



Some Aspects of Chromosome Structure and Functions

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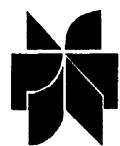
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Dedicated to

Dr. Adayapalam T. Natarajan

Professor Emeritus, Leiden University
Leiden, The Netherlands

in recognition of his contributions to the
fields of Cytogenetics, Radiation Genetics
and Environmental Mutagenesis

Preface

It was at the end of the 19th century that a Swiss biologist, Karl Nageli first proposed the existence of hereditary organelles that carried information from parent to offspring. Ensuing decades experienced vigorous studies that led to the development of discovery that chromosomes are indeed the carriers of genetic information.

Subsequent studies, especially by Morgan and Bridges, established *unequivocally* the chromosome theory of inheritance.

Today, the structure of chromosome is well established. At the physical level, eukaryotic chromosomes are composed of a single, linear, double helix of DNA. The elementary helical structure involves nucleosomes, comprised of histones around which the DNA is wrapped. A hierarchy of higher order helical structures of chromosomal architecture may possibly be responsible for the regulation of gene expression.

The localized condensations of DNA constitute chromomeres. Uncoiled structures sometimes extending from chromomeres, which form loops, is the result of discontinuities in the regular coiling of the DNA in the chromosome.

The focus of the current investigations in this area is on the molecular architecture of the chromosomes, where many aspects are still in process of being unravelled. A total of three billion nucleotide pairs, which correspond to approximately 30,000-50,000 genes, exist in human genome. A draft of the sequence of the nucleotides for the major part of the human genome is now available in the genome databases. The sets of nucleotide sequences in the DNA coding for a specific function, constitute the genes. A gene comprised of a defined number of nucleotides arranged in specific order thus is the unit of inheritance.

Abnormalities at both physical as well as molecular levels in DNA are the cause of many human diseases. Physical abnormalities in chromosome structure and number like polyploidy, aneuploidy etc. are known to result in various syndromes e.g., trisomy of chromosome 21 results in Down's syndrome, monosomy of X chromosome is present in Turner's syndrome. Similarly, many other syndromes with numerical chromosomal abnormality exist. At the molecular level, the irregularities in nucleotide sequence or loss or gain of a nucleotide in the DNA structure is also implicated in causing many diseases. The alterations of single nucleotides have been identified as etiological factors of a number of human diseases. Dreadful diseases such as cystic fibrosis, Xeroderma pigmentosa, Sickle cell anaemia and Graves disease etc. are a few amongst many such diseases. Even cancers like retinoblastoma, cervix, colon-rectum, and of other organs are a result of mutational activation of normal proto-oncogenes into abnormal oncogenes. The proto-oncogenes and anti-oncogenes liable to mutational activation and causing cancer include *myc, ras, src, p53, yes, abl, fes, erh-B, fins, rvs, mos, sis, int-2, myb, fos, met, etc.*

The investigations on the role of chromosomes in causing diseases have made medical genetics as one of the frontier fields in research. The advent of molecular genetics has further revolutionised research. Genetic screening, especially at pre- and neo-natal stages, has significantly contributed to the prevention of human suffering. The scope and versatility of new techniques has made it possible to test ideas previously open only to speculation.

With exception to newborn and some carrier screening, most genetic testing is performed in university laboratories, with medical geneticists often conveying the results directly to family members. But in recent years, there has been a strong trend towards physician performing diagnostic tests. Moreover, scientists, employed by commercial firms are trying to develop simple tests, to identify disease-causing alleles, which people will be able to perform at home.

Another scientific program of recent origin is that of Gene Mapping—Human Genome Project. The human genome contains an estimated 50,000 genes which encode information in DNA. These genes are distributed among 23 pairs of chromosomes. Although most of these genes have been assigned to specific chromosomal location, but only a few genetic diseases out of about 4000, have been understood at molecular level.

This volume was planned to compile the data on various aspects of structure and functions of chromosomes investigated in laboratories around the world. This volume is specially dedicated to honor Professor A.T. Natarajan, a distinguished cytogeneticist, who has dedicated his life to unravel structural mysteries of chromosomes.

R.C. SOBTI
G. OBE
R.S. ATHWAL

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1. Aspects of Tandemly Organized, Repetitive Sequences in Chromosomal DNA

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Abstract: Most tandemly reiterated DNA sequences have not yet revealed their intrinsic properties and secrets, although these elements are abundant and ubiquitously interspersed in most genomes. Some characteristics of these repeats are discussed herein with special emphasis on the chromosomal interaction and medical aspects.

Key words: DNA binding proteins; repetitive DNA elements; trinucleotide diseases.

1. Introduction

The knowledge on sequences from chromosomal DNA has expanded immensely in recent years, refining also our ideas on genetic complexities. For virtually all of the approximately 40,000 genes in the human genome, at least partial sequence data are available today, providing the basis for further functional analyses also on the protein level. It remains still somewhat obscure, however, why coding sequences represent only a minor fraction of eukaryotic chromosomes *e.g.*, about 3% of the entire human genome. Repetitive sequences comprise ~ 50% of this genomic “desert” (Ohno, 1972) and have attracted some interest due to their striking features, perhaps reflecting structural meaning in chromosomes. Repetitive DNA has long been regarded as a characteristic feature of eukaryotic chromosomes, but these elements have also been detected in more and more prokaryotes (Hancock, 1996) with the exception of *Escherichia coli*. Functional meaning of these sequences has not been proven beyond doubt and, therefore, it has generally been questioned, except for some special repetitive genes *e.g.*, the rRNA gene batteries some of which contain several hundred copies in tandem arrays at several loci (Lewin 1980). Studies on the evolution of repetitive sequences, on their possible functions in recombination mechanisms and gene expression as well as recent findings on their roles in certain human diseases, on protein binding and specific DNA secondary structures point to biological relevance of at least some members of this class of sequences—nevertheless a comprehensive theory on the biological meaning of repetitive elements remains to be established.

Here we cover the large group of repetitive sequences comprising different basic motifs in tandem arrays. A comprehensive and broadly accepted classification scheme is basically derived from the lengths of their repetition units (see Tautz, 1993). The names of the different subclasses were chosen unsystematically - following the initial designation of the so-called satellite DNA. These ‘satellites’ to the bulk of other genomic DNA were described historically first in the early era of space enthusiasm more than 3 decades ago - and they were named without knowledge of their exact sequence composition. The so-called microsatellites or simple repeats (short tandem repeats) harbor the shortest basic motifs and the lowest degree of complexity. Their motifs range from 1–6 basepairs (bp) in length with 5 to approximately 100 perfect tandem copies at each locus. Simple repeat loci are scattered throughout

the human genome in about 10^4 – 10^5 copies of the elements with mono- and di-nucleotide motifs and in lower numbers of those with longer motifs. Microsatellites with the most abundant motif (GT)/(CA) are found in 50,000 to 100,000 copies, a number exceeding even the number of genes in the human genome (Nadir *et al.*, 1996). The distribution pattern of simple repetitive sequences is more or less even: one such element is located within 10 kilobases (kb) on average, also within transcriptional units in the 3'-and 5'-untranslated regions of protein-coding mRNAs. In addition to microsatellites, minisatellites (Jeffreys *et al.*, 1985) have sometimes also been named ‘variable number of tandem repeats’ (VNTR).

The repetitive units of minisatellites are 9 to approximately 100 bp long. Two to several hundred copies exist per locus. For each locus the basic units show varying sequence similarities to different core sequences. Thus, larger minisatellite units are practically never completely homogenous: the repetitive motifs exhibit sequence variability within one locus. In the human genome, many thousands of minisatellites with different repetitive units are interspersed with practically all other DNA elements, but they are often clustered in subtelomeric chromosomal regions (Royle *et al.*, 1988). This observation led to speculations about a possible function of minisatellites in the pairing of chromosomes during meiosis or in recombination events. Analysis of crossovers adjacent to a GC-rich minisatellite, which is known to mutate by conversion and crossover within the repeat array, revealed a recombination hotspot centered upstream of the locus. Allele-specific cosuppression of crossovers and repeat instability suggests that the hotspot is responsible for driving repeat turnover and thus that minisatellites might evolve as by-products of localized meiotic recombination in the human genome (Jeffreys *et al.*, 1998). Both, micro- and minisatellites exhibit a high degree of polymorphism. The number of repetitive units at a given locus can be polymorphic to hypervariable between individuals in a population. Generally, minisatellites are by far more polymorphic than microsatellites and can have extremely large numbers of different alleles (Pena *et al.*, 1995). This hypervariability is also due to the slight variations in the sequence make-up of the individual repeat unit.

Classical satellites represent the longest category of tandemly organized repetitive DNA. Originally, they were identified in buoyant density gradients (Skinner, 1977). In these gradients, the main band comprises the bulk of genomic DNA. Several additional minor bands of differing density arise due to their different GC-content. Satellites consist of large areas of highly repetitive DNA. With DNA sequencing methodology growing more efficient, satellites have been recognized as consisting of quite heterogenous sequence entities. Their GC-content (and resulting specific density) does not necessarily have to differ from the rest of the genome, leading to the designation as ‘cryptic’ satellite sequences (Tautz, 1993). Satellites often contain millions of tandem repeats with short motifs. The more perfect the tandem repeats are organized in a satellite, the less amenable is this DNA block to complete sequence analysis. Classical satellites have repetitive units of up to 100 or even 1000 bp. The degree of sequence redundancy amounts to up to 10^7 copies at each locus. In general, there are only one or a few satellites per genome, and no more than one (or two) per chromosome. Satellites are mostly located in heterochromatic regions *e.g.*, at the centromeres or on the sex chromosomes (Jones and Singh 1981; Therkelsen *et al.*, 1997).

