was therefore suggested that otefin may enter the nucleus via NPCs and be directed to the membrane by its hydrophobic C terminus (Ashery-Padan, Weiss et al. 1997). Retention and stability of interaction with the NE certainly required sequences located in the wild-type otefin nucleoplasmic domain (Ashery-Padan, Ulitzur et al., 1997). It is not known whether an alternative mechanism for IMP entry to the nucleus exists in reality. This would liken otefin targeting to the membrane to the lamins. Otefin is expressed in all cells at all stages of development in *Drosophila* (Ashery-Padan, Ulitzur et al., 1997).

IV. ASSOCIATIONS BETWEEN NUCLEAR ENVELOPE MEMBRANES AND UNDERLYING STRUCTURES

A. Nuclear Lamina

The nuclear lamina is comprised of 10 nm filaments that can be visualized *in situ* as a meshwork lining the INM using transmission electron microscopy (Aebi et al., 1986). The filaments themselves represent polymerized lamin subunits, a theme consistent with other cytoskeletal fibres (Geisler et al., 1998). Lamins are classified as type V intermediate filaments. Other members of the larger family include keratin, desmin, and vimentin. The common features, and those necessary for function of the IF proteins, are alpha helical domains placed centrally in the primary structure of the polypeptide. The amino and carboxy termini show less conservation and are used in the classification of the proteins into different groups (Stuurman et al., 1998).

The central alpha helical domain includes hydrophobic and charged residues that bring about the dimerization of two monomer subunits into a two-stranded parallel alpha-helical coiled coil (Geisler et al., 1998). The strands are held together by hydrophobic interaction and the nonpolar residues responsible for these interactions are therefore hidden in the interior of the coil. The polar side chains on the surface of the coil are available for lateral ionic interactions with oligomers in an antiparallel fashion to bring about the higher-order filamentous structure (Stuurman et al., 1996). Of note in the lamin primary structure is the presence of phosphorylation sites for the cell cycle control kinase, cdc2 kinase.

These phosphorylation sites occur either side of the central α helical rod domain. In the C-terminal domain is a nuclear localization sequence required for import of lamin into the nucleus via the NPC. The C-terminus comprises a CaaX motif, which is a consensus sequence for posttranslational modification with lipid moieties, in the case of the lamins that is farnesylation (Lutz et al., 1992).

The lamina is not made up of one protein, but contains different classes of lamin protein that are variations on the structure described above and interacts to form a heteropolymer (Worman et al., 1988). Lamins are therefore divided into two main groups, A-type and B-type. The B-type lamins are acidic in nature, are constitutively expressed at all stages of development (Lehner et al., 1987) and in all cells, and remain associated with the mitotic spindle during mitosis (Georgatos et al., 1997). They conform to the overall structure described above. There is a further subdivision into lamin B1 and lamin B2 (which have two conserved tetrapeptide regions in the alpha helical rod domain).

In contrast, the A-type lamins have a neutral isoelectric status, are expressed only in terminally differentiated cells, and are soluble during mitosis. The A-type lamins contain an internal proteolytic cleavage site for posttranslational processing near the C terminus, that results in the loss of the CaaX motif at the C terminus, and consequently the lipid association at the nuclear periphery. Lamin A is farnesylated and directed to the periphery through this hydrophobic tag but loses the modification through the proteolytic event on the modification site (Lutz et al., 1992). Stabilization at the nuclear periphery is therefore through association with lamin B polymers and, to some extent, the inner membrane proteins. Differential splicing of a common gene results in the A-type lamins being subdivided further into lamin A, lamin C, lamin Adelta10, and lamin C2 (Furukawa et al., 1994; Machiels et al., 1996). All of these variants behave alike as far has been determined and can therefore be generalized to A-type lamins.

The vertebrate lamins are highly conserved in their structure and function (Spann et al., 1997). Therefore, lamins from *Xenopus*, which is a useful system for studying these proteins, may also be divided into the two main groups, i.e., A-type (LA) and B-type (LI, LII, and LIII) (Benavente et al., 1985). Similarly, *Drosophila* lamins, although more closely related to other vertebrate intermediate filament proteins than vertebrate lamins, still maintain the group characteristics. Lamin Dm0 is considered to be the *Drosophila* equivalent of vertebrate B-type lamins and behaves accordingly. *Drosophila* lamin C is an A-type lamine. The developmental expression of the *Drosophila* lamins mimics that seen in vertebrates.

Immunocytochemical studies localize lamins predominantly to the nuclear rim during interphase where they are part of the polymerized lamina. There are, however, discrete intranuclear accumulations of both lamin A and lamin B, which do not localize to the same sites and are not apposed to membrane structures (Moir et al., 1994). Invaginations of the nuclear envelope are invested with a lamina and therefore account for some intranuclear foci of lamin proteins (Fricker et al., 1997).

The sites not associated with invaginations may represent stores of lamin subunit or alternatively sites for posttranslational modification prior to insertion into the lamina. Lamins have been implicated in the proper functioning of replication enzymes offering a further explanation for an intranuclear source of the protein.

B. The Role of Lamins in Differentiation

It has previously been mentioned that the B-type lamins are expressed in all cells throughout differentiation, whereas the A-type lamins are expressed only in terminally differentiated cells. Indeed this has been shown in *Xenopus* (Benavente et al., 1985), chicken (Lehner et al., 1987), and mammals (Worman et al., 1988; Rober et al., 1990). The different isoforms of each lamin type also vary depending on the stage of development. Lamin B2 is expressed at constant levels throughout development, while lamin B1 peaks in early development. A-type lamins are expressed only during or after differentiation of the cell (Lehner et al., 1987; Rober et al., 1990). Depending on the cell type this may be during embryonic development or after birth.

The consistent pattern of differentiation induced expression in A-type lamins leads to speculation over their role in the control of this process. Experiments of embryonal carcinoma cell lines, which do not normally express A-type lamins, reveal that the expression of lamin A can be induced by differentiation of these cells. Lamin A message appears 24 hours (Lanoix et al., 1992) to 3 days (Mattia et al., 1992) after *in vitro* differentiation. It is localized in the nuclear periphery and is targeted to daughter nuclei normally after mitosis (Collard and Raymond, 1990; Peter and Nigg, 1991). Lamin A mRNA appears to be posttranscriptionally degraded in embryonic cells in a process that is reversed by differentiation (Lanoix et al., 1992). The expression of lamin A in embryonic cells by transfection without *in vitro* differentiation of these cells conversely does not bring about such differentiation (Peter and Nigg, 1991).

There are some conclusions that can be drawn from these studies. First, it is clear that B-type lamins can form polymers and exercise the necessary functions for NE maintenance and cell division. Second, it seems that expression of the A-type lamins is the result of, rather than the cause of, differentiation in the cell. In addition, the mechanisms whereby lamin A is targeted and assembled in the cell are not dependent on differentiation, suggesting that they are either inherent to the lamin A molecule or shared by other molecules, likely the B-type lamins. It has been noted that cells induced into quiescence show stronger staining of A-type lamins in the lamina and that this may be used as a proliferative index for lung tumours (Coates et al., 1996). A lung adenocarcinoma cell line has conversely shown defective lamin A incorporation into the nuclear periphery (Machiels et al., 1996). The cause-and-effect relationship of these intriguing observations is not clear.

A model for lamin expression may propose B-type lamins as being suited to rapidly dividing cells requiring a rapidly mobilized lamina. A-type lamins may bring about a more stable lamin polymer with a different set of interactions with the chromatin. The ability of lamin A to influence gene activity has not been demonstrated. The factors controlling the expression patterns of the lamin gene may also shed light on this phenomenon and link the observations in carcinoma cells.

C. Functions of the Lamina

What happens to the nucleus when the lamina is disrupted? Nuclei assembled from *Xenopus* extracts in the presence of a dominant negative mutant of lamin A which lacks the N-terminal domain and cannot form polymers cause both structural and functional changes in the assembled nucleus (Spann et al., 1997). The lamina of these cells was disrupted and lamins formed intranuclear clumps. The resulting nuclei were small and failed to grow in size giving support to the idea of the lamina as a support structure for the NE. It is important to note, however, that the nuclei did manage to form even in the absence of a functional lamina. This suggests that other factors are more important in the assembly of the membrane (Newport et al., 1990; Spann et al., 1997). These nuclei also failed to replicate their DNA, which was shown to be due to a failure of elongation rather than initiation of DNA synthesis. Lamin B has been reported to be coincident with sites of DNA replication (Moir et al., 1994), and implicated in the actual process of DNA synthesis (Newport et al., 1990; Meier et al., 1991; Goldberg et al., 1995; Jenkins et al., 1995). Another study of dominant negative lamins, using a mutant lamin B, showed that nuclei could form in the absence of a polymerized lamina, that the nuclei formed in the absence of lamina were small, and that these nuclei failed to replicate their DNA (Ellis et al., 1997). This study shows however that initiation of replication is dependent on a lamina formation, but that elongation can take place in the face of lamina disruption (Ellis et al., 1997). Previous studies have shown that *in vitro* assembly of the nuclear envelope around chromatin can take place in the absence of lamin (Newport et al., 1990; Jenkins et al., 1993). However, these experimental systems were later shown to have trace amounts of lamin (Lourim and Krohne, 1993), which may be enough for complete assembly.

Immunocytochemical data suggest that replication factors involved in the elongation phase, e.g., proliferating cell nuclear antigen (PCNA) and replication factor C (RFC) are disrupted in lamina defective nuclei (Spann et al., 1997), however this may be a direct result of the particular mutant employed. Yang and colleagues (1997b) microinjected the lamin-binding fragment of LAP2 into the metaphase and G1 cells and showed that nuclear volume increase was inhibited despite the formation of an import-competent nucleus. The lamina in these cells was not disrupted, suggesting that the binding of soluble lamin or the quenching of available polymerization sites inhibited nuclear volume increase. Moreover, these cells did

not replicate their DNA and a cell-cycle checkpoint involving nuclear volume was suggested as one possibility.

Probably the most holistic demonstration of lamin function *in vivo* to date is that of a germline *Drosophila* mutant with greatly decreased expression of lamin Dm0 (the *Drosophila* equivalent of lamin B). An insertion into an intron of the lamin Dm0 gene resulted in a decreased expression of the protein to 20% of normal in homozygotes (Lenz-Bohme et al., 1997). The phenotypic effects included reduced viability, sterility, and impaired locomotion. The ultrastructural features included an increase in annulate lamella (see below), clustering of nuclear pore complexes (by loss of tethering to the underlying lamina), and irregular or even absent nuclear envelopes in a small percentage of cells. Observations by the authors indicate that a total lack of lamin Dm0 expression arrests development following few divisions when maternal stocks are depleted (Lenz-Bohme et al., 1997). It is apparent, therefore, that an organism can achieve full development with a very small amount of lamin present. It should be remembered that *Drosophila* contains lamin C, which is expressed in differentiating cells. The degree to which lamin C can compensate is not known.

V. TRANSPORT ACROSS THE NUCLEAR ENVELOPE

The nuclear envelope separates the nucleoplasm from the cytoplasm, permitting transport between the two compartments only via specialized pores. The nuclear pore complexes (NPCs) are among the largest of cellular protein machines, consisting of hundreds of specific soluble, peripheral, and integral membrane proteins organized with an eightfold rotational symmetry and a set of stoichiometric protein–protein interactions to give a total molecular mass of about 125 MDa. The structural organization, composition, and function of these critically important features of the nucleus have been well reviewed elsewhere (Davis, 1995; Pante and Aebi, 1996a, 1996b; Corbett and Silver 1997; Fabre and Hurt 1997; Goldberg et al., 1997). For the purposes of this review, only a few points will be made concerning nuclear pore complexes.

First, because NPCs are the only route between the nucleus and cytoplasm, their number and distribution determine where on the nuclear surface such interchange can happen, and how much. The density of NPCs per unit area of nuclear surface has been analyzed in the budding yeast *Saccharomyces cerevisiae* (Winey et al., 1997). The number of NPC per nucleus ranged from 65 to 182, with the number increasing steadily through the cell cycle beginning in mid G1. The peak density of NPC occurs during the S phase as the increase in nuclear surface area exceeds the increase in NPC number during G2. Not only does the number of NPCs per nucleus vary during the cell cycle, the distribution of NPC over the nuclear surface also alters. NPC distribution is nonrandom, with a weak tendency to cluster that is enhanced in early mitotic cells. An association of NPC clusters with the spindle

pole body (see below) suggests that this increased clustering is not random (Winey et al., 1997). A similar variation in NPC density during the cell cycle has also been found in plant nuclei (de la Torre et al., 1979). Whether each NPC is continuously active is not known.

Second, because there is a massive flux of material in each direction (i.e., mRNA, ribosomes and ribonucleoprotein complexes from nucleus to cytoplasm and all nuclear proteins, including transcription factors and structural subunits, from cytoplasm to nucleus) there must either be two sorts of nuclear pore complex (i.e., an "in" complex and an "out" complex), or one complex must be able to transport in both directions. Because there is no evidence for stable functional heterogeneity among NPCs, it is more likely that each NPC can mediate bidirectional transport. This raises a second question: can an NPC transport in both directions at once, or does it switch between being an "in" door and an "out" door? If such switching were taking place, then the control proteins determining the timing of switching would be the most critical components for determining nucleocytoplasmic communication. In the absence of such switching, it is difficult to envisage how simultaneous bidirectional transport could be made independent of the counter flux in the opposite direction.

Third, the nuclear pore complex functions at the interface between two highly ordered environments. Not only does substrate for import arrive at the NPC along defined cytoskeletal tracks, at least for the last part of the journey (Shah et al., 1998), but the export of RNA from the nucleus may also involve linear tracks (Meier and Blobel, 1992; Murti et al., 1993; Rosbach and Singer, 1993; Xing and Lawrence, 1993; Moen et al., 1995; Xing et al., 1995). The NPC itself has been shown by high-resolution electron microscopic studies to have both nucleoplasmic (Ris, 1997) and cytoplasmic (Pante and Aeby, 1996a, 1996b) filaments. The association of the NPC at the nuclear envelope with cytoskeletal features that extend both into the cytoplasm and into the nucleoplasm places significant constraints on the mobility of the pore complexes in the plane of the envelope. Indeed, this organization suggests that an additional role of the NPC might be to mediate connections between otherwise separate skeletal networks (Maniotis et al., 1997). Thus, in the same way that the intermediate filament networks of adjacent epithelial cells are connected across a double membrane boundary by a specialized large protein complex called the desmosome, the NPC might be regarded as the equivalent of the desmosome, integrating stresses on cytoplasmic and nucleoplasmic skeletal elements. There is a body of evidence, beginning with the histological studies of Sigmund Freud (discussed in Triarhou and Del Cerro, 1987) that the nuclear contents may rotate in three dimensions with respect to the cytoplasmic organelles of the cell. Coordinate movement of nucleoli and chromatin in the face of stationary juxtanuclear cytoplasmic structures has been described (De Boni and Mintz, 1986), suggesting that the outer nuclear membrane remains immobile. Several groups have concluded that nuclear rotation is saltatory, discontinuous, reversible, and dependent on the metabolic activity of the cell; the consensus shear

plane lies between the ONM and the INM (De Boni, 1988; Fung and De Boni, 1988; Paddock and Albrecht-Buehler, 1988). These observations are very hard to reconcile with our current understanding of the organization of NPC in the NE, and a shear plane within the nucleoplasm seems more plausible.

Fourth, structural analysis of the NPC suggests that there is an aqueous pore connecting nucleoplasm and cytoplasm, through which passive diffusion could take place. Studies using low-molecular-weight fluorescent dextrans as passive tracers suggest that there is indeed passive diffusion across the NPC for molecules below 10 kDa, whereas molecules above 70 kDa are certain to require nuclear localization signal (NLS) recognition and energy consumption in the form of ATP hydrolysis. It remains uncertain whether the passively transported small molecules pass through the same channel as the specifically transported species that require signals and ATP hydrolysis. The behavior of the NPC passive diffusion channel shows a more complex pattern for small charged species such as ions. Such a charge-dependent selectivity has been shown especially clearly for calcium ions (Petersen et al., 1998). Whether NPCs show free permeability to calcium ions remains controversial (Lipp et al., 1997; Gerasimenko et al., 1996), but it still seems likely that NPCs play an important role in calcium signaling in the nucleus. The importance of the nuclear envelope itself in calcium signaling is considered further below.

VI. SPECIALIZATIONS OF THE NUCLEAR ENVELOPE

A. Structures within the Nuclear Envelope

In the majority of mammalian cell types, the nuclear envelope consists of a double membrane studded with NPCs. The space between the INM and ONM remains uniformly narrow and the only additional features are branched membrane bridges to the peripheral ER. However, we have already seen that lower eukaryotes that divide using a closed mitosis require a mitotic spindle apparatus that can span the nuclear envelope (see above). In the case of the budding yeast *S. cerevisiae*, the centrosome, or spindle pole body (SPB), can be found embedded in the NE throughout the cell cycle (Winey and Byers, 1993). A mechanism for insertion of the characteristic electron dense plaque of the SPB into the nuclear envelope must exist, however, as the daughter SPB produced by duplication during G1/S is initially cytosolic (Byers and Goetsch, 1975). Recently, an essential 42-kDa phosphoprotein, Spc42p, has been implicated in the attachment of the SPB to the nuclear envelope (Donaldson and Kilmartin, 1996). Despite the lack of an apparent transmembrane domain, spc42p appears to be critical for the association of the SPB with the ONM.

In contrast, the SPB of the fission yeast *Schizosaccharomyces pombe* is cytosolic for most of the cell cycle, developing an association with the nuclear envelope

only after SPB duplication during G2 (Ding et al., 1997). The position of the cytosolic SPB is far from random, however, as it is always found closely apposed to the nuclear envelope where the nuclear envelope is itself closest to the plasma membrane. Perhaps most strikingly of all, the NE adjacent to the SPB shows electron dense specialisations, especially in the INM. This plaque of electron dense material extends into the subjacent nucleoplasm, where it coincides with gamma-tubulin labeling and with the *in situ* hybridization signal for *S. pombe* centromeres (Funabiki et al., 1993).

In an extraordinary process that precedes prophase, the electron dense area of NE beneath the SPB invaginates to permit incorporation of the SPB into a fenestra, delivering the *S. pombe* SPB to a location within the NE very similar to the location of SPB in budding yeast. The mechanism behind this induced, cell cycle-restricted invagination and fenestration remains very obscure, although two genes in *S. cerevisiae* have recently been implicated in the process of incorporation of daughter SPB (NDC1) or NPC (NUP116) into the NE in budding yeast. Yeast bearing a temperature-sensitive (ts) mutant of the NDC1 gene show defective SPB insertion into the NE at the nonpermissive temperature (Winey et al., 1993), whereas yeast defective for NUP116 show an aberrant NPC organization in which a persistent nuclear envelope blister covers the cytoplasmic face of the NPC (Wente and Blobel, 1993). Recently, the apparent similarity between the insertion of SPB and NPC into the NE has been strikingly confirmed at the molecular level by the finding that ndc1p is an NE membrane protein that colocalizes with both NPC and SPB, suggesting a shared function in the assembly of these organelles into the NE (Chial et al., 1998). The ability of the yeast NE to invaginate locally and specifically during the process of SPB incorporation in *S. pombe* may offer important clues to the morphogenesis of the deep nuclear invaginations that are frequently found in mammalian nuclei (see below).

In *Drosophila melanogaster* interphase nuclei there is clear evidence for nonrandom organization of chromosomes within the nuclear volume (Hochstrasser and Sedat, 1987). This organization varies in a tissue and cell type specific way, and may be related to gene expression in differentiated cells, perhaps much more widely than just in insect cells (Blobel, 1985; Comings, 1968). In addition to nonrandom distribution of chromosomes in the interphase nuclei, Hochstrasser and Sedat found associations between specific regions of chromosomes and the NE (Hochstrasser and Sedat, 1987). These interactions have subsequently been confirmed and mapped in greater detail using fluorescent *in situ* hybridization techniques (Marshall et al., 1996). Specializations within the NE are frequently found at these sites of chromosome association. The most common form of specialized structure is an invagination of the INM alone, often associated with a double-membraned evagination, although double-membraned invaginations were also seen (Hochstrasser and Sedat, 1987). In addition, the intermembrane space created by the invagination of the INM is frequently filled with numerous electron dense particles ranging from 100 nm to over 200 nm in diameter. The identity of these

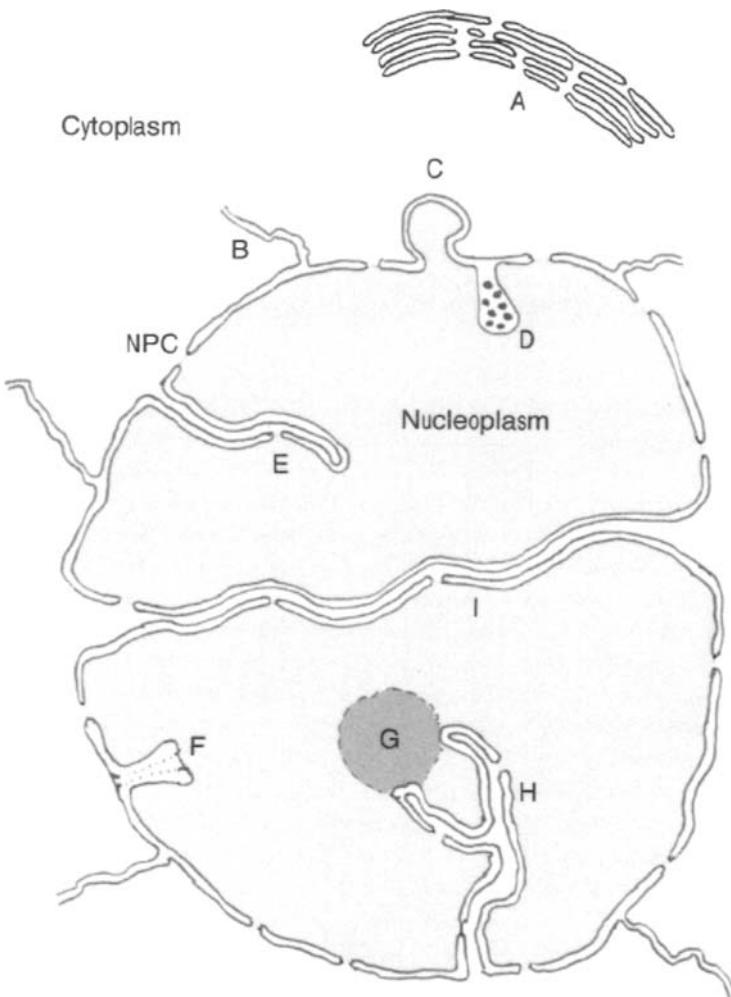


Figure 2. Organization of the nuclear envelope. The schematic shows an interphase nucleus to highlight the morphology and specialisations of the nuclear envelope. The features illustrated are (A) annulate lamellae; (B) connections from the nuclear envelope to the endoplasmic reticulum; (C) a double-membrane evagination of the entire nuclear envelope; (D) a single-membrane invagination of the INM alone (note the electron dense particles in the intermembrane space); (E) a blind-ended double-membrane invagination (note the core of cytoplasm); (F) a short single-membrane invagination characteristic of some plant nuclei (note that the inner end and the connection at the ONM have electron density and the appearance of half rings of NPCs); (G) the nucleolus; (H) a branched double-membrane nuclear channel ending in proximity to the nucleolus; (I) a nuclear channel with two connections to the nuclear surface.

structures remains to be determined; similar structures have not been identified in mammalian cells, suggesting that their function may be unique to gene expression in insect cells. A role in gene amplification, or the high-level transcription and ribonucleoprotein transport that follows, would be plausible except for the fact that the chromosome attachment sites are invariant and do not correspond to amplified puff sites.

B. Modifications of the Morphology of the Nuclear Envelope

Deep Folds and Lobulation

In the majority of cell types, the nuclear envelope forms a smooth uniformly curved interface between the nucleoplasm and the cytoplasm, consistent with the minimum surface area necessary to cover the volume required by the decondensing chromatin at the end of mitosis (Figure 2). However, some cell types, such as the polymorphonuclear leukocyte (PMN) or neutrophil, develop characteristically lobulated nuclei during their maturation, demonstrating that a smooth minimal area NE is not the only possibility. Indeed, studies in cultured cells have shown that major irregularities of the NE, from deep folds to extensive lobulation, can arise entirely as a result of the expression of specific patterns of cytoplasmic intermediate filaments (IFs) (Sarria et al., 1994; Kamei, 1994). The exact role of the IFs is uncertain, however, because in the case of the human adrenal tumor cell line SW-13, it is the absence of IFs that lead to deep folds of the NE (Sarria et al., 1994), whereas in the case of a human pancreatic cell line MIA PaCa-2, the deep folds appear to be related to the presence of large IF bundles indenting the NE (Kamei, 1994). Another rare cause of large irregularities in the mammalian NE is the amplification of gene clusters, such as occurs in the CHO C 400 cell line, where the amplified cluster is located in a distinct bulge in the NE (Dukwel, 1992). This morphology may be the mammalian equivalent of the pods described in *Drosophila* nuclei (Hochstrasser and Sedat, 1987), but electron dense particles were not seen.

Annulate Lamellae

Annulate lamellae consist of extensive, cytoplasmic-flattened membrane cisternae perforated by numerous NPCs (Cordes et al., 1996). The cisternae are often multiple and stacked, and the NPCs in adjacent cisternae are frequently aligned. An association of annulate lamellae with ER or the NE itself has been suggested, but this is based on observations in virus-infected cells that show a marked abnormal expansion of both rough and smooth ER membranes (Marshall, Borg, et al., 1996) (Wang et al., 1997). The NPCs in annulate lamellae have been shown to have a similar protein composition to those in the nuclear envelope, including the NPC-associated membrane protein p210, and will bind labeled nuclear transport

substrates (Ewald et al., 1996; Meier et al., 1995). The presence of additional NE-associated nuclear components such as a lamina in these cytoplasmic annulate lamellae has not been reported. Annulate lamellae are very rare in some cell types, and very frequent in others, suggesting that they may form part of a stable differentiated phenotype (Cordes et al., 1996). Furthermore, the size and number of annulate lamellae shows a cell type specific variation.

The relationship of annulate lamellae to the NE is underscored by observations that they are disassembled and reassembled in a cell cycle dependent manner and in synchrony with the NE. All of these features suggest that annulate lamellae could be regarded as orphan fragments of NE that form in the cytoplasm by a process independent of recruitment of vesicles to chromatin surfaces. If this is the case, they may share many similarities with those intranuclear fragments of isolated, NPC-studded, membrane cisternae that are not attached directly to the NE (Kessel, 1983; see below).

Invaginations and Evaginations of the Nuclear Envelope

In the polytene nuclei of *D. melanogaster* the nuclear envelope undergoes a series of striking morphological modifications to produce both invaginations and evaginations (Park and De Boni, 1992). In these cells, evaginations of the entire NE form double-membrane-bound blebs extending into the cytoplasm (nuclear envelope evaginations, NEE). Frequently, NEE are associated with corresponding invaginations of membrane into the nucleoplasm, which in this cell type appear to consist of the INM only (nuclear envelope invaginations, NEI). The resulting NEI forms a deep, fingerlike, single-membraned projection into the nucleoplasm. The morphology of these structures is highly complex, and can only be appreciated following reconstruction analysis after serial section TEM. In these regions of NE where the space between the ONM and INM is widened to form "pods," abundant electron-dense particles may accumulate (Hochstrasser and Sedat, 1987).

Short invaginations of the NE have also been found in plants, especially during microsporogenesis in *Pinus banksiana* (Dickinson and Bell, 1972; Li and Dickinson, 1987). Invaginations of the NE have also now been found in a wide range of mammalian cell types (Bourgeois et al., 1979; LaVelle and Tank Buschmann, 1983; Stevens and Trogadis, 1986; Fricker et al., 1997). In contrast to the single-membraned NEIs of insect cells, mammalian NE invaginations predominantly involve the entire nuclear envelope, enclosing a finger of cytoplasm extending into the nucleus. To distinguish the double-membraned invaginations in mammalian cells from single-membrane NEIs, we propose the name nuclear channels for these structures.

Mammalian nuclear channels exhibit a range of morphologies from simple tubular invaginations extending perpendicular from the NE toward the nuclear interior, to complex multibranched structures with extensive ramifications throughout the nuclear volume (Fricker et al., 1997). Furthermore, the pattern of

channel morphology in a given cell type is a stable characteristic of that cell type, arguing strongly that these structures have a significance and are not functionless remnants of NE reassembly after cell division. Perhaps most striking of all are channels that make two connections with the NE and thus provide a passageway through the entire nucleus, rendering the nucleoplasm annular in consequence (Stevens and Trogadis, 1986; Fricker et al., 1997; Gant and Wilson, 1997).

Mammalian nuclear channels are highly nonrandom, showing a significant association with the nucleolus (Bourgeois et al., 1979; Fricker et al., 1997). Furthermore, scrapeloading of cultured cells with fluorescent high-molecular-weight tracers that are excluded from the nucleoplasm confirms that the core of the channel is in diffusional continuity with the bulk cytoplasm (Fricker et al., 1997). Taken together with the observation that nuclear channels are studded with NPCs (Fricker et al., 1997) that have appropriate composition and orientation (Shah et al., 1998) and can bind import substrates in nuclear import reactions using isolated nuclei (D. Gorlich, personal communication), these observations are compatible with an important role for channels in nucleocytoplasmic transport.

The significance of the nucleolus in production of ribosomal RNA is well known; recent results have recalled earlier observations that implicate the nucleolus in transport of mRNA as well (Schneiter et al., 1995). Early experiments using UV ablation of the nucleolus in cell hybrids led to the conclusion that the nucleolus played a key role in mRNA export (Harris et al., 1969; Sidebottom and Deak, 1976). The discovery of yeast nucleolar protein temperature-sensitive mutants that accumulate poly A+ RNA in distorted nucleoli at the nonpermissive temperature offer significant support for this view (Kadowaki et al., 1992; Kadowaki, et al., 1995). Thus, an association of nuclear channels with nucleoli may suggest a function in nucleocytoplasmic transport of mRNA as well as rRNA and ribosome subunits.

The nuclear lamina that lies beneath the NE extends over the nucleoplasmic surface of channels as well; EM immunocytochemistry not only confirms that this electron-dense coat contains lamin B (Fricker et al., 1997), but also that the INM of the channel contains the lamin B receptor protein (see above; Ellenberg et al., 1997). Thus, the presence of intranuclear channels may account for previous observations of isolated intranuclear foci of NE components, including members of the lamin family and NPC components (Gant and Wilson 1997). Double-label immunocytochemistry suggests that channels may account for many intranuclear lamin B foci, but at the light-microscope level there are apparently additional isolated lamin B foci that are not associated with detectable channels. Whether these foci represent channels or even isolated vesicles that contain abundant lamin B but undetectable amounts of INM or ONM components (and therefore do not appear double labeled in these experiments) remains to be determined.