Repetitive elements other than tandemly organized structures are not detailed here except for their relationships to the former. Interspersed repetitive sequences are situated as single, isolated copies of a repetitive motif in between other (in some cases repetitive) sequences. Short and long interspersed nucleotide elements [SINES and LINES (Singer, 1982)] are defined according to their lengths of less or more than 500 bp. Each of these types cover about 5–10 % of the human genome. Origin and development appear to result from mobilization and (random) insertion mechanisms - comparable to (retro-) transposition of retroviruses. Alu elements are the most abundant sequences among the

SINES in primates. They were named according to a characteristic restriction enzyme recognition site being present in most members of this family (Deininger *et al.*, 1992). Their length is less than 300 bp and they exist in about 500,000 copies per haploid genome in man. Alu elements can be located within transcriptional units, resulting in their presence in hnRNA (up to 10% of the total amount) and even in mature RNA. The estimated age of these redundant structures is about 30×10^6 years. The sequence of Alu-elements is related to the gene coding 7SL RNA. Alu elements are not completely identical in sequence, but show about 90% identity with the consensus sequence. They are composed of two monomers, each having a poly(A)+ tail. The poly(A)+ tract of the 3' monomer can be followed by tandemly repetitive sequences and can be accompanied *e.g.*, by microsatellites with the (GT) motif. This phenomenon could explain the co-evolution of certain Alu elements and (GT)_n microsatellites (Nadir *et al.*, 1996). Also other classes of repetitive sequences show close interrelationship among each other: Minisatellites are often tightly linked to other hypervariable sequences or Alu elements. Moreover, many DNA regions contain internal repetitions without tandemly organized structure which can be mixed with other repetitive elements. Such regions were called cryptically simple sequences (Tautz 1993). Analysis of eukaryotic genomes with special computer algorithms demonstrates that many genomic sequences have at least traces of such structural features. Thus, the spectrum between homogenously organized simple sequences, regions with less obvious degree of redundancy and entirely random nucleotide sequences could be regarded as continuum.

2. Representation of Simple Repeated DNA Sequences in Databases

Tandem repetitive DNA was regarded as a mere offshoot of eukaryotic genome research by most molecular geneticists until the instrumental character of these elements was fully appreciated as efficient marker system for multilocus DNA fingerprinting (Jeffreys *et al.*, 1985, Ali *et al.*, 1986), DNA profiling (for definition see Tautz, 1993, Pena *et al.*, 1995) and especially chromosome/gene/genome mapping. Only a small subset of the highly abundant simple repeat sequences appear in mature mRNA, and then predominantly in the 3' and 5' untranslated regions (see *e.g.*, Epplen and Epplen, 1994). Inconspicuous expression as well as the futile search for immediate biological functions have inspired interpretations that these elements can be subsumed in the voluminous category of 'junk' DNA (Ohno, 1972). Only on theoretical grounds, it has been postulated that micro-/minisatellites may be involved in the development of complex polygenic traits such as common neuropsychiatric diseases via their potential influence on gene expression (Comings, 1998). But on the other hand, more than a dozen so called trinucleotide diseases have indeed been defined involving different triplet repeat motifs in the causal pathogenesis of human diseases (see below). Furthermore, there is evidence and theoretical reasoning that longer VNTRs are associated with neurodegenerative disorders and other syndromes in addition to the well-characterized trinucleotides (De Fonzo *et al.*, 1998). Hence a number of different simple repeats may also well have functional significance when encountered by chance in the swiftly growing DNA databases.

The EMBL databank was searched using the FASTA algorithm for all blocks of simple (mono- to tetranucleotide) repeats extending for 40 bases in 2001. In Table 1 the representation of perfect simple repeats and composites thereof are depicted in the human (EMHUM) and all combined databases (GEALL). For the simple tetranucleotides *e.g.*, 4^4 different motifs should have been considered, but only 26 basic units are included. All other basic motifs, up to a length of four bases are not listed, because they are represented within the shorter motifs (mono- to trinucleotides) or they are obtained by shifting the phase by one or more positions or by reading the complementary strand of the depicted entities. The incidence of all possible permutations of simple mono- to tetranucleotides is far from being equal. Certainly, some reporting biases do not yet allow extrapolation of the true natural

Table 1 Representation of microsatellites in the EMBL/GENBANK data bank*

Simple repeat motif (length in bp)	EMHUM (25)	EMHUM (40)	GEALL (50)	GEALL (40)
(g) _n	9	1	1	305
(a) _n	4 521	763	178	2 818
(ga) _n	2 633	587	212	9 387
(gt) _n	6 072	2 220	796	>10 000
(gc) _n	6	—	—	4
(at) _n	3 198	2 784	996	9 270
(gga) _n	161	30	12	522
(ggt) _n	181	28	6	287
(ggc) _n	83	18	7	99
(gaa) _n	265	178	151	3 196
(gat) _n	247	50	4	354
(gac) _n	3	1	—	33
(gta) _n	62	21	7	380
(gtt) _n	616	61	1	502
(gca) _n	233	82	43	211
(aat) _n	1 823	569	66	3 642
(ggga) _n	378	79	15	624
(gggt) _n	9	2	—	9
(gggc) _n	—	—	—	—
(ggaa) _n	1 731	1 129	631	6 722
(ggat) _n	714	354	51	2 336
(ggtt) _n	46	17	4	137
(ggtt) _n	63	16	—	237
(ggca) _n	60	5	—	220
(gagt) _n	20	7	—	81
(gaaa) _n	2 033	1 599	1 310	>10 000
(gaat) _n	477	30	—	306
(gaac) _n	—	—	—	9
(gata) _n	1 799	1 327	344	8 580
(gatt) _n	81	—	—	104
(gaca) _n	140	17	2	578
(gact) _n	4	—	—	42
(gtaa) _n	4	—	—	34
(gtat) _n	281	142	17	1 915
(gtta) _n	14	5	2	48
(gttt) _n	1 174	42	—	298
(gcaa) _n	38	9	1	162
(gcat) _n	—	—	—	4
(gcta) _n	11	—	—	45
(gcc) _n	8	—	—	45
(aaat) _n	4 322	1540	248	8 406
(aatt) _n	135	—	—	42

*GEALL contains 11 002 540 sequences and 13 121 308 246 nucleotides as of Febr. 13th, 2001; EMHUM contains 129 978 sequences and 1 150 498 856 nucleotides as of Febr. 13th, 2001.

frequencies of simple repeats. Among these skewing biases in databanks are over representation (i) of human sequences (EMHUM vs. GEALL), (ii) of model organisms, (iii) of genes vs. under representation of spacers, (iv) excess of certain microsatellite motifs because of their increased presence on short restriction fragments used for establishing respective minilibraries. In contrast underrepresentation of very large simple repeat blocks is due to (i) reduced cloning efficiency, (ii) general instability of simple repeat tracts (reduction in size, rearrangements) in the prokaryotic hosts commonly employed for molecular cloning (iii) underreporting due to limited interest of the gene hunters in these elements and (iv) listing exclusively in specialized data bases.

Remarkably, bps forming strong bondage (S, C or G) are less abundant in simple repeats than those held together by only two hydrogen bridges (W, A or T). This fact agrees with the general (A+T) surplus vs. the lowered (G+C) frequencies in the introns and spacers of eukaryotes. Perhaps poly(A)+ blocks are an effective basis for the generation of derived simple repeat tracts as may be inferred *e.g.*, from human Alu repeats. In recent primate evolution, certain Alu repeats harbored a short poly(A)+ block that is apparently the source of a variety of subsequently emerging simple repeats. So far it seemed, that $(GT)_{>20} / (AC)_{>20}$ microsatellites outnumber all other motifs by far, but by monitoring the frequency development of simple repeats, it is obvious that poly(A)+ block derivatives are encountered and reported more and more frequently. $(GATA)_{10}$ stretches are still most abundant among the longer basic motifs. Thus, in general terms, perfect (YN) dinucleotide periodicity appears particularly successful for generating/maintaining a simple repeat block in the genome. This selective effect on the relative frequencies is even more pronounced when the composite simple repeat blocks are inspected more carefully. Whereas $(GT)_n(GA)_m$ repeats are frequently encountered, all possible shifts in the (YN) periodicity at the border of the two perfect tandem blocks are practically never observed. This remarkable phenomenon (that the (YN) periodicity has to be maintained in composite simple repeats) can also be observed for other di- and tetra-nucleotide combinations. It is obvious that motifs containing CpG dinucleotides are virtually never contained in longer simple repeat blocks because they are biologically erased from modern genomes of the eu- and prokaryotes investigated. The general lack of CpG dinucleotides results because these dinucleotides are prone to mutate (after C residue methylation and oxidative deamination).