The channels are stable structures that persist for many hours in cultured mammalian cells, but they are far from static. The use of live-cell, laser-scanning confocal microscopy to study cells labeled with a lipophilic dye such as

DiOC_6 that accumulates in membranes including those of the ER and NE reveals channels to be highly dynamic structures (Fricker et al., 1997). Extensive movement of channels on a time scale of minutes have been observed in several cell types, and the results obtained using lipophilic dyes have recently been confirmed using cells expressing GFP-tagged ER resident proteins (D.J. Vaux, unpublished observations).

The widespread presence of channels in mammalian nuclei raises important issues of the functional significance of these structures. A possible role in nucleocytoplasmic transport has already been mentioned, and the implications for control of transport to and from deep nucleoplasmic sites has been discussed (Fricker et al., 1997). It is clear that the presence of a specific pattern of nuclear channels may function to bring specific groups of genes into close proximity with NPC and a route to the cytoplasm. Such a role in differentiation would be compatible with the cell-type variation seen. A variation in the abundance of channels in certain disease states (see below) indicates that channel dynamics may be linked to overall levels of transcriptional activity in the cell.

Apart from a conceptually simple role in bringing cytoplasmic highways close to deep intranuclear structures for bidirectional transport, several other functional roles for channels may be envisaged (Fricker et al., 1997; Gant and Wilson, 1997). The characteristic morphology of channels suggests that they are well suited to a role in the spatial organization of nuclear functions in addition to transport of substrates or products of nucleic acid metabolism. For example, channels provide deep intranuclear sites where phospholipids are available, potentially involved either in activation of lipid-dependent enzymes, or in providing substrate pools for independent phosphoinositol-signaling pathways within the nucleus (see below). Moreover, because the ER and the attached NE are both important in calcium signaling within the cell, it is possible that the associated nuclear channels may offer a powerful spatial regulation of calcium-dependent nuclear activity (see below).

C. Disease-Related Changes in the Nuclear Envelope

The shape of the NE, as we have seen, is a differentiation-related phenotype that may vary under different physiological conditions. In addition, pathological changes to the cell may be reflected in alterations in NE morphology. An increase in the complexity of the NE is a well-known and common finding in many malignancies (Bernhard and Granboulan, 1963), and this often includes the appearance of deep nuclear invaginations (Hoshino, 1961). The presence of deep NE invaginations in malignant cells has more recently been confirmed by immunocytochemical analysis of glioma cells using a monoclonal antibody to an antigen present on the NE and in elongated intranuclear extensions (Singh et al., 1994). These changes would be consistent with an activity-dependent dynamic control of nuclear channel abundance, but do not provide clues as to how this might be achieved.

In human breast cancer, the protein product of the BCRA-1 gene has been reported to be enriched in or associated with invaginations of the nuclear envelope (Coene et al., 1997). Because BRCA-1 has been implicated in both DNA repair (Scully, Chen et al., 1997a, 1997b) and transcription (Scully, Anderson et al., 1997), this might offer important clues to the function of channels. Unfortunately, the cellular localization of this protein has been extremely controversial, although it now seems likely that a cell cycle dependent movement between nucleus and cytoplasm accounts for some of the controversy (Scully et al., 1997a). While these results would be compatible with a role for channels in the cell cycle dependent nucleocytoplasmic transport of BRCA-1, recent EM immunocytochemical data suggests that the BRCA-1 foci are most likely to be independent of channels (D.J. Vaux, et al., manuscript in preparation).

In inflammatory conditions of muscle, such as dermatomyositis and polymyositis, nuclei may show increased numbers of nuclear invaginations, along with other changes in the nucleoplasm (Tome and Fardeau, 1986). In the autosomal dominant disorder myotonic dystrophy, the morphology of muscle nuclei is often altered, with the appearance of deep invaginations that may be large enough to contain cytoplasmic organelles (Tome and Fardeau, 1986).

Huntington's disease is an autosomal dominant neurodegenerative disorder caused by polyglutamine-encoding CAG repeats in the first exon of the coding region for the huntingtin gene on human chromosome 4. A transgenic animal model of this disease has been produced by expressing part of the promoter region together with a CAG-expanded pathological version of exon 1 (Mangiarini et al., 1996). The transgene-bearing animals develop a progressive neurological disorder and die prematurely. A striking feature of the neuropathology in these animals is the presence of a single amorphous intranuclear inclusion in cortical and striatal neurons. In addition, the NE of these damaged neurons develops deep invaginations (Davies et al., 1997). The correlation between transgene expression, disease phenotype, and neuronal nuclear channel appearance is extremely tight. The significance of these observations for the human disease remains to be confirmed (Saudou et al., 1998). However, it is clear that intranuclear inclusions are a hallmark of several human CAG-repeat neurodegenerative diseases (including spinocerebellar ataxias, X-linked spinocerebellar muscular atrophy, and dentatorubralpallidolysian atrophy; Sisodia, 1998), and a common role for deep nuclear channels in all of these conditions remains an exciting possibility.

VII. PHOSPHOLIPID AND SIGNALING IN THE NUCLEAR ENVELOPE

Biochemical evidence for the presence of large quantities of phospholipids within the nucleus has been available for many years. Although the functional subdivisions of the nuclear interior have independent composition as well as

distinctive localized functions, they are not demarcated by phospholipid bilayer membranes as cytoplasmic organelles are. Thus, the majority of the intranuclear phospholipid cannot be in the form of bounding membranes of nuclear subcompartments. The presence of membrane-bound invaginations of the NE (see above) may account for some of this nuclear lipid, but it is likely that a large part of this phospholipid does not exist in the form of conventional membrane bilayers within the nucleus (Divecha et al., 1993; Mazzotti et al., 1995).

The presence of nonmembrane intranuclear lipid would be an interesting curiosity were it not for the demonstration that such lipids may mediate a range of critical signal transduction processes elsewhere in the cell. The cytoplasm contains a family of kinases with specificity for phospholipid headgroups such as phosphatidylinositol-3-kinase (PI-3 kinase) (Zvelebil et al., 1996). These PI-3 kinases have been implicated in the control of a wide range of processes involving cytoplasmic signaling, including chemotactic migration, intra-Golgi transport, and exocytosis (Shepherd et al., 1996). These results implicate phospholipid phosphorylation and dephosphorylation as important modulators of membrane-dependent processes in the cytoplasm. A significant advantage of such systems is that signals may be localized to specific membranes by prior recruitment of the relevant phospholipid.

A role for phosphoinositide signaling within the nucleus was first suggested by the identification of diacylglycerol, phosphatidylinositol (PI), and PI phosphate kinase activity in preparations of NE (Smith and Wells, 1983). The spatial organization of the phosphoinositide signaling within the nucleus is uncertain, although the persistence of PI-3 kinase activity after detergent removal of the NE membranes suggests that they are not associated with nuclear membranous structures (Divecha et al., 1991). It has been suggested that these PI, diacylglycerol, and PI kinase activities are associated with the nuclear matrix, based on biochemical (Payrastre et al., 1992) and electron microscopic (Maraldi et al., 1994) data. Whether or not these components formed part of a lipid bilayer was not certain, but a localization in association with a proteolipid complex was considered more probable.

These localization studies suggested that PI is not associated with the membranes of the nuclear envelope, although the presence of deep intranuclear invaginations was not taken into account. If the focal collections of phospholipids are not part of major nuclear membrane profiles the question arises as to what biophysical form they take. In the absence of a lipid bilayer, PIs are unstable in the polar environment of the cell. The exception to this statement is when they are bound to specific phospholipid binding and carrier proteins. The prototypical member of this expanding family is the yeast phospholipid transfer protein, the product of the essential SEC14 gene in the yeast *S. cerevisiae* (Bankaitis et al., 1989). This protein has been shown to be required for PI transfer between membranes in yeast and consequently plays an important, if possibly indirect, role in the many cellular processes that require specific localization of PI. SEC14 was initially isolated as a critical component of the secretory pathway in yeast (Skinner et al., 1993), but subsequently shown to be identical to PIP1, a gene isolated in a screen for phospholipid-associated proteins

in yeast (Cockcroft, 1998). Sec14p has not been localized to the nucleus in yeast. However, there are clear SEC14 orthologes in other species, including human. It is now clear that the human SEC14 ortholog is only the first member of a family of related genes that encode proteins capable of binding phospholipids (Salama et al., 1990; Chinen et al., 1996). The family is known as the PI transfer proteins (PITP) (Cockcroft, 1998; Kearns et al., 1998).

Very recently, the presence of at least two members of this family in the mammalian nucleus has been demonstrated immunocytochemically and by micro injection of tagged purified proteins (De Vries et al., 1996). In this study, the confocal data presented shows that for PITP-beta a perinuclear accumulation is accompanied by small elongated intranuclear foci that appear in several consecutive optical sections (Figure 4 in De Vries et al., 1996). This is very similar to the appearance of deep nuclear channels, and may represent the first evidence to couple these structures to intranuclear PI signaling. It thus remains an intriguing possibility that focal accumulations of PI, PITPs, and PIP-3 kinase colocalize at fine terminal branches of a complex intranuclear mesh of fine membrane figures.

If these components are found together in one intranuclear site, then it is interesting to ask what other nuclear components are colocalized with these foci. This is because such a colocalization might offer clues to the nuclear function that is being controlled by signals transduced via a PI signaling pathway. Obvious candidates include the control of replication, transcription, or RNA processing and transport. An initial report of the association of nuclear PIP-3 kinase foci with accumulations of splicing components is potentially very exciting (Boronenkova et al., 1998). In this study, no association with nuclear channels (defined by lectin labeling) was seen. However, it should be remembered that EM analysis is needed to follow fine-branches from the main trunks of nuclear channels (see above), so it remains possible that fine branched membrane channels provide the physical substrate for localizing these signaling molecules. The key unresolved issue is whether this nuclear signaling pathway is independent of similar cytoplasmic signaling pathways, or whether this offers a means of coupling signaling pathways in the cytoplasm and the nucleus in order to orchestrate gene expression, mRNA processing, or transport and so on with processes in the cytoplasm. Further immunocytochemical studies at the EM level will be required to clarify the physical basis for localization of the nuclear PI signaling components, and may shed light on communication with the cytoplasm.

VIII. CALCIUM SIGNALING AND THE NUCLEAR ENVELOPE

The concentration of calcium ions is a critical second messenger system within most, if not all, eukaryotic cells. Calcium has many advantages as a signaling system including speed, versatility (depending on calcium-responsive proteins

that can be differentially expressed depending on the cell type), and simplicity of signal generation. Signal generation is simple because of the very large calcium concentration gradient across the plasma membrane from an external concentration that is closely maintained in the 2.1 to 2.6-mM range to a cytosolic concentration in the micromolar range. This is a much bigger gradient than for other asymmetrically distributed ions (e.g., sodium outside 137 to 145 mM, intracellular 5 mM, potassium inside 135 to 145 mM and outside 4 to 5.5 mM). Another major advantage of a calcium-based signal is that the presence of high concentrations of buffering proteins enables the signal to be spatially regulated even when there is no membrane between a responding and a nonresponding region. This capability explains coordinated muscle contraction, segmental membrane activation in phagocytosis, and specific leading-edge effects during cell motility, among many other processes. A further advantage that is closely related to the presence of calcium-binding systems is that the signal may be made very transient before calcium-binding proteins, or more usefully, calcium pumps restore the presignal state.

A corollary of the importance of calcium signaling within the cell is that the mechanisms for establishment of homeostasis must be capable of rapidly controlling calcium concentration within a very small range in the face of very large fluxes across the plasma membrane. The paradigm for this process is the restoration of low intracellular calcium within muscle fibers by calcium ATPases that consume ATP to pump calcium ions up a concentration gradient. These ATPases are found at the plasma membrane as well as at high concentration in the membrane of the specialized intracellular calcium storage organelle of muscle, the sarcoplasmic reticulum (SR). In nonmuscle cells a similar internal store for calcium is provided by the ER. The regulation of the calcium storage activity of the endoplasmic reticulum is a complex process, involving mechanisms for release (ryanodine receptor and IP₃-dependent release) as well as a specific calcium ATPase characterized by inhibition by low concentrations of the drug thapsigargin.

The relevance of the calcium-storage behavior of the ER to the NE structure and function is clear in several contexts. First, the NE is continuous with the ER, so that the simplest expectation would be that the luminal space between the INM and ONM would serve as a single storage site for calcium in diffusional continuity with the remainder of the ER lumen. Thus, many of the concerns of calcium storage and its control would be shared by the NE and its specializations (Petersen et al., 1998). Evidence that this is the case comes first from immunocytochemical confirmation that the normal content of the ER is also found in the lumen of the NE, and that this extends to calcium-binding proteins such as calreticulin and protein disulphide isomerase (Fricker et al., 1997). Second, the ER can channel calcium movement rapidly across the entire diameter of the cell (Mogami et al., 1997). Furthermore, the calcium channel proteins and receptor proteins of the endoplasmic reticulum are also found on the NE (see below).

The enormous bidirectional traffic between the nucleoplasm and the cytoplasm proceeds via the controlled-gate structure of the NPC (see above). Some of the NPC proteins are membrane proteins, with cytosolic domains contributing to the structure of the pore itself. The luminal domains of these proteins lie within the lumen of the NE and are thus poised to sense conditions inside this organelle and modify transport through the pore accordingly. Direct experimental evidence for an interaction between the transport activity of NPCs and the calcium level in the NE has now been provided by experiments using drugs such as thapsigargin to prevent refilling of ER calcium stores (Lee et al., 1998). As described above, passive diffusion accounts for transport of molecules < 10 kDa, whereas above 70 kDa ATP-consuming active transport is needed. For molecules in the range 10 to 50 kDa, transport may be via passive diffusion, but this depends on the NE luminal calcium concentration. Depletion of cisternal calcium leads to a block in transport in this molecular weight range (Lee et al., 1998). This is accompanied by a conformational change in the NPC, involving a large movement of the central plug toward the cytoplasmic ring (Perez-Terzic et al., 1996). ATP-dependent NLS signal-mediated nuclear import is also sensitive to the change in NPC conformation that follows loss of cisternal calcium (Greber and Gerace, 1995). Calcium dependence of both passive and active nuclear transport provides a rather general control mechanism, permitting the simultaneous modulation of mRNA export and protein import, for example.

The nuclear envelope is in a unique position to make *independent* contributions to the calcium homeostasis of the nucleoplasm and the cytoplasm. This could be achieved by the asymmetric expression of calcium channels in the two membranes, or in differential concentrations of receptors responsible for sensing in nucleoplasm and cytoplasm the level of those compounds responsible for the triggered release of calcium from the ER stores (Petersen et al., 1998). Evidence for differential expression of proteins between the INM and the ONM is now clear, and specific INM proteins have been identified (see above). Evidence is now accumulating that only the ONM contains the calcium ATPase uptake pump (Humbert et al., 1996), whereas the INM expresses both high levels of IP₃ receptors (Humbert et al., 1996; Hennager et al., 1995) and a cADP-ribose responsive calcium channel, most probably belonging to the ryanodine receptor family (Santella and Kyozuka, 1997; Gerasimenko et al., 1995). Despite the small size of the luminal space in the NE, the calcium concentration is held at a resting level >100 μM and the NE lumen is continuous with the massive calcium stores of the ER, making this a very potent source of calcium for intranuclear signaling.

These issues are germane to the control of differentiation and cellular activity because nuclear calcium controls many aspects of gene expression via multiple pathways (Hardingham et al., 1998). It is plausible to suggest that rapid changes in transcription rates of specific genes could be obtained via calcium signaling; it is probable that calcium spike frequency, amplitude, and duration can be discriminated by different isoforms of calcium/calmodulin-dependent protein kinases to

give still further subtleties to the responses that may be obtained (Heist and Schulman, 1998). The control of nucleoplasmic calcium concentration then takes on a new significance. Furthermore, the presence of membrane channels invaginating the nucleus offer an additional mechanism for the spatial integration of control of gene expression. In such a model, the NE would mediate calcium transients responsible for local changes in gene expression within the peripheral nucleoplasm, whereas deep nuclear channels would act analogously to the T-tubule system of muscle to propagate these signals in a spatially specific manner deep into the nuclear interior.

The effectiveness of such deep intranuclear calcium signaling would be entirely dependent on the regions of chromatin that were brought into proximity with a channel. Thus, this mechanism would probably only be useful if specific chromosomal regions could rely on being close to the nuclear envelope, either at the nuclear surface or close to a channel deep in the nucleoplasm. This prediction generates the testable hypothesis that the structure of the interphase nucleus should be sufficiently organised for certain loci to be always close to the nuclear boundary. Evidence from *Drosophila* embryo nuclei suggests that there are at least some systems in which the organization of each chromosome follows a pattern that is statistically predictable across a population of synchronised cells (Marshall, Dernburg et al., 1996). Similar analyses of chromosomal location in mammalian interphase nuclei will be required before the generality of this organization can be assessed. The known associations between the intranuclear position of the nucleolus and certain chromosomal regions (nucleolar organizer regions, or NORs, (Ozen et al., 1995; Schwarzacher and Wachtler, 1991)) offer some reason for optimism that the chromosomal distribution throughout mammalian interphase nuclei will turn out to have considerable organization.

IX. CONCLUSION

The view that the NE simply represents an inert double-membrane barrier between the nucleoplasm and the cytoplasm is no longer sufficient. Although the NE does form such a barrier, it also contributes to the structure, distribution, and transport behavior of the NPC that mediate bidirectional nucleocytoplasmic transport. Furthermore, the NE is increasingly recognized to play a role in organizing underlying nucleoplasmic structures. Specific proteins of the INM have been identified that may offer selective, controllable interactions between the NE and the underlying structures. As this includes domains of chromatin, an indirect role in the control of gene expression and differentiation is therefore also possible.

The role of the intermembrane luminal space is now better understood, and the part played by the calcium-stores within the NE is now being established. A complex role in calcium mediated signaling now seems very likely, adding further to the contribution of the NE itself to the control of gene expression. This complexity

may be further enhanced if the possible contribution of NE membrane to a nuclear PI kinase pathway is added.

Finally, the recognition that the NE may be morphologically specialised with deep invaginating channels offers a further spatial sophistication to the signalling activities of NE structures. The altered expression of these structural features in disease states may tell us much more about their normal roles, as well as providing useful diagnostic tools.

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MEMBRANE PORES

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I.	Introduction	300
II.	Endogenous Pores	300
III.	Induced Pores	304
	A. Viruses	304
	B. Microbial Toxins	306
	C. Protozoa	310
	D. Animal Toxins	310
	E. Immune Molecules	311
	F. Common Features	312
IV.	Synthetic Pores	315
V.	Conclusions	318
	Acknowledgments	318
	References	319

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I. INTRODUCTION

Fifty years ago, methods for the sequencing of proteins, and for the determination of their three-dimensional structure by X-ray crystallography, were being developed. Elucidation by X-ray crystallography of the three-dimensional structure of DNA—from which insight into how DNA is replicated emerged almost incidentally—followed shortly thereafter. But a quarter of a century was to elapse before the structure of membrane proteins began to be clarified. Indeed, in 1950 the very nature of biological membranes was still in doubt. Today, we know not only the structure of several membrane proteins—although many still elude us—but we can express particular proteins by gene technology in cells that do not contain them and are thus able to study their unique properties. A review of membrane proteins might therefore be appropriate, were it not for the fact that many excellent ones, that describe recent technologies, exist (see, e.g., Jennings, 1989; Reithmeyer, 1995; Shao et al., 1995; von Heijne 1995; Watts et al., 1995; Danielson and Falke, 1996; Fearnley and Walker, 1996; Gennis and Ferguson-Miller, 1996; Jones 1998).

Instead, we concentrate on those membrane proteins that form a pore across a membrane. Such pores range from transient, short-lived ones that enable a molecule like glucose to cross a membrane to stable, long-lived pores such as the ion channels of muscle and nerve. [We use the word *pore* to indicate any structure that allows water-soluble molecules to cross a biological membrane, but follow current practice in referring to a pore that allows ions to flow freely down their concentration gradient as a *channel*.] This discussion includes pores created by infectious agents. The mechanisms by which infectious microbes subvert the normal machinery of plant and animal cells are essentially those of molecular mimicry. A virus binds to a certain molecule on the surface of a host cell because a part of the viral surface happens to have the appropriate shape; sometimes this is because it resembles a normal ligand such as a hormone, neurotransmitter, or cytokine. Binding is usually followed by entry of the virus and the start of its replication. The same strategy is followed by numerous bacterial toxins: binding to a surface receptor as a result of an appropriate fit or resemblance to a natural ligand. In this instance the outcome is pathogenic because entry of the toxin—or a part of it—disrupts cell metabolism through further molecular mimicry, as in the case of diphtheria, cholera, tetanus, and botulinum toxin, or because the toxin forms a pore stable and large enough to cause leakage of essential molecules and ions, as in the case of pneumolysin and *Staphylococcus aureus* α toxin.

This review focuses particularly on bacterial toxins of the second kind—those that form stable pores across the plasma membrane of cells. This is partly because the subject has received relatively less attention than other topics, and partly because it happens to be the field of the authors' own researches over the past decade or so. We begin with a brief review of endogenous pores and follow with

a discussion of induced pores and the ways in which they resemble endogenous ones. More recently our studies of membrane pores have led us to explore the properties of pores created across synthetic, nonbiological, membranes: this forms the final part of our review.

II. ENDOGENOUS PORES

Biological membranes have evolved in order to separate the substrates and enzymes, as well as the RNA and DNA, of living processes from their environment: biological membranes are at least as old as the first cells that emerged on earth almost 4 billion years ago. Without membranes the molecules of life are too dilute to function effectively: enzyme-catalyzed reactions do not occur in air, in rivers, in lakes or the oceans, nor do they take place within the soil. In all these environments, in contrast, there are cells—chiefly microbes—in all of which enzyme-catalyzed reactions occur: the production of ATP from nutrients, and the utilization of adenosine triphosphate (ATP) to drive biosynthetic reactions like the synthesis of DNA, RNA and proteins. The membranes surrounding cells serve not only to keep biological molecules at high enough concentration—up to the mM range—for enzyme-catalyzed reactions to occur at reasonable rates, they also serve to maintain an internal ionic milieu different from that of the environment: this means, in general, higher K⁺ and phosphate, lower Na⁺ and Cl⁻, and much lower Ca²⁺. Just why such a milieu evolved as the more efficient for biological catalysis is not clear. Perhaps it approximates to the milieu inside certain zeolites or clays—which we now know are able to catalyze organic reactions (Creyghton, 1998)—and from which life may have evolved (Nisbet and Fowler, 1996). It certainly does not approximate to the milieu of the oceans in which metazoan life is supposed to have originated: today oceans are, and probably were, 4 billion years ago, high in Na⁺ and Cl⁻ (around 0.5 M).

Because all biological reactions take place in a watery milieu—whether high in K⁺ and phosphate or not—and because the extracellular environment is generally also of a watery nature—biological membranes have evolved as molecular structures that are insoluble in water and hence able to separate two watery solutions. In most cases these structures are bilayers of phospholipids, with the polar, water-soluble moieties making contact with the two liquids that they keep apart, and the apolar moieties in the middle providing a barrier to the flow of water-soluble molecules across them; the apolar middle part also lowers the energy of the system through hydrophobic interactions and results in the stable assembly of phospholipids as a two-dimensional sheet or bilayer.

If biological membranes were no more than phospholipid bilayers, however, how would the nutrients essential to the propagation and maintenance of life pass from the environment to the inside of a cell? For a gaseous nutrient like oxygen, this does not present a problem, as oxygen is hydrophobic enough to

permeate a lipid bilayer. For water-soluble nutrients like glucose and amino acids to be able to cross a lipid bilayer, however, a special mechanism has evolved. This is based on a class of protein that is able to insert into a lipid environment through hydrophobic interactions. The substrate is bound close to the lipid barrier; as a result of subtle changes in protein conformation, the bound substrate becomes exposed to either the extracellular or the intracellular side of the plasma membrane. Such a mechanism accounts for the movement of molecules like glucose—whether by facilitated diffusion or by Na^+ -linked cotransport (Henderson, 1991; Silverman, 1991), as well as amino acids (Malandro and Kilberg, 1996), across biological membranes. Similar proteins are responsible also for generating and maintaining the ionic composition within cells: high K^+ , low Na^+ and even lower Ca^{2+} . This class of proteins moves K^+ and Na^+ , and Ca^{2+} , against their respective concentration gradients through the utilization of ATP—the so-called sodium pump or Na^+/K^+ -ATPase (Skou, 1998) and calcium pump or Ca^{2+} -ATPase (de Meis, 1995; Carafoli, 1997), respectively. In none of these cases does a molecule like glucose or an ion like K^+ permeate freely: passage across the membrane is dependent on binding to a specific site on the respective membrane protein, and the porous nature of the protein assembly is essentially transient. Nevertheless, such structures do appear to allow a certain amount of nonspecific leakage to occur: the leakage of Na^+ through the Na^+/K^+ -ATPase—in either direction—is one example; the fact that the glucose transporter contains more water associated with it than might be expected of a protein buried in a lipid environment (Alvarez et al., 1987), is another.

The synthesis of ATP is dependent on a second type of transmembrane protein: a channel—across the plasma membrane in the case of prokaryotic microbes, and across the mitochondrial and chloroplast membranes in the case of eukaryotic organisms—that allows protons to move down their electrochemical gradient. Such proteins adopt a three-dimensional structure resembling a doughnut or polo mint: the outside of the structure is sufficiently hydrophobic to make contact with the hydrophobic middle of phospholipid bilayers; the inside is sufficiently hydrophilic to allow ions to flow freely down their concentration gradient to drive the synthesis of ATP (Racker, 1976; Collinson et al., 1996; Boyer, 1998). As metazoan organisms capable of muscular movement and nerve transmission developed, other types of ion channel evolved: those through which ions like Na^+ (Trimmer and Agnew, 1989; Marban et al., 1998), K^+ (Latorre et al., 1989; Nichols and Lopatina, 1997; Ashcroft and Gribble, 1998; Durrell et al., 1998; Sansom, 1998; Vergara et al., 1998) and Ca^{2+} (Bean, 1989; de Meis, 1995; Dolphin, 1998; Randall, 1998; Walker and de Waard, 1998) leak at fast rate ($>10^6$ ions/second) (Hille, 1992; Catterall, 1995). Although it is assumed by many physiologists that ion channels for K^+ are essential for the generation of a transmembrane electrical potential—negative on the inside—across the plasma membrane of animal cells because that is the situation in muscle and nerve, this is not correct. Other cells, like neutrophils (Bashford and Pasternak, 1985) and certain tumor cells

(Bashford and Pasternak, 1984), set and maintain a transmembrane potential through an ion pump—the Na^+/K^+ -ATPase, which is electrogenic; anion leaks restrict the potential to around -60 mV (Bashford and Pasternak, 1986). The situation is similar to that of mitochondria and chloroplasts that pump out protons at the expense of oxidative energy coupled to a return of protons through the proton channel or $\text{F}_0\text{/F}_1$ -ATPase (Boyer, 1998); when ATP synthesis is not required, non specific proton leaks restrict the potential to around -180 mV. Because many cells lack K^+ and Na^+ channels, it appears that cation ion pumps preceded cation channels in evolution as well as during ontogeny. Just what roles a transmembrane potential fulfills in nonexcitable cells is not clear; Na^+ -linked amino acid entry into cells may be one.

In addition to membrane proteins that are responsible for the movement of ions and molecules like glucose and amino acids across membranes, there exist proteins—similar to those of ion channels—that form larger, stable membrane pores. The proteins termed connexins constitute the so-called gap or communicating junctions between adjacent cells that are large enough (some 1.5 to 2 nm in effective diameter) to allow molecules of up to around 750 to 1000 Da to pass (Goodenough et al., 1996; Perkins et al., 1997; Zhou et al., 1997; Finbow and Pitts, 1998). Somewhat larger-sized pores (around 2 nm in diameter) are present in the outer membrane of mitochondria: one such type of pore allows nucleotides like ATP to pass (Blachly-Dyson et al., 1993; Rostovtseva and Colombini, 1996, 1997), another enables certain proteins synthesized in the cytoplasm of cells to cross the outer mitochondrial membrane (Schatz, 1998).

Finally, we should mention a class of transient pore, similar to that involved in the passage of glucose across a membrane through the glucose transporter or the passage of Na^+ and K^+ —in opposite directions—through the Na^+/K^+ -ATPase. These structures enable peptides and proteins to somehow wriggle across a membrane as a result of binding to a specific site on a membrane protein. The synthesis of proteins on the endoplasmic reticulum of eukaryotic cells is one example (Rapoport et al., 1996); the import into mitochondria of proteins synthesized in the cell cytoplasm across their outer (Schatz, 1998) and inner (Pfanner et al., 1994) membrane is another. In the latter case a transmembrane voltage gradient and ATP are required to pull proteins through the pore. In other transient pores, like those of the glucose transporter or Na^+/K^+ -ATPase, heat (kT) alone provides the activation energy necessary for the conformational change in protein structure that presents the molecule to be transported to either side of the hydrophobic lipid barrier. Flow through ion channels requires little activation energy, with Q_{10} values considerably less than 2 and approaching the “diffusion limit” of 1.2.

The membrane proteins that have been discussed are stable entities, regardless of whether the transport they catalyze is transient or continuous. Once formed across a membrane, the proteins remain in place for the lifetime of that particular cell, save only for the fact that turnover may replace, add, or remove a particular pore-forming protein. Glucose transporters, especially those of insulin-sensitive

Table 1. Transport Across Membranes

Protein Involved	Type of Compound	Rate	Reference
None	O ₂ , CO ₂ , anesthetics	Slow	—
Diffusion-limited	Water, fatty acids		
Carrier	Glucose, amino acids	Fast	Silverman (1991)
Diffusion-facilitated	Nucleosides		Malandro and Kilberg (1996)
Pump	Na ⁺ , K ⁺ , Ca ²⁺ , H ⁺	Fast	Skou (1998); de Meis (1995)
Diffusion-facilitated and ATP-energized			Carafoli (1997)
Channel	Na ⁺ , K ⁺ , Ca ²⁺ , H ⁺	Very fast	Hille (1992); Bean (1989)
Diffusion-facilitated			Boyer (1998)

Note: Selected references to some of the proteins involved are given. Further references may be found in the text.

cells like muscle and fat, are a good example of this situation (Simpson and Cushman, 1986). Turnover of a different kind occurs in cells through injury. For example, in multiple sclerosis and other demyelinating diseases, Na⁺ channels at the nodes of Ranvier of certain myelinated axons become randomized along the length of the axon (Moll et al., 1991; Waxman et al., 1994; England et al., 1996): to transmit fast and effective action potentials, a given number of channels needs to be concentrated at the nodes of Ranvier. Although stably inserted into a membrane, pores *function* for only a limited time during the life of a cell: when the concentration of glucose or K⁺, for example, is sufficiently high to bind to the respective protein (glucose transporter or Na⁺/K⁺-ATPase), or when the concentration of acetyl choline or transmembrane voltage is sufficiently high to open a cholinergic or voltage-gated channel.