Some sequence-based rules may be proposed for the generation and/or maintenance of simple repeats in present-day genomes (databanks). These rules were delineated already some time ago (Epplen *et al.*, 1998) and seem to hold true. The mere representation of simple tandem repeats in the databanks argues for mechanisms that allow several motifs to expand considerably more efficiently than others, both with respect to primary establishment (incidence) and the secondary elongations (increased length). Subsequently, the stability of simple repeats appears also to be dictated by their sequence contents and especially their chromosomal environments.

Frequent simple repeat blocks in databanks contain -W (A/T) >> S (G/C) bases -perfect YN dinucleotide periodicity - no CpG dinucleotides

3. Human Diseases Associated with Tandem Repetitive Elements

In the last decade, certain examples of repetitive elements have been recognized to be involved in the causal pathogenesis of human disease (for review see Ashley and Warren, 1995, Paulson and Fischbeck, 1996). Trinucleotide repeat disorders are caused by a newly defined mechanism of mutation, expansion and meiotic instability of simple trinucleotide repeat loci (*op. cit.*). Some of the first and essential

steps toward the identification of this class of disorders came from chromosome research. Fragile X (FraX) syndrome, a frequent genetic cause of mental retardation, is associated with a cytogenetically visible folate-sensitive fragile site on human chromosome Xq27.3 (Ashley and Warren, 1995). The molecular basis for this fragile site is an expanded (CCG)_n stretch within this chromosomal region. To date, similar triplet expansions have been identified in a growing number of other human disorders *i.e.*, Huntington's disease (HD), spinocerebellar ataxias types 1, 2, 3 (Machado-Joseph disease), 6, 7, 8, 12 (SCAI, -2, -3, -6, -7, -8, -12), spinal and bulbar muscular atrophy (SBMA), dentatorubral-pallidoluysian atrophy (DRPLA), myotonic dystrophy (MD) and Friedreich's ataxia (FA) (Paulson and Fischbeck, 1996, Brice, 1998, Ross *et al.*, 1998). These disorders share striking clinical and genetic features. They all affect one or more regions of the central nervous system (CNS). Most of them are progressive neurodegenerative diseases. There is often remarkable interindividual variability in the clinical presentation, even within families. The trinucleotide stretches within the respective genes are also present as shorter, polymorphic alleles on normal chromosomes. In affected individuals, the trinucleotide blocks are elongated beyond precisely definable thresholds. Age of onset and severity of symptoms correlate statistically with lengths of the expansions in many of the disorders, though a prognosis for the individual with respect to clinical course is impossible based on these data (Paulson and Fischbeck, 1996, Ross *et al.*, 1998). While normal alleles are transmitted relatively stably, expanded disease-associated alleles show increased mutability when transmitted to the following generations, often resulting in growing numbers of triplet units. Due to this meiotic instability, the triplet expansions are also named dynamic mutations (Ashley and Warren 1995). The increasing lengths of triplet stretches explain the clinical phenomenon of 'anticipation': in contrast to classical mendelian inheritance there is a tendency to lower age of manifestation and more severe symptoms in subsequent generations (Paulson and Fischbeck, 1996).

A subgroup of triplet repeat diseases is characterized by expansion of microsatellites with CAG as the basic unit and by specific clinical features. This subgroup includes HD, SBMA, DRPLA and the SCAs. They are progressive neurodegenerative diseases, symptoms are (almost completely) restricted to the CNS. Most of them manifest in the fourth to fifth decade. Inheritance is autosomal dominant or X-chromosomal (SBMA). The (CAG)_n tracts are located within the coding regions of the underlying genes, and are translated into longer stretches of glutamines in the respective proteins (Ross *et al.*, 1998). Generally, the number of (CAG)_n units do not exceed 30 on normal chromosomes. On disease chromosomes, moderate expansions are found, up to -2 or 3 times of the normal lengths at maximum. An exception is represented by SCA6 as only minor expansions from 4-16 to 21-27 repeat units are observed (Zhuchenko *et al.*, 1997). Large increases in repeat numbers are almost completely restricted to paternal transmission in (CAG)-diseases. Patients with disease manifestation in childhood or youth often exhibit large expansions and have in most cases inherited the disease-causing alleles from their fathers. Intermediate allele sizes of 30-37 (CAG) units in the *Huntington* gene, responsible for HD, have increased meiotic instability and can expand into disease-associated alleles on transmission, thus causing new mutations underlying sporadic cases of HD. Most of the gene products in (CAG)_n-type disorders are proteins with unknown cellular functions. In SBMA, the androgen receptor is affected, making plausible certain signs of androgen insensitivity often observed in SBMA patients (Paulson and Fischbeck, 1996). The SCA6 gene encodes a subunit (α_{1a} -isoform) of a calcium channel (Zhuchenko *et al.*, 1997). The pathogenetic mechanisms leading to neurodegeneration remain unclear. Despite the expression in many regions of the CNS and other tissues, cell loss is restricted to specific populations of neuronal cells in each disorder. Mutations change certain features of the respective proteins and seem to result in new, deleterious metabolic effects (gain of function). Insights into cellular function and dysfunction of the disease associated proteins are expected from identification and characterization of interacting proteins (*e.g.*, Huntington associated protein, HAPI, Huntington

interacting protein, HIPI, and others in HD). In several (CAG)_n-type disorders neuronal intranuclear inclusions have been identified, containing aggregates of a large number of different proteins or protein fragments with elongated polyglutamin stretches. Their formation appears central in the pathological process (Brice, 1998; Ross *et al.*, 1998), although the cytoplasmic changes should not be neglected (Hackam *et al.*, 1999).

In FraX, MD and FA, the expanded trinucleotide blocks are located outside the coding regions of the responsible genes. They are clinically variable multisystem diseases not only with neurological symptoms but with putative additional manifestations outside the CNS (*e.g.*, heart affection; endocrinopathies in FA, MD; dysmorphic features in FraX). In addition to neurodegeneration, disturbed developmental processes seem to be involved. In FraX syndrome, expansion of a (CCG)_n block is found in the 5' untranslated region of the *FMRI* gene (also termed FRA XA; Fragile X chromosome, A site). In rare cases, FraX syndrome is caused by point mutations within the *FMRI* gene or deletion of the entire gene. MD is transmitted autosomal-dominantly and results from expansions of a (CTG)_n stretch within a gene cluster on chromosome 19 (Harris *et al.*, 1996). The (CTG)_n block is located within the 3'-untranslated region of a gene encoding a protein kinase, but additional flanking genes are supposed to be involved in the pathogenesis. Both in MD and FraX syndrome, massive expansions from normally less than 50 to 1000 (and more) trinucleotide units are demonstrable (Paulson and Fischbeck, 1996). Alleles with intermediate repeat lengths are called premutation (FraX) or protomutation (MD), respectively. These do either not result in manifestation of disease or they are associated with mild symptoms, but exhibit increased meiotic instability (Paulson and Fischbeck 1996). Massive expansions, underlying most severe expression of disease (*e.g.*, congenital MD), arise almost exclusively in maternal transmission. In contrast to (CAG)_n-type diseases, not only meiotic, but also mitotic instability is frequently observed in MD and FraX. The resulting somatic mosaicism seems to contribute to the remarkable phenotypic variability observed in these disorders. As of now, FA is the only example of autosomal recessive inheritance among triplet repeat disorders. FA is caused by homozygous expansion of (GAA)_n blocks within intron 1 of the *frataxin* gene, encoding a protein involved in mitochondrial iron homeostasis (Brice, 1998). In isolated cases, point mutations were identified within the *frataxin* gene. In FraX syndrome, MD and FA, expansion mutations do not seem to exert effects on protein level, but to reduce gene expression on the level of transcription and/or translation.

In addition to the FraX syndrome, other chromosomal fragile sites have been correlated with instable repetitive DNA elements (Ashley and Warren, 1995, Yu *et al.*, 1997). A second folate sensitive fragile site on the X-chromosome is located 600 kb telomeric to FMR1 (FRA XA) and is termed Fragile XE (FRA XE). On the molecular level, expansion of a (CGG)_n microsatellite in the 5'-untranslated region of the *FMR2* gene is observed in FRA XE individuals. Expansions of the (CGG)_n tract (and in some cases deletions within the FRAXE region) are associated with mild mental impairment. There are other inducible sites of chromosome fragility in man that have not (yet) been associated with noticeable disease phenotypes (Ashley and Warren, 1995, Yu *et al.*, 1997). To this group belong the folate-sensitive fragile sites FRA XF and FRA16A both of which harbor expanded (CCG)_n repeats. The folate-insensitive fragile site FRA16B is also not associated with a known phenotype and contains an expanded AT-rich minisatellite repeat (Yu *et al.*, 1997). The fragile site FRA 11B contains an expanded (CGG)_n motif which can lead to chromosome breakage. This was demonstrated by its association with Jacobsen (11q-) syndrome, a chromosomal deletion syndrome. Expansion of the triplet repeat seems to cause instability and predispose to chromosome rearrangements resulting in 11q-syndrome (Ashley and Warren, 1995, Yu *et al.*, 1997). Thus by analysing tandem repeat expansion diseases, the gap is bridged between classical cytogenetics and genomics as well as molecular medicine.