These, then, constitute the various protein assemblies (several are made up of more than one specific protein) that facilitate the movement of ions and other molecules across biological membranes (Table 1). The movement of molecules *with*—as opposed to *through*—membranes, as occurs during endocytosis (Mellman, 1996), exocytosis (Almers, 1990) and transport across endothelia (Anderson, 1998), falls outside the scope of this review, except insofar as the processes involve membrane fusion, which is discussed briefly in the following section.

III. INDUCED PORES

A. Viruses

Certain enveloped viruses—the paramyxoviruses in particular—induce the formation of pores across the plasma membrane of cells to which they attach. The

mechanism of induction involves fusion between the envelope of the virus—made up of a phospholipid bilayer containing membrane proteins—and the plasma membrane of the host cell. The viral phospholipid bilayer not only resembles that of host cells, it *is* a part of the bilayer of the cell that the virus last infected, and from which it was released by budding off. The membrane proteins are virus-specific molecules that were synthesized in the infected cell and then inserted into its plasma membrane. Such proteins (1) bind to receptors on the host cell plasma membrane and then (2) induce fusion between the viral and cell membrane. As a result, the rest of the virus enters the cell and continues the infective process. The best-studied example of this situation is the Sendai virus, also called HVJ (haemagglutinating virus of Japan) because of the country of its origin and because one of its membrane proteins cross-links erythrocytes (and other cells to which it binds); the protein is known as HN as it has haemagglutinating, and also neuraminidase, activity (Okada, 1969). Because the envelope of several such paramyxoviruses already contains a pore, fusion between it and the host cell introduces the pore into the latter, initiating leakage of ions and small molecules into and out of the cell; only at high concentrations of virus are pores large enough to allow macromolecules like proteins to leak out (Pasternak, 1984). Because the leakage of ions across the plasma membrane alters internal osmotic pressure, erythrocytes are ruptured (hemolyzed). Nonerythroid cells do not lyse—except at high concentrations of virus—because such cells are covered with microvilli and other membrane structures, which can unfold to accommodate the extra pressure (Knutton et al., 1976); in addition, such cells are able to shed proteinaceous pore assemblies (Ramm et al., 1983) and to recover (Bashford et al., 1985).

The clinical significance of the Sendai virus is limited, although it can induce pathology in an experimental animal model (Mahadevan et al., 1990). On the other hand, it has proved to be a valuable research tool because of its ability to cross-link

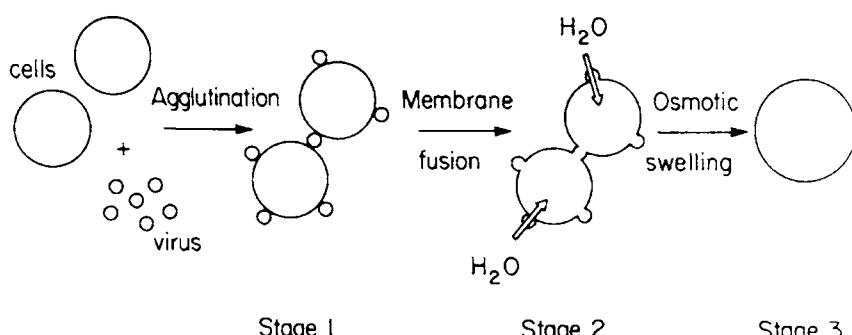


Figure 1. Virus-induced pores drive the formation of giant cells. For further information, see Knutton and Pasternak (1979).

two cells (which can be different), and then to fuse with each. Because the act of fusion introduces a pore between cell-virus-cell, which then expands and forms a single giant cell containing two nuclei in a common cytoplasm (Figure 1), it allows one to study nuclear control of cell function (Harris, 1970). For example, by fusing a malignant cell with a nonmalignant one, Harris was able to show that malignancy in this situation is recessive because the hybrid cells were not malignant (Harris, 1970). The pores introduced into the two cells by the Sendai virus are crucial to the formation of a single giant cell. If a type of the Sendai virus that does not contain pores is used, cell-virus-cell fusion still takes place, but the "dumbbell" structure that is formed does not expand into a giant cell: it is the internal osmotic pressure created by leakage of ions through virus pores that causes the expansion of the two cells into a single one (Knutton and Pasternak, 1979).

Haemolytic paramyxoviruses like Sendai are the exception, rather than the rule. Clinically important paramyxoviruses such as mumps or measles are not hemolytic and do not form pores in host cells (Foster et al., 1983; Gray et al., 1983), but Newcastle disease virus, which is an important pathogen for poultry and cattle, is hemolytic and forms pores (Klemperer, 1960; Poste and Pasternak, 1978). Most other viruses—whether enveloped or not—do not fuse with the plasma membrane of cells to which they attach. Instead, binding is followed by endocytosis, sometimes called viropexis, as a result of which the virus enter the cytoplasm and begins to replicate. In the case of enveloped viruses, of which the myxoviruses—and influenza virus in particular—are the best-studied examples, membrane fusion does take place, but it is between the viral envelope and the endosomal membrane; the low pH of endosomes is required to trigger the process by initiating a conformational change in the viral "fusion" protein. If the external pH is artificially lowered, the viral envelope fuses with the plasma membrane and, as with hemolytic paramyxoviruses, introduces a pore into the cell membrane (Patel and Pasternak, 1985). In summary, it would appear that pore formation by viruses is not a prerequisite for the entry of enveloped viruses into host cells through membrane fusion. However, the criterion for the absence of pore formation was lack of leakage of ions and cellular metabolites (Foster et al., 1983; Gray et al., 1983), and it is quite possible that transient, small pores are formed whenever fusion takes place—as in exocytosis (Almers, 1990)—and that more sophisticated methods (Albillios et al., 1997) are necessary to observe them.

B. Microbial Toxins

Transient Pores

Several toxins released by microbes cause pathogenesis by introducing an inhibitory protein into the cytoplasm of host cells. The best-characterized examples are cholera and diphtheria toxins (Olsnes et al., 1993; Lesieur et al., 1997; Mekalanos et al., 1997; Sandvig et al., 1997; Raufman, 1998), and tetanus and botulinum

toxins (Montecucco and Schiavo, 1993; Brin, 1997)—the last-mentioned of which is the most toxic naturally occurring substance known to man. The active form of these toxins is a dimeric protein, with a disulphide bond linking the subunits. The two disulphide-linked fragments—called A and B in the case of cholera and diphtheria toxin—are derived from a single precursor protein that is cleaved by proteolysis during maturation, just as occurs during the biosynthesis of insulin. On binding to the surface of a sensitive cell, the B fragment becomes inserted into the plasma membrane. This facilitates insertion of the A fragment, which, following reduction of the disulphide bond, is released into the cell cytoplasm where it interferes with the normal metabolism of the host cell. The way that the B fragment enables the A fragment to cross the plasma membrane bears some resemblance to that by which eukaryotic proteins cross the endoplasmic reticulum (Rapoport et al., 1996) or inner mitochondrial membrane (Pfanner et al., 1994). Neither the A nor B protein alone is able to elicit cell damage, but the B protein on its own forms a small, transient pore.

The ability of such microbial toxins to induce pore formation is based largely on experiments with planar lipid bilayers (Montal and Mueller, 1972). A bilayer made up of natural or synthetic phospholipids is spread across a small orifice—typically less than 10 µm in diameter—that separates two salt solutions, into each of which an electrode is placed. A potential difference is then applied across the two electrodes: very little current is recorded, showing that such a bilayer does not permit the movement of ions across it. As mentioned in the Introduction, this is precisely the reason why biological membranes evolved as phospholipid bilayers. If now some pore-forming protein is added at a sufficiently high concentration, current flows across the bilayer; the reader will note that pores large enough to be detected by the flow of an ion current are formed even in the absence of the relevant receptor molecule, that serves to concentrate the pore-forming protein at the surface of a host cell in the natural situation. The B proteins of cholera, diphtheria, tetanus, and other toxins all form pores of varying size (in terms of conductance) across lipid bilayers.

An interesting point to note is that current does not flow continuously. Instead, periods of high conductance alternate with periods of low conductance, just as in endogenous ion channels (Hille, 1992): Figure 2 shows some examples. Although the patterns of fluctuation vary considerably, largely for technological reasons, none shows a constant current when analyzed at sufficiently fast time resolution. In fact, *any* protein that forms a pore across a membrane produces fluctuations of current when studied by the lipid bilayer technique. What does this mean? In the case of endogenous ion channels, the fluctuations in current result from changes in protein conformation between an “open” and a “closed” state (Hille, 1992; Catterall, 1995; Unwin, 1995); the open state allows permeation of ions through it, the closed state does not. It would, therefore, seem that all pore-forming proteins oscillate between similar open and closed conformations. However, experiments discussed below in relation to pores formed by *S. aureus* α toxin and to pores

across synthetic membranes, suggest that this assumption is unwarranted, and other explanations are possible. It may be noted that although pores across lipid bilayers do disappear after awhile, they cannot be said to be "transient" in the way that pores formed by A-B toxins across the plasma membrane of susceptible cells are; in lipid bilayers, there seems to be little difference between pores that are transient and those that are stable in the natural situation.

Stable Pores

Several bacterial toxins (Jeljaszewicz and Wadstrom, 1978) form pores that result in cell damage—not because an inhibitory protein is introduced into the cell, but because the pore itself is stable and large enough to cause intracellular metabolites and K⁺ to leak out and extracellular Na⁺ and Ca²⁺ to leak in, just as with the hemolytic paramyxoviruses discussed above. As with the Sendai virus, the size of the pore depends on the concentration of toxin. Only at high concentration are the pores large enough to allow leakage of intracellular proteins, and cytolysis, to occur (Bashford et al., 1986). At lower concentrations of toxin, nonerythroid cells are able to recover from the insult (Korchev et al., 1995a). Some of these toxins are secreted by bacteria—the colicins are an example—in order to kill other bacteria; when studied in the lipid bilayer system, the pores resemble those induced by the toxins discussed below. The important point is that the structure of such pore-forming agents has been obtained at greater resolution than in other cases, and therefore constitutes a good model for conformational changes involved in pore formation (Cramer et al., 1995).

The size of pore that different bacterial toxins induce has been studied by leakage of metabolites and dyes across the membrane of host cells and out of liposomes (artificial vesicles made up of phospholipids), by conductivity measurements across planar phospholipid bilayers—assuming a cylindrical pore whose length is equivalent to the thickness of the bilayer—and by electron microscopy of negatively stained preparations. Although conductance is not necessarily proportional to size—high conductance potassium channels are probably no larger than low conductance channels—in several cases the sizes of toxin-induced pores obtained by the three methods are in quite good agreement. They vary from around 1 to 2 nm diameter for *S. aureus* α toxin (eg Korchev et al., 1995b) to more than 10 nm for *Clostridium perfringens* θ toxin (e.g., Menestrina et al., 1990) and other toxins belonging to the family of thiol-activated cytolysins like streptolysin, pneumolysin, and listeriolysin (Smyth and Duncan, 1978), all of which share a common amino acid sequence—WEWW—that appears to be involved in pore formation (Paton et al., 1993). The larger types of pore show several discrete conductance states in lipid bilayers; pneumolysin, for example, exhibits small (less than 50 pS), intermediate, and large (greater than 1 nS) fluctuations in 0.1 M KCl (Korchev et al., 1992), and the same is true of perfringolysin (Menestrina et al., 1990).

Current across pores induced in lipid bilayers by all these toxins fluctuates between high- and low-conductance states in the same way as current through transient pores and endogenous ion channels (Figure 2). Moreover, current is voltage-dependent (in most cases, voltage promotes the low-conductance state). It might therefore be supposed that in each case the pore oscillates between an open and a closed configuration. However, when the size of the high- and low-conductance states induced by one microbial toxin, namely *S. aureus* α toxin, was measured by an exclusion technique, the low-conductance state was found to be substantially "open." The technique (Krasilnikov et al., 1992) depends on introducing uncharged molecules like glycerol, sugars, polyethylene glycols, and dextrans of various sizes into the solutions separating a lipid bilayer: if the molecule is small enough to penetrate the pore, current decreases because of viscosity changes; if it is too large to enter the pore, current remains unchanged. In the case of *S. aureus* α toxin, molecules as large as sucrose penetrate the low-conductance state. In fact the difference in pore size between the high-and low-conductance state is less than 2 fold, whereas the difference in conductance is more than 10-fold (Korchev et al., 1995b). In short, conductance is not a reliable measure of pore size, and "closed" states may actually be "open." A similar conclusion has been reached in regard to the voltage-dependent anion channel of the outer mitochondrial membrane (that has other features in common with the *S. aureus* α channel [Menestrina, 1986]), insofar as the low-conducting state allows small ions to pass (Rostovtseva and Colombini, 1996). Insofar as other endogenous ion channels are concerned, it has long been assumed that "large" (in terms of conductance) K⁺ channels are not actually large in terms of size: the selectivity of "maxi" K⁺ channels, for example, is the same as that of low-conductance channels, which speaks against a physically large pore. No satisfactory molecular explanation for the high conductance of such channels has as yet been advanced: multibarreled low-conductance channels that open and close in unison is one interpretation of the data; a negative site near the mouth of a maxi channel that maintains high selectivity has also been noted (e.g., Latorre et al., 1989), and the possibility that negative sites themselves are responsible for an abnormally high conductance (see below) is another possibility (Lauer and Gage, 1997). Regarding the low-conductance state of endogenous ion channels, it is generally ascribed to experimental deficiencies of the technique (an insufficiently tight seal in the case of patch pipet measurements on intact cells, e.g.) and is assumed to reflect a closed configuration of the channel through which the ions pass.

If the difference between high-and low-conductance states, at least as far as *S. aureus* α toxin is concerned, cannot be explained merely by a difference in effective pore size, what other interpretation might be advanced? Based on studies with synthetic pores that are discussed shortly, we propose that fluctuations in current can arise because of fluctuations in surface charge. When fixed charges are present along the surface of a pore, counter ions are attracted, and the conductance is higher than it would be through an uncharged pore; the phenomenon has been

termed "surface conductance," a concept recognized almost a century ago (Smoluchowski, 1903). Surface conductance contributes to the overall current whenever current is not proportional to ionic strength; fluctuations of ion selectivity in concert with ion current is another clue. Both criteria are fulfilled in the case of *S. aureus* α toxin (Korchev et al., 1997). In short, ion flow through membrane pores is determined not merely by the physical dimensions of a pore, but by the nature of its surface chemistry as well.

C. Protozoa

Several species of protozoa secrete protein toxins. Although not as well characterized as those of bacterial origin, it is clear that several have pore-forming properties. The amebapore produced by *Entamoeba histolytica* (Gittler et al., 1984) is an example. In contrast to the pores discussed above, however, intracellular metabolism seems to be involved; in that sense such pores fall into a different category, more akin perhaps to the pores formed during exocytosis.

Yet another type of pore that does not resemble those induced by bacterial toxins is one that is induced across the erythrocyte membrane by malarial parasites like *Plasmodium falciparum*. These facilitate the transport of sugars and other nutrients required for growth of the internalized malarial parasite (Ginsburg et al., 1983; Ginsburg, 1990). Again, they have little similarity to the pores induced by bacterial or animal toxins, which are all rather nonspecific, but have more in common with endogenous pores like the glucose transporter. Their interest lies in the fact that inhibitors of the uptake process may have potential as antimalarial drugs (Ginsburg and Stein, 1987; Ginsburg, 1994).