4. Protein Binding to Simple Repetitive Sequences Depends on DNA Secondary Structure(s)

As mentioned above, simple repetitive DNA sequences are widely distributed in most eukaryotic chromosomes, not only in the genomic 'desert' but also in promoters, attenuators and autonomous replicating regions (Epplen *et al.*, 1998). In order to approach the general questions on the biological meaning of simple repetitive DNA located in or close to transcription units, it has been proposed that simple repeats themselves possess moderate enhancer, co-silencer or co-enhancer or attenuator activity (*op. cit.*). In addition, perfect di- and trinucleotide repeats bind nuclear proteins as revealed in studies with synthetic oligonucleotide targets (Richards *et al.*, 1993). Intron 2 of class II transplantation antigens, the *HLA-DRB* genes, contains a composite $(GT)_n(GA)_m$ block. This structure is preserved in this position from artiodactyls to man and it coevolves with exon 2 sequences encoding the β -sheets of the antigen-presentation protein (Schwaiger and Epplen, 1995). The reasons for evolutionary conservation of the microsatellite are not clear and a biological function of this sequence has not been excluded. In addition, *HLA-DRB* genes show allele specific polymorphisms of the $(GT)_n(GA)_m$ microsatellite concerning the length and the composition of the repeat (Epplen *et al.*, 1997b).

Protein binding and structural features of $(GT)_n(GA)_m$ stretches from different *HLA-DRB1* alleles (Mäueler *et al.*, 1994, 1999) and polymorphic $(GAA.TTC)_n$ tracts in intron 1 of the *frataxin* gene (Campuzano *et al.*, 1997) were studied in their original genomic environments. Electrophoretic mobility shift assays revealed that HeLa nuclear proteins bind to DNA fragments containing these simple repeat blocks. The major retarded protein/DNA complexes comprise in both cases zinc dependent proteins present in nuclear extracts from different cell types. Competition experiments using various simple repeats differing in length and flanking regions demonstrate specific interactions. DNase 1 footprinting show protein binding sites located either within the repeats alone or within the repeats as well as their flanking regions, often with preference for one strand. Comparing different $(GT)_n(GA)_m$ alleles, a regular pattern of footprints was not detectable in the $(GT)_n$ part indicating that the zinc dependent protein recognizes structural rather than sequence specific features. O₅O₄ (Osmium tetroxide) and diethyl pyrocarbonate (DEPC) modifications followed by electrophoretic and electron microscopical analyses demonstrate that the $(GT)_n$ tract harbors a Z-DNA like conformation (Herbert and Rich, 1996) and that the homopurine blocks often form different types of intramolecular triple helices. A similar situation was evident using $(GAA.TTC)_n$ blocks of different lengths within *frataxin* intron 1 as targets. Whenever a critical length of the trinucleotide block is exceeded $(GAA)_{>60}$ primary RNA transcription and replication may be hindered or even abolished as evidenced already *in vivo* (Oshima *et al.*, 1998). Mature mRNA and frataxin protein is not observed in the tissues of Friedreich ataxia patients (Campuzano *et al.*, 1997). This phenomenon of a critical expansion length of $(GAA)_{>60}$ correlates astonishingly well with the minimal expansions observed among the large collection of Friedreich ataxia patients (Epplen *et al.*, 1997a, unpublished data).

5. Conclusions

Not all tandem repetitive elements are generally without any biological significance. The biological meaning of individual repetitive loci may be situated somewhere between the extremes of 'junk' DNA without any functional relevance and structures essential for the maintenance of life processes as evidenced by their role in the pathogenesis of human diseases. As anticipated, genomic 'desert' territory keeps a few secrets among which (simple) tandem repetitive sequences are not only outstanding because of their impressive make-up. In addition to their insurmountable informativeness on the

basis of partially exorbitant variability, individual investigative dissections of such repeat loci in and around genes entertains not only the ‘simple’ enthusiast, but clarifies also medically relevant topics.

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2. Mapping and Organization of Highly-Repeated Sequences by Means of Fluorescence *in situ* Hybridization Against Synaptonemal Complex-Associated Chromatin of Rye

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Abstract: A method of preparing two-dimensional surface spreads of plant synaptonemal complexes (SCs) associated with fluorescent *in situ* hybridization has been applied to analyze the location and organization of five different highly-repeated DNA sequences in rye. Our observations indicate that, depending on the type of sequence, the chromatin has different properties with regard to anchorage to the SC. While the organization of telomere sequences is maintained in different organisms, species seem to differ with respect to the organization of centromeric satellites.

1. Introduction

Fluorescence *in situ* hybridization (FISH) facilitates that fluorescent molecules can be deposited in chromatin at the sites of specific DNA sequences. Nucleic acid probes are hybridized *in situ* to chromosomes or nuclei and detected using fluorochromes that are either directly conjugated to nucleotides incorporated into the probe or indirectly through conjugation to a second molecule (typically an antibody or avidin) which binds to a probe label.

FISH has demonstrated to be an effective technique for chromosome and genome identification and physical mapping of defined sequences in plant and animal species. Although FISH is usually performed on mitotic metaphase chromosomes, there are reasons to believe that pachytene (meiotic) chromosomes may be better substrates. Surface-spread pachytene chromosomes are several times the length of metaphase chromosomes and the chromatid fibers extend perpendicular to the lateral elements of the synaptonemal complex (SC), forming loops in such a way that the chromosome has a brush-like appearance (Weith and Traut, 1980). This arrangement makes DNA loops more accessible to *in situ* hybridization methodology and, therefore, to detailed DNA sequence localization (Moens and Pearlman, 1990a; Solari and Dresser, 1995; Barlow and Hultén, 1996; Peterson et al., 1999).

Studies on the organization displayed by some repetitive DNA sequences at pachytene have been carried out in mammals (Moens and Pearlman, 1989; 1990b; Heng et al., 1994, 1996) but are scarce in other species (Moens and Pearlman, 1988; Cuñado and Santos, 1998 and references therein). Recently, we have developed a method of preparing two-dimensional surface spreads of plant SCs associated with fluorescent *in situ* hybridization (Cuñado and Santos, 1998). This technique is used here to analyze the location and organization of five different repetitive DNA sequences in rye, *Secale cereale*.

2. Materials and Methods

Five plants of the Japanese cultivar "JNK" and two of the Korean population Puyo, carrying one standard accessory chromosome, were used.

2.1 Preparation of SCs spreads

Fresh unfixed anthers containing pollen mother cells potentially in prophase I of meiosis were removed from inflorescences. One anther of each floret was stained and squashed in acetic orcein to establish the approximate stage of the remaining anthers, which were then prepared for synaptonemal-complex isolation as indicated by Holm (1986) with minor modifications: namely, the presence of 0.03% "Triton X-100" detergent in the swelling medium, and with the fixative solution containing 4% paraformaldehyde and 1.7% sucrose in distilled water, adjusted to pH 8.9 with borate buffer. For silver impregnation, a few drops of 50% AgNO₃ solution were placed on the preparations which were then covered with a patch of nylon cloth at 60°C until they turned pale yellow in colour. Slides were rinsed, air dried, and stored at 4°C until *in situ* hybridization.

2.2 DNA probes and labeling

The probes employed in FISH analyses included: (i) A rDNA probe, pTa71, containing the 18S, 5.8S, and 25S genes and spacer sequences (Gerlach and Bedbrook, 1979). (ii) A highly repeated DNA sequences of 120 base pairs (bp), pSc119.2, derived from *S. cereale* (Bedbrook et al., 1980). (iii) The probe CCS1 represents a 260bp region within a previous studied clone (Hi-10), which was originally isolated from *Brachypodium sylvaticum L.* ($n = 9$) DNA by HindIII digestion and cloning into pUC18 vector (Abbo et al., 1995; Aragón-Alcaide et al., 1996). iv) The probe D1100 contains highly repetitive specific sequences of the standard rye B chromosome. v) A telomeric synthetic probe. The pTa71 and D1100 clones were labeled by nick translation and the pSc119.2 and CCS1 were amplified and labeled by PCR, all of them with digoxigenin-11-dUTP (Roche). Telomeric probes were labeled with biotin-16-dUTP by nick translation of PCR-amplified products using the oligomer primers (5'-TTTAGGG-3')₅ and (5'-CCCTAAA-3')₅ in the absence of template DNA (Cox et al., 1993).

2.3 DNA:DNA *in situ* hybridization

FISH against synaptonemal complex-associated chromatin was made following the procedure described by Cuñado and Santos (1998). Digoxigenin-labeled probes were detected with two successive incubations, 5 µg/ml monoclonal anti-digoxigenin (Roche) and 10 µg/ml antimouse Ig-fluorescein (Roche) in blocking buffer in a humid chamber at 37°C, 45 min each. The biotinylated probes were detected with incubations in fluorescein isoliocyanate (FITC)-ExtrAvidin 1:100 (Sigma) in blocking buffer, 45 min at 37°C. After each incubation, slides were washed in TNT (0.1M Tris, 0.15M NaCl, 0.05% Tween 20) for 3 × 5 min. Finally, slides were dehydrated in an ethanol series (70, 95 and 100%, 1 min each) and air-dried. Vectashield mounting medium (Vector Laboratories) was applied beneath a coverslip. Slides were studied with a Olympus BX-60 epifluorescence microscope and photographs were taken by a double exposure with the fluorescent image followed by a dark field image of the SC using Fujicolor Provia-400 color print. Chromatin loop size emerging from the SC was defined as the distance from the SC attachment site to the tip of the average loop. As the fluorescent signals are quite strong in silver stained prophase I nuclei, the combination of transmitted-light and epifluorescence images in the same micrograph allows the assignment of each signal to specific chromosome regions.

3. Results and Discussion

3.1 The rDNA probe pTa71

The signal dots were always associated with only one bivalent and clustered on and near the axis and radiated away from there (Fig. 1a). Measurements of the loop size were prevented in many nuclei by the presence of the nucleolus. So, only eight nuclei were amenable for analysis. The mean loop size was $4.95 \pm 0.2\mu\text{m}$. The position of the hybridization signal, relatively near the telomere, is in agreement with that reported previously in mitotic chromosomes (Cuadrado et al., 1995). Unfortunately, the lack of preservation of centromeres on SCs of Grammineae and the similar length displayed by the rye chromosomes prevent the identification of the chromosome bearing the nucleolar organizing region.

3.2 The 120bp family

This sequence was detected by the pSc119.2 probe at subterminal (a mean of five hybridization signals per nucleus) and interstitial (a mean of four signals per nucleus) regions of rye chromosomes (Fig. 1b). In well-spread nuclei, the individual chromatin strands and loops are discernible and it is apparent that there is a beaded pattern of fluorescence along the chromatin strands. The mean loop size in subterminal regions was $6.43 \pm 0.8\mu\text{m}$, while in interstitial regions it was $6.16 \pm 0.9\mu\text{m}$ (measurements from ten nuclei). It has been proposed that DNA sequence determines the potential for loop formation (Heng et al., 1994), while the size of the loop is controlled by chromosome position (Heng et al., 1996). Thus, sequences located at chromosome termini in mice formed shorter loops than identical interstitial sequences. The results shown here do not indicate any clear relationships between the loop size displayed by the 120bp repetitive family and the chromosome location of these sequences. However, these observations must be considered with caution because in the case of rye it is not possible to identify the chromosomes carrying the hybridization signals as occurs in the experiment performed by Heng et al. (1996). Furthermore, differences in the amount of sequences of the 120bp family in different chromosome regions may also play a role in the loop size.

3.3 The CCS1 family

Hybridization with these sequences produces clear signals in the form of spot aggregation without definite shape, that were usually not associated with SC, in positions which concur with centromere location (Fig. 1c). The diameter of these aggregates ranged from 2.13 to $5.61\mu\text{m}$ (fifteen nuclei analyzed). These observations support the hypothesis that species differ with respect to the organization of centromeric satellites. Thus, in mice centromeric minor satellite DNA is tightly associated with the SCs (Moens and Pearlman, 1990b) but the α -satellites in human and the satellite DNA I in rats (Moens and Pearlman, 1989), and the EcoRI family in gilthead seabreams (Cuñado et al., 2000) form different sized loops that are associated with SCs only at their bases. The hybridization pattern shown by the CCS1 sequences in rye indicate that hybridization does not occur with whole loops, but with only a part of the loops, to produce spots. The high number of dot signals of CCS1 probe may indicate that these sequences are not distributed in continuous tandem arrays. Also, differential packaging of the 30nm fiber of the target DNA resulting in the apparent splitting of the signals cannot be excluded.

3.4 The D1100 family

A B chromosome-specific repeat family D1100 (Sandery et al., 1990) localized at the distal region of the long arm was used as a probe against SC associated chromatin of rye meiocytes from Puyo population. This technique revealed that a high proportion of synapsis displayed by the B univalent

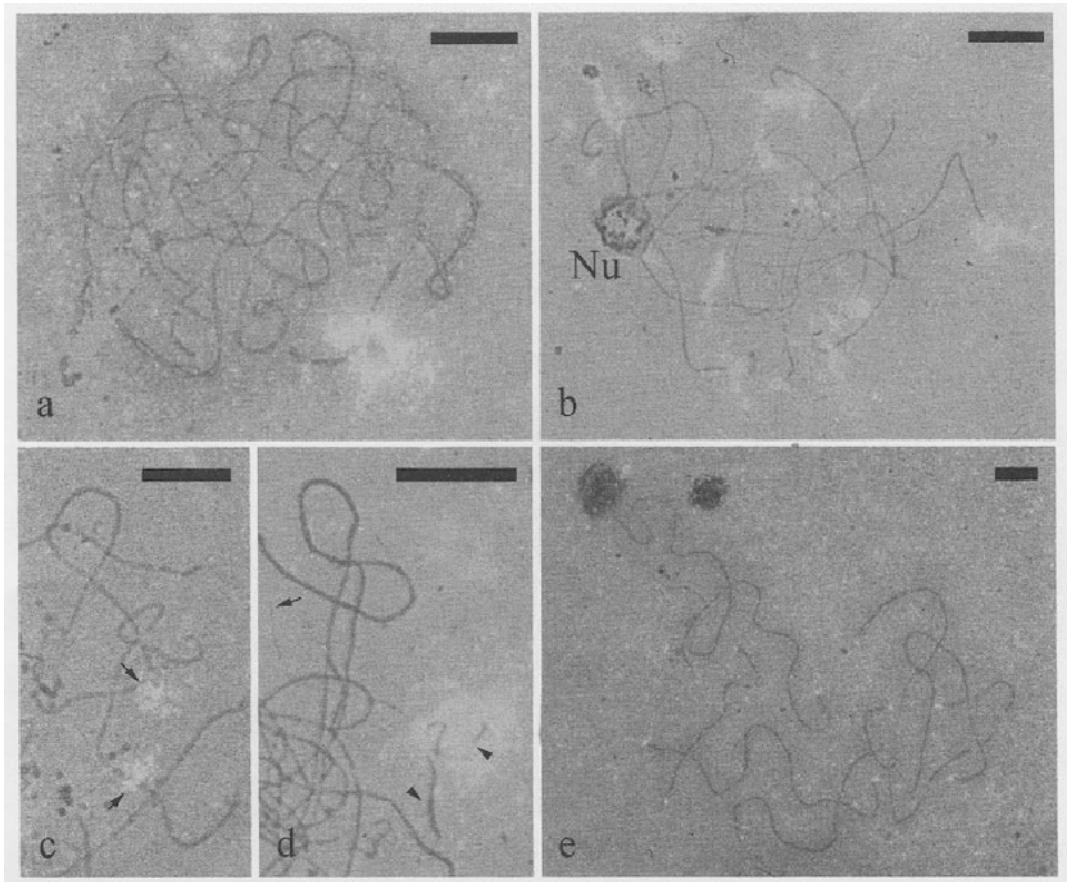


Fig. 1. Fluorescent *in situ* hybridization to whole-mount, surface-spread pollen mother cells at late zygotene-pachytene of rye. Yellow-green fluorescing cloud or dot-like signals visualize sites of hybridization, while the synaptonemal complexes (SCs) remain red-brown due to silver staining. In the pictures, SCs appear black and hybridization signals appear white (a) rDNA is clearly visualized in absence of nucleolus. (b) Location of the 120 bp sequence (Nu = nucleolus). (c-d) Partial views of pachytene nuclei. (c) Hybridization sites of CCSI sequences appear as spot aggregation without definitely shaped, one per bivalent, at positions that concur with centromeric regions (arrows). (d) Selected B chromosome. Arrow indicates one of the ends while arrowheads indicate two self-synapsed regions, the sequence D1100 being located in one of them. (e) The location of the telomere probe. This sequence is associated with the two ends of each SC. Bar represents 10 μ m.

could be of non-homologous nature (Fig. 1d). The organization of the B chromatin forming loops with a mean value of $4.14 \pm 0.52 \mu\text{m}$ (twenty nuclei analyzed), and the beaded pattern of fluorescence along the chromatin strand resembles that displayed by some other repetitive sequences of rye A-chromosomes analyzed in this work.

3.5 Telomere DNA

The $(\text{TTTAGGG})_n$ sequence hybridizes to the telomeres in most of the plant species analyzed to date (Fuchs et al., 1995). In pachytene nuclei of rye, telomeric DNA sequences are tightly associated with the ends of SCs (Fig. 1e). Telomere chromatin loops appeared very condensed with a size, $1.2+0.27 \mu\text{m}$ (ten nuclei analyzed), smaller than that displayed by the other repetitive sequences analyzed in this work. The size of the signals within the same nuclei varied widely, the maximum difference found was about $3 \mu\text{m}$, indicating that individual telomeres may contain different number of repeats of the

telomeric sequence. The organization of telomere sequences observed in rye is similar to that observed in rodents (Moens and Pearlman, 1990b), humans (Barlow and Hultén, 1996), the wild wheat *Aegilops uniaristata* (Cuñado and Santos, 1998) and fishes (Cuñado et al., 2000), suggesting that the distribution of these types of sequences in relation to the SC components seems to be maintained in very different organisms. In view of evolutionary conservation of telomeric sequences, these findings are not unexpected.