D. Animal Toxins

Many animals, like snakes and spiders, sea urchins, and insects, incapacitate their prey through the secretion of toxic molecules. These are introduced into the host organism through a bite (snakes and spiders), a sting (insects), or a prick (sea urchins). Many of the toxins are hemolytic; some, like the phospholipases of snake venom, because they literally dissolve away the membranes of erythrocytes and other cells; others, like a toxin called latrotoxin from black widow spiders (Chanturia and Lishko, 1992; Krasilnikov and Sabirov, 1992), cytolsins from sea anemones, and other echinoderms (Belmonte et al., 1993; Macek et al., 1994), melittin—a well-defined 26 amino acid peptide—from bees (Habermann, 1972; Tosteson and Tosteson, 1981; Alder et al., 1991) and mastoparan—a 14 amino acid peptide—from wasps (Mellor and Sansom, 1990) because they form membrane pores. Where the properties of such pores have been studied in the lipid bilayer and other systems, they have been found to resemble the properties of pores formed by microbial toxins: melittin, for example, forms pores (around 10 pS) akin to those induced by A-B toxins, whereas the sea anemone cytolsins

form pores (around 500 pS; 1 to 2 nm diameter) similar to those induced by *S. aureus* α toxin. Moreover, several animal toxins form pores that are blocked by divalent cations (see Table 2).

In addition to the synthesis of such obviously toxic proteins, several animals secrete pore-forming proteins and peptides that seem to play a defensive, rather than an aggressive, role. Various such peptides called magainins, for example, are secreted by toad skin (Duclohier, 1994) and similar molecules called cecropins have been isolated from moths (Christensen et al., 1988) and the intestine of pigs (Agerberth et al., 1993). The pores formed by these molecules resemble those induced by melittin (Bechinger, 1997): like melittin, they bind largely to the surface of membranes (Gazit et al., 1996), with only occasional incursions through the middle of the bilayer to form a transient pore, as opposed to the more stable pores of toxins like *S. aureus* α toxin (see Section F). Insofar as these peptides secreted by animals have a protective role, they may be considered as part of their nonspecific immune system. In addition, there are two types of more specific immune molecules, i.e., those of humoral origin (complement proteins) and those of cellular origin (leukocyte-derived proteins), which will now be discussed.

E. Immune Molecules

Two types of immune molecule of animals—the “membrane attack complex” of activated complement (Mayer, 1972; Bhakdi and Tranum-Jensen, 1984) and the cytolysin from cytotoxic T cells (Henkart, 1985)—form pores across the plasma membrane of cells destined for destruction. In the first case, such cells are recognized by complement proteins because they are coated with an antibody elicited against some invading microbe; in the second case, they are recognized by a membrane protein on the surface of a cytotoxic T cell because they display a peptide derived from an invading microbe, often a virus, within the groove of a major histocompatibility complex (MHC) or human leucocyte antigen (HLA) protein that is present on the surface of the cell to be destroyed. In each case, the formation of a pore, similar to the large, stable pores formed by microbial toxins, is part of the destructive process. The two types of protein are not only similar in action, they also resemble each other in structure (Young et al., 1986). As in other situations, nonerythroid cells possess mechanisms for recovery (Ramm et al., 1983; Bashford et al., 1988a): whether affected cells recover or are killed depends on several factors, one being the amount of pore-forming agent, and hence the number of pores induced. Although both types of pore-forming agent—microbial toxin and immune molecule—cause the same sort of damage to host cells (indeed, there is homology between part of the complement protein C9 and melittin; Laine et al., 1988), the outcome insofar as the whole organism is concerned is different: in the case of microbial toxins it is detrimental; in the case of immune molecules (that act to rid the body of infectious microbes) it is beneficial.

F. Common Features

Attention has already been drawn to the fact that pores formed by agents as diverse as viruses, microbial toxins, and immune molecules have certain features in common. All, for example, show fluctuations in ion current similar to that displayed by endogenous ion channels (Figure 2).

The induction of pores also shares common features, insofar as several pore-forming agents display cooperativity when leakage of ions and small molecules from cells is measured; when two agents are added together, there is synergy between different agents (Bashford et al., 1986). The reason for this is unclear. It may be related to the fact that in many cases pore-forming proteins aggregate before, or during, insertion into the membrane. It is also the case that as the concentration of pore-forming agent is raised, progressively larger pores are formed: at low concentrations of *S. aureus* α toxin, for example, ions alone leak across the membrane; at higher concentrations, phosphorylated metabolites leak out; at even higher concentrations, trypan blue and other dyes leak into cells; only at the highest concentrations do proteins leak out and cells become lysed. (It is also the case, as might be expected, that as the concentration of pore-forming agent is raised, a progressively larger percentage of cells is affected.) An observation that is relevant to the induction of pores is that when pore-forming proteins bind to the plasma membrane of cells or to a lipid bilayer, most of the protein lies along the surface, with very little penetration into the interior of the membrane (e.g., melittin: Kuchinka and Seelig, 1989; cecropin: Gazit et al., 1996): only a small percentage of bound molecules form transmembrane pores (e.g., *S. aureus* α toxin: Bashford et al., 1996). In the case of the endogenous protein annexin V (Voges et al. 1995), Ca^{2+} channel-like activity appears to be induced without *any* penetration by the protein (Voges et al., 1994). Such situations are reminiscent of the interaction between other lipophilic molecules, such as local anesthetics, and membranes: most molecules are bound at the surface, with few penetrating the interior of the membrane (Conrad and Singer, 1981). The difference between stable and transient pores probably reflects no more than the extent to which subtle changes of conformation, between one that leads to a lower energy state when inserted into the lipid milieu of a membrane and one that does not, occur. It is such structural changes that need now to be explored by novel techniques.

Another common property is that flow through pores is inhibited by divalent cations (Pasternak, 1987a) and protons (low pH) on the extracellular side of cells (Bashford et al., 1989), with $\text{H}^+ > \text{Zn}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+}$ in order of efficacy (Bashford et al., 1988b) (Table 2). Where ion flow is measured as current in the bilayer system, the effect of protons and divalent cations is to promote voltage-dependent "closure" of pores (Bashford et al., 1988b); in some cases this is reversible, in other cases not (Pasternak, 1991).

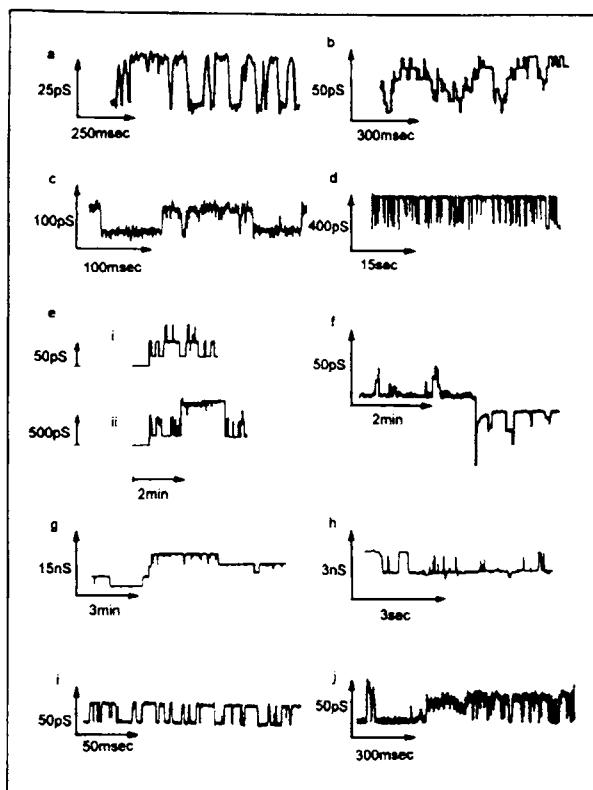


Figure 2. Ion currents through pores. (a) A sodium channel reconstituted into a PE/PS bilayer membrane bathed in 0.15 M NaCl, pH 7, in the presence of 120 nM batrachotoxin. Applied voltage -90 mV (adapted from Cukierman et al., 1988). (b) Diphtheria toxin fragment CRM45 in an asolectin bilayer membrane bathed in 0.1 M NaCl, pH 5.5. Applied voltage -120 mV (adapted from Kagan et al., 1981). (c) Tetanus toxin in an asolectin bilayer membrane containing ganglioside on one side only and bathed in 0.5 M KCl, pH 7. Applied voltage 100 mV (adapted from Borochovneori et al., 1984). (d) *Staphylococcus aureus* α toxin in a DiPhyPC bilayer membrane bathed in 0.1 M KCl, pH 4.5. Applied voltage 20 mV (authors' unpublished experiments). (e) Pneumolysin in a DOPC:ergosterol bilayer membrane bathed in 0.1 M KCl, pH 7.4. Applied voltage 100 mV (i) or 50 mV (ii) (adapted from Korchev et al., 1992). (f) Heat shock protein Hsp-70 in a DiPhyPC bilayer membrane bathed in 0.1 M KCl, pH 7. Applied voltage +/- 80 mV (adapted from Alder et al., 1990). (g) Complement poly C9 in an asolectin bilayer membrane bathed in 0.1 M NaCl, pH 7. Applied voltage 120 mV (adapted from Young et al., 1986). (h) Perforin (from cytotoxic lymphocytes) in a PC/PI bilayer membrane bathed in 0.1 M NaCl, pH 7. Applied voltage -7 mV (adapted from Bashford et al., 1988a). (i) Current between borosilicate glass and SylgardTM bathed in 0.4 M NaCl, pH 7. Applied voltage -300 mV (adapted from Sachs and Qin, 1993). (j) Current through a single pore in a PETP membrane bathed in 0.1 M KCl, pH 7.4. Applied voltage -500 mV (adapted from Bashford, 1995).

Table 2. Agents that Form Divalent Cation-Sensitive Pores in Membranes

Viruses	Sendai	Pasternak and Micklem (1974)
	Newcastle disease	Burnet (1949); Poste and Pasternak (1978)
	Influenza	Patel and Pasternak (1985)
Bacterial Toxins	<i>S. aureus</i> α -toxin	Bashford et al. (1984); Harshmann and Sugg (1985)
	<i>S. aureus</i> δ -toxin	Mahadevan et al. (1990)
	<i>C. perfringens</i> θ -toxin	Menestrina et al. (1990)
	Streptolysin O	Thelestam and Mollby (1980)
	<i>S. pneumoniae</i> pneumolysin	Bouhois et al. (1991)
	<i>E. coli</i> hemolysin	Menestrina et al. (1990)
	<i>A. hydrophila</i> hemolysin	Wilmsen et al. (1990)
	<i>C. lacteus</i> cytolyisin	Liu and Blumenthal (1988)
	<i>B. thuringiensis</i> δ -endotoxin	Knowles et al. (1989)
	Various	Avigad and Bernheimer (1976)
Animal toxins	Melittin (bee venom)	Bashford et al. (1986)
	Cytolysin (sea anemone)	Pasternak et al. (1989)
	Latrotoxin (spider venom)	Mironov et al. (1986)
Immune proteins	Activated complement	Gotze et al. (1968); Yamamoto and Takahashi (1975); Boyle et al. (1979)
	Cytolysin (perforin)	Bashford et al. (1988a)
Synthetic Compounds	Polycations	Bashford et al. (1986); Elferink (1991)
	Triton X-100	Avigad and Bernheimer (1976); Bashford et al. (1986); Madigan et al. (1990)

Note: For further references, see the text and Bashford (1995).

Although the induction of pores by some agents—like the perforin of cytotoxic lymphocytes—requires Ca^{2+} , this is at a relatively low concentration; higher concentrations prevent leakage. While low pH can have little effect physiologically, except in modulating virus-induced leakage across the endosomal membrane, this is not the case for extracellular Ca^{2+} and Zn^{2+} . Indeed, cell damage through leakage of ions and metabolites occurs only when the protective effect of plasma Ca^{2+} is overcome (Pasternak, 1987b), and a slightly increased concentration of intracellular Ca^{2+} that leaks through the induced pore aids recovery; too high an intracellular Ca^{2+} concentration leads to cell injury (Pasternak, 1986). Divalent cations prevent leakage not only through pores formed by proteins and peptides, but also through pores induced by synthetic agents such as polylysine or detergents at sublytic concentration (Bashford et al., 1986; Rostovtseva et al., 1994). What might the mechanism of their action be? At present, the answer is not yet clear. Moreover, because the induction and function of pores are separate processes, it is likely that divalent cations and protons act at more than one level: prevention of insertion (although not of binding) may be one; closure of pores may be another (although it is also possible that the two events are two sides of the same coin).

In order to examine the mechanisms underlying some of the common features, such as fluctuation of ion current and inhibition by divalent cations and protons, we have turned to a study of pores across synthetic membranes that contain no lipid or protein.

IV. SYNTHETIC PORES

The common properties of pores induced across lipid membranes suggest that small, water-filled pores in *any* material might behave in the same way (Lev, 1990). Track-etched membranes made of plastic such as polycarbonate or polyethyleneterphthalate (PETP) provide a system in which pores of defined shape can be studied. The plastic material is first rolled out to a thickness of 10 to 50 μm . It is then bombarded at right angles with a beam of high-energy ions, which generate "tracks" of damaged material. Subsequent etching with warm alkali enlarges the tracks into quasi-cylindrical pores; the longer the etching time, the larger the diameter of the pores. The smallest pores appear to have an effective diameter of about 2 nm: in other words, they are of the same size as endogenous pores like gap junctions or the voltage-dependent anion channel of the outer mitochondrial membrane, or as induced pores such as that formed by *S. aureus* α toxin. The fact that they are some 1000 times longer seems to matter rather little insofar as their properties are concerned, which turn out to be remarkably similar to those of the pores we have discussed so far.

Track-etched PETP membranes have several favorable features. (1) They are robust: the same piece can be used over and over again for a series of flow measurements. (2) They are chemically well-defined; for example, the negatively charged surface (etching hydrolyzes the ester bonds to produce hydroxyl and carboxylic acid groups) can be re-esterified by treatment with diazomethane (Rostovtseva et al., 1996). (3) They can be produced with pore densities ranging from 1 pore/cm² to 10⁹ pores/cm²; this means that a 1-cm² piece, which fits well into the apparatus used for lipid bilayer experiments, can be used to assess ion current through a single pore or through an ensemble-averaged large number of pores. With the high pore-density material one can also measure the flow of suitably tagged ions or small molecules by radioactive assay in a bilayer apparatus or in a modified flow dialysis cell. Ion current through single pores can be measured in high pore-density material by use of a patch pipet.

The properties that pores through synthetic membranes share with endogenous and induced pores may be summarized as follows: (1) ion current through single pores shows fluctuations between high and low conductance states; (2) ion flow is selective; and (3) ion flow is inhibited by divalent cations and protons.

1. Ion current through single small pores in PETP and other synthetic membranes fluctuates between high-and low-conductance states (Figure 2), just as it does through endogenous and induced pores across biological membranes. It does

not matter whether the pores are present at low density and studied in a bilayer apparatus (Lev et al., 1993) or whether the pores are present at high density and studied with a patch pipet (Korchev et al., 1997). However, the diameter of pores is important: large pores (1 μm in diameter, e.g.) do not exhibit fluctuations. Independent confirmation of these results was obtained by Sachs and Qin (1993) who observed fluctuations of ion current when a patch pipet was pressed against the material normally used by electrophysiologists to achieve a tight seal, but in the absence of any biological membrane (Figure 2).

2. Despite their very similar size and mobility, K^+ and Cl^- ions do not pass through small pores in PETP membranes with equal facility. At neutral or alkaline pH, K^+ permeates such pores much more freely than Cl^- . The difference in diffusion can be assessed electrically as the reversal potential; that is, the voltage required to prevent current from flowing across a pore that separates solutions of differing strength: 0.1 M and 1.0 M KCl, for example. If the pore were nonselective, the reversal potential would be zero. With PETP pores that bear a negative surface charge as the result of the etching process, the reversal potential can be as high as 56 mV in a 10-fold gradient, indicating a strong preference for K^+ over Cl^- . The same result is obtained if diffusion of K^+ and Cl^- is measured directly with radioactive tracers (Rostovtseva et al., 1996). Ion selectivity, like fluctuation of ion current, disappears as pores become progressively larger.