It can be concluded that the *in situ* hybridization method used here permits a novel approach to the analysis of chromatin organization in plant meiotic prophase chromosomes. Moens and Pearlman (1988) stated that the folding mechanism seems precisely regulated and the loop sizes are roughly the same for the chromosomes of a given species, but they can vary from species to species. However, the observations shown here indicate that different repetitive DNA sequences display different loop sizes at pachytene. Whether or not the size of the loops reflects the number of sequences of each repetitive family remains to be ascertained. The chromatin packaging observed at the tip of rye chromosomes is conserved in other organisms such as rodents, humans and fishes. On the contrary, the organization of centromeric satellites seem to differ between species. It remains to be established whether or not the loop size organized by a given DNA sequence shows variation at different stages of meiosis and also, whether or not the same DNA sequence is associated with the SC from the onset of axial core formation until the dissolution of the SC at diplotene. In addition, consistent involvement of specific DNA sequences with the SC between nuclei has not been demonstrated. It is evident that further research is needed to elucidate the mechanisms governing the relationship between chromatin and SC at early stages of meiosis.

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3. Some Insights on the Mechanism of *in situ* DNA Digestion by Restriction Enzymes and on Fluorochrome Banding: Case of the *Argentata* group

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Abstract: We discuss the mechanisms involved in *in situ* DNA digestion by restriction enzymes and fluorochrome banding using cytogenetic and molecular data obtained from research developed in the species of the *Callithrix argentata* group (Platyrrhini, Primates). Additional evidence was obtained from the Neotropical bat species *Carollia perspicillata* (Phyllostomidae, Chiroptera). With regard to the restriction enzymes it is quite clear that occurrence of the restriction site is not enough for banding, as the digestion of DNA depends on access to the restriction site by the enzyme. The data on fluorochrome banding suggest that some sequences of base pairs are very important for binding of the fluorochrome, especially with CMA³. These sequences are more important than the long-range variation in the base pair composition along the chromosome, as previously supposed.

Key Words: Primates, bats, heterochromatin, chromosomes, RE banding, fluorochromes.

1. Introduction

According to Hershkovitz (1977) the South American primate genus *Callithrix* comprises two species groups, *jacchus* and *argentata*. This proposal has broad approval (Mittermeier *et al.*, 1988; 1992; Vivo, 1988; 1991). The *jacchus* group occurs in the Atlantic forest of eastern Brazil, while the *argentata* group inhabits the Amazon forest. The species or subspecies status of the taxa of these groups is presently under discussion (Hershkovitz, 1977; Coimbra-Filho, 1984, 1990; Mittermeier *et al.*, 1988, 1992; Vivo, 1988, 1991; Natori, 1990; Rylands *et al.*, 1993).

The first detailed karyotypic description from a species of the *argentata* group was made by Barros *et al.* (1990). They showed the occurrence of distal heterochromatic blocks on many chromosomes of the species *Callithrix emiliae* (Fig. 1). Latter Nagamachi (1995) and Nagamachi *et al.* (1996) confirmed this trait on all the *argentata* group species so far analyzed. For a better characterization of these heterochromatic blocks, Pieczarka (1995) and Pieczarka *et al.*, (1996) analyzed the karyotypes of three species of the *argentata* group with *in situ* DNA digestion using seven restriction enzymes (*Hinf*I, *Mbo*I, *Alu*I, *Rsa*I, *Dde*I, *Hae*III and *Msp*I). They also performed fluorochrome banding using CMA³ and DAPI. In the same year Alves *et al.* (1995) sequenced the satellite DNA from the heterochromatic blocks of the karyotype of *Callithrix argentata*. They found a repetitive sequence of

1528 base pairs. Using FISH, they confirmed that this DNA was located on the distal heterochromatic blocks.

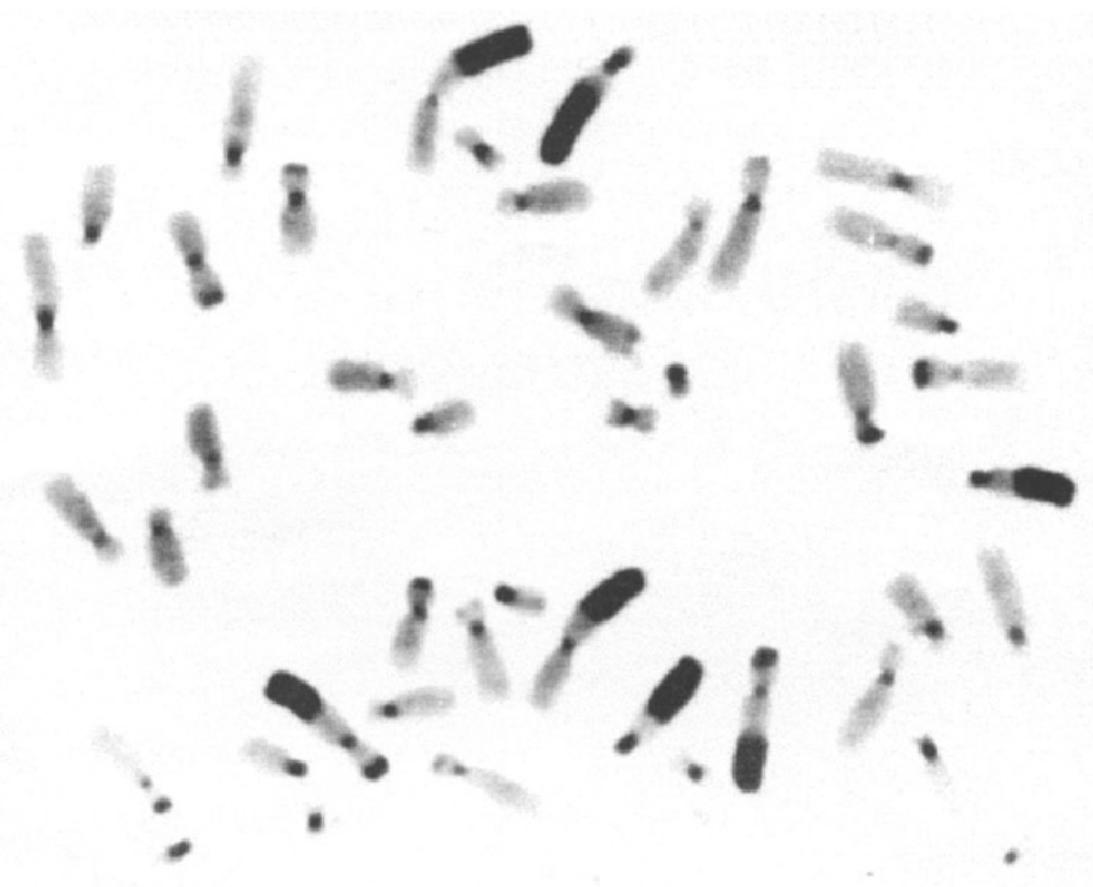


Fig. 1. C-banded metaphase of *Callithrix emilae*

For a better understanding of how the restriction enzyme digests the DNA *in situ*, as well as the mechanism involved in the fluorochrome banding, it would be necessary to compare the banding patterns with the base sequence of the DNA. This is quite difficult to do in human chromosomes, as the heterochromatic regions have a mix of different types of satellite DNA (Babu, 1988). Since the distal heterochromatic blocks of the *argentata* group have already been sequenced and RE and fluorochrome banded, this study becomes possible.

2. Mechanism of *in situ* DNA Digestion by Restriction Enzymes

After 1983 it became clear that restriction enzymes could detect specific fractions of DNA *in situ*, for they induce banding in chromosomes, most of the time C-banding. This banding is the result of a differential extraction of DNA (Mezzanotte *et al.*, 1983; Miller *et al.*, 1983; Bianchi *et al.*, 1985). All researchers agree that restriction enzymes cut the DNA at specific sites, and that the digested chromosome regions are rich at these sites. However, the reason why a restriction enzyme does not cut the DNA of some regions is the major doubt. The first hypothesis suggested that the banding was a consequence of the restriction sites' distribution patterns along the chromosome (Miller *et al.*, 1983, 1984; Bianchi *et al.*, 1985; Ludeña *et al.*, 1991). Bianchi *et al.* (1985) showed that enzymes with higher frequencies

of restriction sites generate DNA fragments with smaller shapes and a high loss of material. Only the enzymes with restriction sites of 4 or 5 base pairs can induce differential staining with Giemsa, for these sites have a higher probability of occurrence than the sites with 6 base pairs. As a consequence, the DNA is cut in smaller pieces. Under this view, the non-digestion would be result of few restriction sites, leaving the DNA pieces too long to be extracted (Miller *et al.*, 1984). In opposition to this view, Mezzanotte *et al.* (1983, 1985) suggested that the structural conformation of the chromatin could be important for chromosome banding. Pieczarka and Mattevi (1999) reviewed the hypothesis in literature related to this possible importance of the structural conformation of the chromatin on the differential extraction of DNA:

(a) *The higher condensation of heterochromatin when compared with euchromatin:* Lica and Hamkalo (1983) believed that the resistance of centromeric chromatin to digestion by EcoRI and AluI is due, at least in part, to protection supplied by the highly condensed structure, since the isolated DNA, without the proteins of the chromatin, can be cut by these enzymes.