3. Flow of ions, whether measured as current or as diffusion of radioactively tagged tracers, is reduced by low pH or by the addition of divalent cations. The same is true of selectivity: at sufficiently low pH, for example, PETP pores become nonselective. The order of efficacy in each case is $\text{H}^+ > \text{Zn}^{2+} > \text{Ca}^{2+} \geq \text{Mg}^{2+}$. A detailed assessment of the inhibition of ion current across PETP membrane pores by divalent cations indicates that the effect is to promote or stabilize the low-conductance state (Lev et al., 1993). Inhibition of ion flow by protons or divalent cations is reduced in large pores.

It may be noted that the order of efficacy of protons and divalent cations is precisely that found for reducing the voltage-dependency of Na^+ channel activation (Hille et al., 1975) or *S. aureus* α toxin channel (Menestrina, 1986; Bashford et al., 1988b) closure. However, caution in interpreting this result is necessary, as it may reflect no more than the fact that both PETP and proteins contain carboxyl groups to which protons and divalent cations bind in the order observed; in the case of proteins, of course, there are additional groups that bind Zn^{2+} (histidine) and protons (histidine and other N-containing side chains).

From this brief description of the properties of track-etched synthetic membranes it is clear that such materials constitute a simple and potentially useful model for elucidating some of the properties of endogenous ion channels and toxin-induced pores. One difference with regard to the latter is that flow of uncharged molecules through PETP pores is unaffected by low pH or by divalent cations, whereas flow through toxin-induced pores across biological membranes is. The reason for this is not yet clear; at this stage we merely note that if lipids are

added to PETP pores, flow of water and uncharged molecules *is* inhibited by low pH and divalent cations (Pasternak et al., 1993), just as it is in pores induced across biological membranes. Before considering the usefulness of PETP pores as a model for such pores, mention must be made of the surprising observation that the magnitude of the conductance through narrow PETP pores is similar to that through endogenous ion channels and pores induced by certain bacterial toxins, namely in the order pS (Figure 2): because the diameter of such PETP pores (around 2 nm) approximates to that of biological pores, yet the length (and hence the resistance) is some 1000-fold greater, one would have anticipated conductances in the fS, not the pS, range.

Our explanation for this discrepancy is in terms of simple electrochemistry (Smoluchowski 1903) and the phenomenon of “surface conductance” (Lev et al., 1993). Because the surface of etched PETP—including the lining of the pores—is negatively charged, it will attract counterions such as K⁺ (and will bind divalent cations and be protonated by H⁺). The result of the attraction of K⁺ means that K⁺ is accumulated (and Cl⁻ excluded) within a narrow pore and hence its concentration within the pore is much greater than that in the bulk solution outside the pore: the conductance is therefore much higher, by several orders of magnitude, than that anticipated; of course, this is true only when the carboxyl groups within the pore are fully ionized: if ionisation is depressed by lowering pH, conductance falls sharply (Lev et al., 1993). Again, it is true only in narrow pores, where the contribution of surface conductance is relatively large; in wide pores, where the contribution of surface conductance is small in relation to conductance of the bulk solution, the conductance that is observed is that anticipated from the geometry of the pore, regardless of the chemistry along its surface. In short, negative charges in, or near the mouth of, a channel or pore affect not only ion selectivity, but the magnitude of the conductance itself. As mentioned earlier, this may provide an explanation for the high conductance of maxi K⁺ channels (e.g., Latorre et al., 1989).

Another lesson to be learned from studying pores across PETP membranes concerns the origin of fluctuations. It is unlikely that a structural change, such as those underlying the opening and closing of proteinaceous channels (Unwin, 1995; Durell et al., 1998), accompanies transitions between high- and low-conductance states in PETP pores. This is because the diffusion of water and uncharged solutes is unaffected by low pH or divalent cations, whereas both these conditions reduce ion flow by stabilizing the low-conductance state. In other words, the effective pore size is not altered during transitions between high- and low-conductance states. What, then, accounts for such changes? We believe that oscillations in surface charge between protonated and ionised carboxylic acid groups are the cause. Such oscillations would, of course, lead to fluctuations in ion selectivity, and this is exactly what is observed (Korchev et al., 1997). The fact that protonation-deprotonation times are many orders of magnitude faster than the fluctuations of current and selectivity that are observed in PETP pores is explained by the strong cooperativity between adjacent surface charges in a confined space such as a

narrow pore (Edmonds, 1998). Confirmation that surface charge indeed oscillates in PETP pores is being obtained with cationic fluorescent dyes (G.M. Alder and C.L. Bashford, unpublished observations). The main point is that an ionic explanation for transitions between high- and low-conductance states appears to apply also to proteinaceous pores across a biological membrane, such as those induced by *S. aureus* α toxin, (Korchev et al., 1997). It may also provide an explanation for observations with endogenous ion channels, such as the phenomenon of "open channel noise" and that of transitions between different "substates" (Hainsworth et al., 1994; Moss and Moczydlowski, 1996).

V. CONCLUSIONS

This review has stressed the similarities displayed by pores across biological membranes—between those of endogenous origin and those induced by exogenous agents as diverse as viruses, bacterial and animal toxins, or proteins produced by the immune system. This should surprise no one. The common origin of life means that the structures adopted by biological molecules are likely to follow a common pattern, with specializations—such as distinct channels for Na^+ , K^+ , and Ca^{2+} , and distinct transporters for glucose and amino acids—evolving therefrom. We also know that the strategies adopted by viruses, bacteria, and other infectious agents are no more than variations on the structure and function of molecular assemblies developed by their hosts. The fact that many types of pore induced by infectious agents are sensitive to zinc—currently sold in various preparations as a complementary medicine—suggests that well-designed clinical trials are likely to prove rewarding.

Attention has also been drawn to the fact that it is possible to produce pores across synthetic membranes that display several of the basic properties of pores across biological membranes. The utility of such materials lies not only in the finding that their properties offer novel explanations for certain biological phenomena, but also in their development for a variety of technological applications (Patent No. 0678051 [Europe]; 5736050 [US]; 515814/94 [Japan]).

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INDEX

- aceruloplasminemia, 212, 227, 229–230
adenosine triphosphate (ATP), 66, 67–70, 71, 76, 80
 cell membranes and, 301–302
 polyamine metabolism and, 118
aerobic metabolism, 67–69
African trypanosomiasis and
 polyamines, 140–142
aging and neurodegeneration, role of
 reactive gliosis, 171, 173–174
Akt (protein kinase B), insulin signaling, 50–51
Alzheimer's disease
 ceruloplasmin and, 229–230
 oxidative injuries and, 172–177
amino acids
 analysis of 56 ssDBP, 252
 ceruloplasmin sequence, 213–219
 GAG (glycosaminoglycan) side chains, 106–107
AMP1 antigen, reactive astrocytic marker, 165
anchorage dependence and cancer, 131–132
aneuploidies
 detection with FISH, 17–18
 in sperm (FISH), 29
animal toxins and membrane pores, 310–311
annulate lamellae, 284–285
ansimorphic gliosis (*see* glial scars)
antioxidants
 astrocyte defense of CNS, 159–160, 175–177
 ceruloplasmin, 232
apoptosis, 66
 polyamines and apoptotic death, 127–131
Arbeitgemeinschaft für Osteosynthesefragen (AO), 84–85
archival specimens and FISH, 24–27
ascorbate oxidase vs. ceruloplasmin, 220
astrocytes
 aging and neurodegeneration, 171, 173–174
 AMP1 antigen, 165
 antioxidant activities of, 159–160, 175–177
 ceruloplasmin, membrane-bound form, 222–225
 CNS repair, 177–179
 cyst fluids, 166
 cytokines, 167, 174–175
 cytoplasmic antigens, 157–159
 enzymatic metabolism in, 159

- fibrous astrocytes, 149–152
 glial fibrillary acid protein (GFAP),
 152–156
 glial hyaluronate-binding protein
 (GHAP), 166
 glial scars, 156, 170–171
 glial tumors, 172–173
 gliosis reduction strategies, 177–178
 IGFBP-2 insulinlike growth factor
 binding proteins, 166
 intermediate filament-associated
 proteins (IFAPs), 152–157,
 165–166
 J1-31 antigen, 160
 M22 antigen, 163
 markers for reactive astrocytes, 159–
 166
 membrane-anchored molecules,
 168–170
 morphology, 149–152
 nestin, 156–157
 neuronal markers expressed, 160
 physiopathology of human brain,
 171–177
 pp25 protein, 166
 protection of neurons by, 191
 protoplasmic astrocytes, 149–152
 radial glial cells, 149–152
 reactive astroglia and CNS injuries,
 167–177
 6.17 antigen, 163–165
 spinal cord injuries and, 204
 transplantation of astroglial cells,
 178–179
 trophic factors, 167
 vimentin, 152–156, 163
 astrogliosis, 168
 inflammation and, 167
 S100 β overexpression, 157
 ATP (adenosine triphosphate), 66, 67–
 70, 71, 76, 80
 calcium signaling, 291
 polyamine metabolism and, 118
 autosomal marker chromosome identi-
 fication with FISH, 13–14
 axonal elongation, 169
 axons, 188–207
 collateral reinnervation and, 198
 reinnervation problems, 195
 (see also axotomy)
 axotomy, 206–207
 membrane dimensions and proper-
 ties after, 194
 motoneurons responses to, 190–195
 peripheral axotomy, 202
 azurin vs. ceruloplasmin, 219
 bacterial toxins and membrane pores,
 300–301, 308–310
 Barr body analysis and FISH, 24
 Bassoon (zinc finger protein), 243
 blood supply
 bone healing, 111–112
 sepsis, 66–67
 blue copper oxidases vs. ceruloplas-
 min, 219–220
 bones
 animal models, suitability of, 85–86
 blood supply and healing, 111–112
 bony calluses and fracture healing,
 86, 87, 92, 99–107
 cartilage, role in fracture repair,
 110–112
 cell differentiation during healing,
 107–110
 collagen and healing, 99–107
 defect healing, 94–96
 distraction osteogenesis, 85, 96–99,
 111
 endochondral fracture repair, 112
 fracture healing, 86–94, 110–112
 growth factors, 112–113
 macromolecular synthesis during
 callus development, 99–107
 marrow transplants, 21
 proteins and healing, 105–106

- brain
DNA binding proteins and development of, 240–258
(*see also* astrocytes)
- breast cancer, 25–26
nuclear envelope changes, 288
oncogene amplifications, 28
- C-banding (constitutive heterochromatin banding), 7
- calcium, extracellular environment, 205–206
- calluses, bony and fracture healing, 86, 87, 92
- origin and differentiation of cells, 108–109
- cancers
breast cancer, 25–26, 28, 288
chromosomal aneuploidy detected with FISH, 18–20, 27–28
chronic myelogenous leukemia, 20
DFMO (α -difluoromethylornithine) as preventive agent, 135
- FISH for detection and diagnosis, 18–20
- FISH for tumor analysis, 21–22
- gene deletions detected with FISH, 23
- glial tumors, 172–173
- interphase cytogenetics, 24–27
- oncogene amplifications detected with FISH, 28
- polyamines and, 123–124, 131–135
- carbon reinforced epoxy resin (CFRE) plates, 87–89
- cartilage
bone defect healing, 94–96
cellular environment and formation, 111–112
distraction osteogenesis and formation, 98–99
fracture repair and, 110–112
proteoglycans and formation of, 107
- cell cycle and polyamines, 125–126
- cell death
apoptosis, 66
polyamines and, 127–131
- cell differentiation
calcium signaling and, 292–293
“dedifferentiated” motoneurons, 195
lamins, role of, 277–278
osteoblasts vs. chondrocytes, 109–110
- polyamines, role of, 118, 121, 136–137
- cell growth, role of polyamines, 118, 121, 136–137
- cell membranes, nuclear envelopes, 261–294
- cellular hypoxia, 66–71
(*see also* cytopathic hypoxia)
- cellular O₂ consumption (in vitro), 74–75
- cellular oxidation state (in vivo), 75
- central nervous system (CNS)
brain development, 240–258
brain-specific DNA-binding proteins, 240–258
- cerebral spinal fluid (CFS), 222
- ceruloplasmin and, 229–230
- neuroplasticity, 187–207
spinal cord, 187–207
- centrosomes (spindle pole bodies), 281–282
- ceruloplasmin, 212
- aceruloplasminemia, 227, 229
- amino acid sequence, 213–219
- antioxidants, 232
- vs. ascorbate oxidase, 217, 220
- vs. azurin, 219
- vs. coagulation factors V and VIII, 220–221
- copper centers, 216–217
- evolutionary pathways, 220
- free radicals, 212, 227–229, 232
- functions of, 212, 227–232

- genetic mutations, 226–227
 histidine residues as copper ligands, 217
 iron binding, 217–219
 vs. laccase, 217, 220
 membrane-bound form, 222–225
 Menkes disease, 231
 neurodegeneration and lack of, 229, 230
 neurological disorders, 229–230
 vs. plastocyanin, 219
 protein structure, 213–219
 triangular arrangement of, 214–216
 truncated form, 225–226
 Wilson's disease and copper toxicity, 230–231
 vs. yeast Fet3p, 221–222
- CFRE (carbon reinforced epoxy resin) plates, 87–89
- CGH (comparative genomic hybridization), 30–31
- channels
 adenosine triphosphate (ATP) and, 302
 nuclear channels, 285–287
 pores vs., 300
- cholera toxin, 200, 306–308
- chondrocytes, 108–110
- chromosomes
 aneuploidies, 17–20, 27–28, 29
 harvest methods, 3–4
 identification via Q-banding, 6–7
 microdeletions and microduplications, 15–16
 Philadelphia (Ph) chromosome, 20
 STAT chromosomes and FISH, 18
- chronic myelogenous leukemia (CML), 20
- clonality, 22–23
- coagulation factors V and VIII, 220–221
- collagens in bone healing, 99–107
- comparative genomic hybridization (CGH), 30–31
- conductance states, 309–310
 surface conductance, 317–318
- connexins, 152, 303
- constitutive heteromatin banding (C-banding), 7, 9
- constitutive transcription factors (CTFs), 240
- copper centers, ceruloplasmin, 216–217
- cyst fluids and astrocytic invitation, 166
- cytogenetics, conventional
 banding based, 2–9
 constitutive heteromatin banding (C-banding), 7, 9
- Giemsa banding (G-banding), 4–6, 9
- high-resolution banding (G-banding), 5–6
- nucleolar organizer regions (NOR) staining, 8
- quinacrine banding (Q-banding), 6–7, 9
- reverse banding (R-banding), 4–5
- solid staining, 4
- cytokines
 astrocytes and, 167, 174–175
 tumor necrosis factor- α (TNF- α), 56–57
- cytopathic hypoxia
 vs. classic, 68, 72–76
- defined, 66
- electron transport chain dysfunction, 77–78
- mitochondrial permeability transition, 80
- oxygen conformance, 79
- in sepsis, 76
- substrate delivery failures, 78–79
- uncoupling of oxidative phosphorylation, 80
- (*see also* cellular hypoxia)