(b) *The shape of the enzyme molecule:* If the molecule is large, it could not penetrate the compacted structure of heterochromatin. Tagarro *et al.* (1991) found that *Taq I* intensely digests the heterochromatic regions of chromosomes # 1, 9, 15, 16 and Y, and less intensely digests the euchromatic DNA. According to these authors, the higher grade of compaction of heterochromatin could not explain the banding. Since *Taq I* has a small molecular weight (27 kDa) when compared with *Alu I* (52 kDa), its smaller shape would be responsible for its ability to penetrate the chromatin structure and make cuts in the restriction sites. Gosalvez *et al.* (1989) used isoschyzomere enzymes (meaning that they cut the DNA at the same restriction sites, but these have completely different molecular structures and biological origins). The authors found different reaction among enzymes that theoretically should produce the same banding, because they cut the DNA at the same sites. Gosalvez *et al.* (1989) suggest that differences in the molecular shape of enzymes and its interaction with the chromosome organization could be involved in the differential ability for cutting DNA.

(c) Molecular interaction between DNA and the other chromosomal components, usually proteins, would avoid the extraction of the DNA from the chromosome. Burkholder (1989) used restriction enzymes for the digestion of unfixed mice chromosomes. From each tip where the DNA was digested, he obtained samples and then fixed them on slides and stained with Giemsa. When the material on the slide had the typical restriction enzyme C-banding pattern (*Hae III*, *Alu I* ou *Bst NI*), the supernatant was analyzed in gel and the shape of the extracted DNA fragments was determined. The same was done with the pellet retained in the chromosomal matrix. In disagreement with the hypothesis put forth by Bianchi *et al.* (1985), the shape of the extracted fragments ranged from 200 base pairs to 4 kilobases. The DNA on the pellet ranged from 500 base pairs to more than 4 kilobases.

Mezzanotte *et al.* (1992) analyzed the DNA solubilized after *in situ* digestion by restriction enzymes and found that the fragments ranged from 2 to 9.5 kb. The DNA which was retained on the slide ranged from 3 to 21 kb. The conclusion is that the size of the fragments of DNA was not really important, the molecular interactions that this segment has with the other chromatin parts is more meaningful as previously suggested by Burkholder (1989).

(d) The occurrence of molecular interactions among the DNA and the other components of the chromosomes, mainly proteins, that would avoid the cut at the enzyme site. Antequera *et al.* (1989) observed that the enzyme *Msp I* is able to cut CpGs sites in purified DNA, whether or not the sites were methylated. However, this enzyme was incapable of cutting methylated DNAs in chromatin. The results suggest that the protein MeCP is linked to methylated DNA *in vivo*, in methylated CpGs sequences, which would avoid the cut by the *Msp I* enzyme.

In our research with species of the *Callithrix argentata* group (Pieczarka *et al.*, 1996), the distal

heterochromatin had a homogeneous response to the enzymatic action, which was fully digested by *RsaI*, partially by *DdeI* and unchanged by *Hinfl*, *MboI*, *AluI*, and *HaeIII*. No banding was obtained with the enzyme *MspI*, as in humans (Miller *et al.*, 1983). We retrieved the nucleotide sequence for this distal heterochromatin (Alves *et al.*, 1995) on GenBank (Access Code L07927). We searched for the restriction sites of the enzymes that we used in our research and we found 9 restriction sites for *RsaI*, 8 for *MspI*, 6 for *DdeI* e *HaeIII*, 3 for *AluI*, 2 for *Hinfl* and no sites for *MboI* (Fig. 2). The distribution of the sites in the sequence is random. In fact *RsaI*, which has most cutting sites, where the longest DNA fragment having has 645 base pairs (bp). If all of *MspI* eight sites were cut, the longest DNA fragment produced would have 729 bp, i.e. slightly longer than the one generated by *RsaI*. *DdeI* and *HaeIII* have fewer sites, but their distribution is more even, which generates shorter DNA fragments: the longest would have 629 bp in *DdeI* and 640 bp in *HaeIII*. Our conclusion is that the amount and distribution of restriction sites are not enough to explain the RE banding. If they were enough, *DdeI* and *HaeIII* would produce banding similar to *RsaI*, but this did not occur.

Enzyme	<i>In situ</i> digestion	Restriction sites localization on the sequence
<i>MboI</i>	No	[REDACTED]
<i>AluI</i>	No	825 1082 1253
<i>Hinfl</i>	No	290 494
<i>DdeI</i>	No	114 198 404 858 951 1015
<i>HaeIII</i>	No	420 830 894 999 1130 1308
<i>MspI</i>	No	543 971 1201 1292 1342 982 1224 1312
<i>RsaI</i>	Yes	38 95 341 986 1419 1473 1 55 1448

Fig. 2. Distribution pattern of restrictions sites in the repetitive sequence of 1528 base pairs found by Alves *et al.* (1995) in the distal heterochromatic blocks of *Callithrix argentata*.

Among the different hypotheses described above for the differential extraction of DNA as a result of the structural conformation of the chromatin, a higher condensation of the heterochromatin when compared with euchromatin seems unacceptable. Comings *et al.* (1973) stated that "even though constitutive heterochromatin seems almost synonymous with tightly condensed chromatin, this refers to its status in the interphase cell. The situation is possibly different during mitosis. The densitometry tracings of mouse chromosomes stained with Feulgen suggested that, if anything, the concentration of DNA in the centromeric heterochromatin was actually less than the concentration in the arms. This

implies that the major feature of C-band heterochromatin is that its degree of condensation is fixed and is thus relatively more condensed during interphase and less during metaphase". These observations do not agree with the hypothesis that the higher condensation of heterochromatin would prevent the penetration of the enzymes in the chromosome structure. The suggestion that the size of the enzyme molecule would affect its ability of penetration on the chromosome also seems unacceptable. Most of the enzymes have a size which ranges from 25 to 30 kd (Gingeras, 1991). Among the enzymes used in the chromosomes of *C. argentata*, *AluI* weights 52 kd, *DdeI* 27 kd (Antequera *et al.*, 1989), *HinfI* 30 kd and *MspI* 29.9 kd (Gingeras, 1991). The weights of *RsaI*, *HaeIII* and *MboI* are unknown. Although *AluI* is almost twice the size of most enzymes, it caused an intense digestion in the centromeric heterochromatin, even more so than *HinfI*. Our results do not support the hypothesis that the larger restriction enzymes would have more difficulty in penetrating the constitutive heterochromatin.

Research using *in situ* nick translation made it clear that despite the fact that a dark band retained its DNA after digestion by *AluI*, it was intensively cut by the enzyme (Bartnitzke *et al.*, 1990). This happens because molecular interactions among DNA and chromosomal proteins would prevent the extraction of the DNA, even if cut. This explanation does not agree well with our data, for there would be no reason for a extraction be made after digestion with *RsaI*, but not after *MspI*. An alternative suggestion is that not all the restriction sites were accessible, which shows the importance of the chromosome structure. At least for *MspI*, there are some evidences that support this hypothesis. Antequera *et al.* (1989) demonstrated that the protein MeCP covers the CCGG sites, which hinder the action of *MspI*.

Mechanism of Fluorochrome Banding

The technique of chromosome banding using fluorochromes was introduced by Caspersson *et al* (1968, 1969) using Quinacrine (Q-banding). This technique revolutionized cytogenetics, allowing the precise identification of the chromosomes. However, Q-banding was soon substituted by G-banding, because Quinacrine, as is the case with most fluorochromes, has low band contrast and fast fading of fluorescence with even a slight excitation by light. For these reasons fluorescent banding techniques were underused for many years. Recently, new methods have been developed to bypass these difficulties. The use of glycerol substituting saturated sucrose or buffer in the mounting media gives stability to the fluorescence, reducing fading of the bands (Sahar and Latt, 1978, 1980).

When one tries to explain the mechanism of fluorochrome banding, one of the most popular hypotheses is that the differential fluorescence is produced by the uneven distribution of base pairs on the chromosome. However, one must keep in mind that Schweizer (1981) suggests that this is an inconclusive hypothesis, for no one knows the possible role of the other chromosome components related to the variation in base pair composition (differential accessibility to DNA by fluorochromes, substitution of base by analogs, etc.). There are two types of fluorochromes used in cytogenetics: the group specific for A-T base pairs and the group specific for C-G base pairs. Fluorochromes like Hoechst 33258 and 4'-6-diamidino-2-phenylindole (DAPI) bind specifically to A-T pairs, while fluorochromes like Quinacrine and Daunomycin have low binding specificity, but fluoresce when binding to A-T pairs. In both situations, the banding pattern obtained is Q-banding, where the brightness is on the base pairs blocks rich in A-T. Fluorochromes specific to C-G base pairs like Chromomycin A3, Mitramycin and Actinomycin D produce R-banding, where the brightness is on the base pair block regions rich in the C-G (Schweizer, 1981).

The technique called counterstaining was developed by the end of 1970's and was extensively reviewed by Schweizer (1981). When a fluorochrome is used to stain chromosomes, the contrast

obtained can be improved with the help of a second agent, sequentially applied on the chromosomes (Schweizer, 1976a; 1976b; Jorgenson *et al.*, 1978; Sahar and Latt, 1978). Usually the effect of the contrasting agent is stronger when it is added after the fluorochrome. For this reason, the fluorochrome is called the “first stain” and the contrasting agent is called “counterstain” (Schweizer, 1981). If the counterstain is fluorescent, it must have a fluorescence spectrum sufficiently different from the first stain. If the counterstain has binding specificity for the same base pair than first stain, it must have a different affinity of binding (Schweizer, 1981). The better contrast obtained by the use of counterstaining can be the result of the competition for binding between the different stains, and in this case the banding is a result of the different affinities the different dyes have for the chromosomes. It is also the result of energy transfer between the dyes. This transfer occurs when the emission spectrum of one stain superimposes on the excitation spectrum of the other. The fluorescence of the first fluorochrome is faded while the fluorescence of the second fluorochrome is highlighted (Sumner, 1982). For the energy transfer the fluorochrome molecules must be close to each other (Latt *et al.*, 1980). This could improve the contrast among the chromosome regions, mainly if they are rich in some base pairs (Sumner, 1982).