- cytoplasmic antigens, astrocytic markers, 157–159, 163–165
- deafferentation
 peripheral nerve injuries, 201
 primary afferents, 198–203
- DFMO (α -difluoromethylornithine), 122, 135
- diabetes, type 2
 Akt (protein kinase B), 50–51
 animal models, 54–55
 described, 42
 Glut4, 51–52, 55–58
 human obesity and, 55–56, 58–59
 insulin receptors, 48
 insulin signaling and glucose metabolism, 42–48
 IRS proteins, 48–50, 53–55
 PI 3-K, 50
 pregnancy and, 54–55
- α -difluoromethylornithine (DFMO), 122
- diphtheria toxins, 306–308
- distraction osteogenesis, 85, 96–99
 cartilage formation during, 111
- DNA binding proteins, 244–258
- dorsal horn of spinal cord
 extracellular environment in, 205
 functional consequences of injury, 206
- injuries to vs. peripheral nerve injuries, 196–198, 206
 non-neuronal responses in, 204–205
- Down's syndrome
 astrocytes and, 173–174, 177
 rapid aneuploidy detection of, 17–18
- electron transport chain dysfunction, 77–78
- emerin, 274
- endochondral fracture repair, 112
- endogenous membrane pores, 301–304
- Fenton reaction, 227–228
- ferroxidases
 Fet3p, 221–222
- Fet3p vs. ceruloplasmin, 221–222
- fibrous astrocytes, 149–152
- Fluorescent *In Situ* Hybridization (FISH)
 aneuploidy detection, 17–18, 27–28, 29
- archival tissues, 24–27
- autosomal marker chromosome identification, 13–14
- bone marrow transplants, 21
- cancer, 18–20, 21–22
- clinical applications, 12–23
- comparative genomic hybridization (CGH), 30–31
- gene mapping, 28
- genotoxicity studies, 29
- methodology, 10–12
- microdeletions and microduplications, 15–16
- mosaicism, detection, 13–15
- oncogene amplifications, 28
- prenatal diagnosis, 17–18
- sequential flow cytometry, 29
- sex chromosome identification, 14–15
- spectral karyotyping (SKY), 30–31
- STAT chromosomes, 18
- telomeres, 32–34
- “fracture disease,” 84
- fracture immobilization, 84–85, 86–94
 carbon reinforced epoxy resin (CFRE) plates, 87–89
- fracture, bone (*see* bones)
- free radicals, 175–177
 ceruloplasmin and, 212, 227–229, 232
- G-banding (Giemsa banding), 4–5, 9
 high-resolution banding, 5–6

- African trypanosomiasis treatment, 140–142
 cancer treatment, 135
 hypertension and, 138–139
 immune disorders, 136–137
GAG (glycosaminoglycan) side chains, 106–107
ganglion cells, 195–196
 ceruloplasmin expressed in, 225
 transganglionic transport, 198
(see also dorsal horn of spinal cord)
gap junctions, 152, 168, 172
gastrointestinal mucosa, septic shock, 71, 73
genes
 insulin signaling knockout models, 52–54
 mapping with FISH, 28
GFAP (glial fibrillary acid protein), 152–156
Giemsa banding (G-banding), 4–5, 9
glial cells
 ceruloplasmin expressed in, 225
 perineuronal glia, 193–194
 Sox proteins and development, 244
(see also astrocytes)
glial fibrillary acid protein (GFAP), 152–156
glial hyaluronate-binding protein (GHAP), 166
glial scars
 posttraumatic glial scars, 152, 170–171
 vimentin and, 156
 X-radiation and formation of, 177–178
glial tumors, 172–173
gliosis
 Alzheimer's disease and, 173–174
 reactive, 148
 strategies to reduce, 177–178
(see also astrocytes)
glucosamine, 57–58
glucose
 aerobic metabolism, 67–69
 insulin signaling and metabolism of, 42–48
 membrane pores and, 302, 303–304
Glut4, 51–52, 55–56
 glucosamine and, 57–58
Gluts (glucose transport proteins), 42, 51–52, 55–58
glycoproteins, ceruloplasmin, 212–233
glycosaminoglycan (GAG) side chains, 106–107
Golgi fragmentation, 263–264
Golgi membranes, 267–268
growth factors
 brain-derived neurotrophic factor (BDNF), 203
 CNS injury responses, 167–168
 nerve growth factor (NGF), 204
GTG karyotypes, 5

Haber-Weiss reaction, 227–228
healing
 bones, 84–113
 spinal cord, 187–207
healing stage, bone fractures, 86
helix-loop-helix transcription factors, 241
helix-turn-helix (homeoboxes), transcription factors, 240–241
high-resolution banding (G-banding), 5–6
histidine residues as copper ligands, 217
HMG proteins, 243–244
homeobox (helix-turn-helix) transcription factors, 240–241
hormones, polyamines and secretion, 126–127
Huntington's disease, nuclear envelopes changes, 288
hypertension and polyamines, 138–139

- hypoxia (*see* cellular hypoxia; cytopathic hypoxia)
- IFAP (intermediate filament-associated proteins), 165–166
- IGFBP-2 insulinlike growth factor binding proteins, 166
- immune-mediated disorders and polyamines, 135–137
- immune molecules and membrane pores, 311
- in situ* hybridization, 9
- induced membrane pores, 304–315
- inducible transcription factors (ITFs), 240
- infection, septic shock, 66–80
- insulin
- effects of, 44–45
 - mRNA and polyamines, 126
 - muscle cells and, 42–60, 303–304
- insulin signaling
- abnormalities, mechanisms of, 56–59
 - Akt, 43, 50–51
 - glucosamine, 57–58
 - glucose metabolism and, 42–48
 - Glut4, 51–52
 - insulin-resistant states, 52–56
 - IRS proteins, 43–44, 48–50, 53–55
 - PC1, 59
 - phosphatases (PTPases), 58–59
 - phosphatidylinositol 3-kinase, 50
 - receptors, 47–48
 - in skeletal muscles, 42–60
 - tumor necrosis factor- α , 56–57
- intermediate filament-associated proteins (IFAPs), 165–166
- interphase cytogenetics, 24–27
- (*see also* Fluorescent *In situ* Hybridization (FISH))
- ion pumps, 302–303
- iron binding, ceruloplasmin, 217–219
- iron-induced free radical formation, 227–229
- iron metabolism, 212
- IRS proteins, insulin signaling, 43–44, 48–50, 53–55
- ischemia
- astrocytes and nestin production, 157
 - polyamines and, 127
- J1-31 antigen, reactive astrocytic marker, 163
- knockouts
- insulin signaling, 52–54
 - type II collagen, 110
 - vimentin gene, 154–156
- laccase vs. ceruloplasmin, 220
- lactic acidosis, 69–70
- lamin B receptor (p58), 271–273, 286
- lamina-associated polypeptides, 273–274
- lamins, role in differentiation, 277–278
- leucine zippers, 242
- leucocytes, 135–136
- licensing factor (protein), 264
- LIM class homeobox motif, 244
- lobulation of nuclear envelope, 284
- M22 antigen, reactive astrocytic marker, 165
- malaria, induced pores and, 310
- mast cells, 135–137
- membrane-anchored molecules, 168–170
- membranes
- adenosine triphosphate (ATP) and, 301–302
 - compartments of nuclear envelope, 268–271
 - Golgi membranes, 267–268
 - inner nuclear membrane, 271–275

- “membrane attack complex,” 311
- nuclear envelopes, 261–294
- proteins of, 271–275, 300
- synaptic stability and changes in, 194
- synthetic membranes, 315–318
(see also pores, membrane)
- Menkes disease, 231
- microbial toxins
 - stable pore formation, 308–310
 - transient pore formation, 306–308
- microdeletions and microduplications
- Fluorescent *In Situ* Hybridization (FISH) and, 15–16
- microglia, 204
- MIRKO (Muscle Insulin Receptor Knock-Out) Mice, 52–53
- mitochondrial permeability transition, 80
- mitochondrial respiration, 73–75
- mitogenesis, activated by insulin, 46
- mitosis, open, 262–268
- mosaicism, detection with FISH, 13–15
- motoneurons, 189
 - responses to axotomy, 190–193
- motor axons, 189–190
- MS, astrocytes and, 175
- MTT assay (*in vivo*), 73–74
- muscles, skeletal
 - cellular hypoxia in septic shock, 71
 - complications of reinnervation, 195
 - insulin signaling in, 42–48, 52–56, 303–304
 - pO₂ and sepsis, 73
- necrotic death and polyamines, 127–131
- nestin, 156–157
- neurodegeneration
 - aging and astrocytes, 171, 173–174
 - lack of ceruloplasmin, 229, 230
- neuroglia (*see* astrocytes; glial cells)
- neuronal markers, astrocyte expressed, 160
- neuroplasticity, defined, 187
- nitric oxide, role in sepsis, 77–78
- nitric oxide synthases (NOS), 77
- nitrogen intermediates, reactive, 77–78
- NO• (nitric oxide) role in sepsis, 77–78
- nonhistone chromosomal (NHC) proteins, 244–258
- nuclear envelopes
 - annulate lamellae, 284–285
 - breakdown of, 264–265
 - calcium signaling in, 290–293
 - deep folds and lobulation of, 284
 - described, 262
 - disease-related changes in, 287–288
 - form during mitosis, 265–268
 - invaginations and evaginations of, 282–284, 285–287
 - membranes of, 268–275
 - mitotic vesiculation, 264–267
 - modifications of morphology, 284–288
 - organization of, 283
 - phospholipids and signaling in, 288–290
 - structures within, 281–284
 - transport across, 279–281
- nuclear lamina, 275–277
 - functions of, 278–279
 - lamins and cell differentiation, 277–278
- nuclear pore complexes (NPCs), 279–281
- nucleolar organizer regions (NOR) staining, 8
- obesity
 - diabetes, type 2: 54, 55–56, 58–59
 - tumor necrosis factor-α, 56–57

- ODC (ornithine decarboxylase) and polyamine metabolism, 118–136
oligodendroglia, 205
oncogene amplifications detected with FISH, 28
ornithine decarboxylase (ODC) and polyamine metabolism, 118–136
orthogonal assemblies, 151–152
osteoblasts, differentiation from chondrocytes, 109–110
osteoprogenitor cells, 94–96, 107–109 bone defect healing and, 94–96
otefin, 274–275
oxidative metabolism, 67–69
oxidative phosphorylation, 69 uncoupling and cytopathic hypoxia, 80
oxidative stress neurological disorders, 229–230
oxygen, cytopathic hypoxia in septic shock, 66–80
oxygen conformance in cytopathic hypoxia, 79

paired box motif, 244
Papancolaou smears and FISH, 24
paramyxoviruses, 304–306
parasitic diseases African trypanosomiasis and polyamines, 140–142
 malaria and induced pores, 310
parkinsonisms, 160, 171–172 ceruloplasmin and, 229–230
PARP (poly(ADP)-ribose polymerase), 78–79
PDH (pyruvate dehydrogenase), 78–79
perineuronal glia, 193–194
peripheral axotomy, 202
peripheral nerve injuries vs. central sensory axon injuries, 203–206

deafferentation and primary sensory afferents, 201
vs. dorsal root injuries, 196–198, 206
PETP (polyethyleneteraphthalate) membranes, 315–318
Philadelphia (Ph) chromosome, 20
phosphatases (PTPases), 46–48, 58–59
phosphatidylinositol 3-kinase, insulin signaling, 50
phospholipid bilayers, 288–290, 304–308
PI 3-K, insulin signaling, 43–44, 50
Pima Indians, 58–59
plastic membranes, 315–318
plasticity, neuroplasticity, 187
plastocyanin vs. ceruloplasmin, 219
plectin, 165–166
pO₂, *in vivo* measurement, 72–73
poly(ADP)-ribose polymerase (PARP), 78–79
polyamines African trypanosomiasis, 140–142
 cancer, 131–135
 cell cycle and, 125–126
 hormone secretion and, 126–127
 hypertension, 138–139
 immune-mediated disorders and, 135–137
 necrotic and apoptotic death, 127–131
 ornithine decarboxylase (ODC) and metabolism of, 118–136
 structure and metabolism of, 118–121
 synthesis inhibitors and degradation, 121–124
polyethyleneteraphthalate (PETP) membranes, 315–318
pores, membrane animal toxins and formation of, 310–311
 vs. channels, 300

- common features of induced pores, 312–315
described, 300
endogenous, 301–304
immune molecules and formation of, 311
induced pores, 304–315
microbial toxins and formation of, 310
of nuclear envelope, 269
protozoa and formation of, 310
stable pores and microbial toxins, 308–310
synthetic pores, 315–318
transient pores and microbial toxins, 306–308
pp25 protein, gliosis marker, 165
pregnancy, type 2 diabetes and, 54–55
prenatal diagnosis with FISH, 17–18
presynaptic terminals, 190–195
functional consequences of loss, 194–195
loss of, 192–195
membranes, changes, 194
probes, DNA/cancer, 20
prokaryotes, mitosis, 262–264
prostatic secretions, 118
protein kinase B (Akt), 50–51
proteins
animal toxins, 310–311
bone healing, 105–106
connexins, 152, 303
membrane proteins, 300
polyamines and, 126–127
(see also specific proteins)
proteoglycans, 106–107
protoplasmic astrocytes, 149–152
protozoa and membrane pores, 310
putrescine, 118–120
pyruvate dehydrogenase (PDH), 78–79
quinacrine banding (Q-banding), 6–7,
9
- radial glial cells, 149–152
rapsyn, 243
reactive gliosis (*see* astrocytes)
reactive nitrogen intermediates (RNI), 77–78
remodeling stage, bone fractures, 86, 91, 93
reverse banding (R-banding), 4–5
Sendai virus (HVJ), 305–306
septic shock, described, 66–67
sequential flow cytometry, 29
sex chromatin analysis and FISH, 24
sex chromosome identification with FISH, 14–15
single-strand DNA binding protein (ssDBP), 245–258
6.17 antigen, reactive astrocytic marker, 162–164
skeletal muscles
cellular hypoxia in septic shock, 71
complications of reinnervation, 195
insulin signaling in, 42–48, 52–56, 303–304
pO₂ and sepsis, 73
SKY (spectral karyotyping), 30–31
sleeping sickness and polyamines, 140–142
Sox proteins, 244
“spared root” model, 199–200
spectral karyotyping (SKY), 30–31
sperm, polyamines in, 118–120
spinal cord
connectivity and motor axon injury, 190–195
deafferentation and primary afferents, 198–203
dorsal horn of, 196–198, 203–206
lesions, types of, 189–190
neuroplasticity, implications of, 188–190
organization of sensory input, 195–196

- peripheral vs. dorsal root injuries, 196–198
presynaptic terminals, 190–195
removal of primary sensory input, 195–206
sensory axons, evaluating changes of, 198
spindle apparatuses, 263, 281–282
spindle pole bodies (centrosomes), 281–282
Sry-boxes, 244
ssDBP (single strand DNA binding protein), 245–258
STAT chromosomes and FISH, 18
substrate delivery failures in cytopathic hypoxia, 78–79
synthetic membrane pores, 315–318
telomeres, 32–34
tissue O₂ consumption (*in vitro*), 74
track-etched synthetic membranes, 315–318
transcription factors in CNS, 240–244
 helix-loop-helix (HLH) motif, 241
 HMG proteins, 243–244
 homeobox (helix-turn-helix), 240–241
leucine zippers, 242
zinc finger proteins, 242–243
- transplants
 astroglial cells, 178–179
 bone marrow, 21
- trisomies
 chromosome 8: 5
 detection and analysis with FISH, 18–20, 22–23
- trophic factors, CNS injury and, 167
- tumor necrosis factor- α (TNG- α), 56–57
- vesicles, mitotic vesiculation, 264–267
- vimentin, 152–156, 163
- viruses and membrane pores, 300, 304–306
- Wilson's disease, 230–231
- winged helix motif, 244
- X-rays and lesion-induced gliosis, 177–178
- Y chromosomes, identification via Q-banding, 6–7
- yeasts, *S. cerevisiae*, 221–222
- zinc, induced pores and, 318
zinc finger proteins, 242–243
zippers, leucine, 242

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