The associated use of three stains (Schweizer, 1980) can selectively highlight two banding patterns in the same metaphase, if the fluorochromes have little or no fluorescence superimposition. These patterns can be visualized with the use of the right excitation of light on the proper wave length. e.g. tri-stain with Cromomycin A³ (fluorescent)/ Dystamycin A (non-fluorescent)/ DAPI (fluorescent) allows the analyses of R-banding and DA-DAPI C-banding on the same metaphase simply by changing the filter set (Schweizer, 1980). The Dystamycin A can be substituted by Methyl Green without loss of quality (Donlon and Magenis, 1983).

Saitoh and Laemmli (1994) stated that the mechanisms responsible for the fluorochrome banding are not those accepted by most researchers, meaning the accessibility of the stains to DNA and the differential composition of bases in the Q- and R-banding. According to these authors, the accessibility must play a lesser role in banding, because all the fluorochromes that have no base specificity (for instance, ethidium bromide or propidium iodide) stain the chromosomes in homogeneous fashion. Regarding the differential base composition in Q and R-banding, Saitoh and Laemmli (1994) note the fact that the G-/Q-banding is only 3.2% richer in AT bases than the R-banding, too small a difference to explain such a large difference in the fluorescent emission among those bands, such as the ones observed with Daunomycin, for instance. They suggest instead that the banding must have an explanation based on chromosome organization. The chromosome banding would be a consequence of non-casual folding of the DNA sequences with higher amounts of AT pairs. The regions richer in AT would be more intensively folded, while the R-bands would be more extended, making their DNA more accessible to transcription. The visual pattern produced by this folding would be enough to generate the bands.

A difficulty with this hypothesis is that the amount of DNA would be different in R- and Q-/G-bands, where the density of DNA would be smaller in the R-bands. If this should be the case, the non-specific fluorochromes would stain the chromosomes in a heterogeneous way, for the intensity of staining is related to the amount of DNA. The authors acknowledge the problem and suggest that the open structure of the R-bands would allow the accessibility to more stain, which would compensate the lower amount of DNA. However, this is simply a suggestion without any kind of supporting evidence. It is hard to accept that the compensation would be so precise that the R-bands would be non-differentiable from G-bands.

In our research with three species of the *argentata* group it was possible to observe that the distal heterochromatic blocks were fluorescent with CMA³ and negative with DAPI (Pieczarka *et al.*,

1996). The analyses of the base sequence described by Alves *et al.* (1995) show that, of the 1528 bases, 833 (54.5%) were A or T and 695 (45.5%) were C or G. This means that most of the sequence is made of A or T. However, the heterochromatic block were CMA³⁺, DAPI-. This is the first well-documented result where a chromosomal region is richer in AT than CG, but stains with CMA³ and not with DAPI. These data are in opposition to the hypothesis which suggests that differential staining is only the result of the unequal distribution of base pairs on the chromosome. An alternative hypothesis, which is in agreement with our data, would be that the fluorochromes link to a specific sequence of base pairs. This would be the case for CMA³. These sequences are probably quite simple, for they could be easily be formed in DNA segments where there was a higher concentration of CG base pairs.

Santos and Souza (1998) have recently described the banding pattern obtained with CMA³ and DAPI in the Neotropical bat *Carollia perspicillata* ($2n = 20$, XX e $2n = 21$, XY₁Y₂). Studies using *in situ* hybridization of telomeric probes (TTAGGG)_n show terminal and interstitial patterns for this probe (Meyne *et al.*, 1991; Pieczarka *et al.*, unpublished data). The interstitial distribution of the telomeric sequences agrees well with the R-banding pattern obtained with CMA³. It must be noted that the probe is not especially rich in CG base pairs; in fact, it shows 50% of each kind of base pair. This result suggests that CMA³ binds to the sequence GGG. Although the sequence of satellite DNA from *Callithrix argentata* found in the heterochromatic blocks is richer in AT bp, it shows the GGG sequence 29 times, where one is GGGG and two are GGGGG. The CCC sequence also appears 8 times CCC, with two being CCCCC. It is known that CMA³ is not an intercalating agent on DNA, and that the binding is done with double helix DNA, where the Mg²⁺ ions are part of the complex DNA-CMA³ (in Sumner, 1982). It is possible that three radicals guanine or cytosine may be necessary for the complex building. In this sense, we suggest that CMA³ binds to a specific sequence of DNA, probably GGG. Molecular studies on CMA³ may demonstrate how binding occurs between this fluorochrome and DNA.

The suggestion on DAPI is that it binds to AT base pairs groups (in Sumner, 1982), but is not possible to say which groups are these, as there are no data on the sequences of regions where the chromosome is stained with DAPI. It is known, however, that these sequences must not be found in the heterochromatic blocks of *Callithrix argentata*, because this regions do not stain with DAPI, despite being rich in AT.

Conclusions

The data here presented suggest the following hypotheses for the mechanisms of *in situ* DNA digestion by restriction enzymes and on fluorochrome banding:

- (a) **Restriction Enzymes:** The occurrence of the restriction site is not enough for the *in situ* extraction of DNA by restriction enzymes. These sites can be protected from enzymatic reaction by chromosomal proteins.
- (b) **Fluorochromes:** In spite of the generally accepted hypothesis which states that fluorochrome banding is a consequence of long-range variation in the base pair composition along the chromosome, the data shown here suggest that some base pair sequences are responsible for the binding of fluorochromes, at least for CMA³. These sequences would have a higher probability of occurrence in chromosomal regions where the base pairs involved were more frequent. For CMA³ the GGG sequence is suggested.

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4. Structural Differences of Chromosomes in Plants Detected by Fluorescence *in situ* Hybridization Using Probes of rDNA, *Arabidopsis*-type Telomere Sequence Repeats and pCrT7-4

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Abstract: The chromosomal sites of 5S rDNA, 45S rDNA and *Arabidopsis*-type telomere sequence repeats characterize individual species and population constitutions in chrysanthemums and cycads and those of *Sau3A* in spruces. The sites of the telomere sequence repeats in the cycad chromosomes occur not only in the usual terminal regions of chromosome-arms as small dots, but also in several centromeric to proximal regions as relatively large signals. Large signals of the 278 bp DNA segment with respect to *Cycas revoluta* involved with pCrT7-4. The Y-chromosome of *Cycas revoluta* was distinct by lack of the large signal of pCrT7-4 and the *Arabidopsis*-type telomere sequence repeats. A highly conserved chromosomal location of these genes lead to speculation on the karyotype evolution and species property of the chrysanthemum and cycad groups.

Key words: *Arabidopsis*-type telomere sequence repeats, Chrysanthemum and cycad chromosomes, Fluorescence *in situ* hybridization, pCrT7-4, pTa71, *Sau3A*, 5S and 45S rDNA

1. Introduction

High repetitive DNA sequences which occupy the great portions of the genomes of the higher plants (Flavell, 1980) are important to contrast structural differences in chromosome as identification markers or genome mapping of specific chromosomes and chromosome sites. Ribosomal DNA and telomere sequence repeats are instances of the highest tandem repeat sequences in chromosomes of plants (e.g., Lubaretz et al., 1996; Schmidt et al., 1994; Fuchs et al., 1995). However, origin, evolution, function, and so on of these repetitive DNA sequences have not been clearly understood in sense of biological significances including the hypotheses of “junk DNA” and “selfish DNA” (Doolittle and Sapienza, 1980; Charlesworth et al., 1994).

Ribosomal DNA consists of two sites as follows: (1) 45S rDNA contained 18S, 5.8S and 26S; and (2) 5S rDNA, which is generally found in different chromosome or different region of chromosomes from 45S rDNA because of differences in site of gene expression in the nucleus. The 45S rDNA region corresponds to the secondary constriction or the nucleolar organizing region (NOR) and is located in heterochromatic region (Kondo et al., 1996a, b). The 5S rDNA region is not corresponding or associated to any specific regions such as the NORs and sex chromosomes, but has been used to

compare differences in chromosome correlated with species within certain genera studied (Alonso-Blanco *et al.*, 1994; Lubaretz *et al.*, 1996; Thomas *et al.*, 1996).

The telomere sequence repeats are generally placed in the terminal region of the chromosomes to conserve their chromosomes during the DNA replication and the cell division. The probe of telomere sequence repeats may screen certain translocation and centric fission and fusion (Cox *et al.*, 1993).

This paper describes mainly our general methodology of FISH using the pTa71, the 5S and the *Arabidopsis*-type telomere sequence repeats in chromosomes of some chrysanthemums, cycads and piceas.

2. Materials and Methods

2.1 Preparation of Somatic Chromosomes

Meristematic tissues of cycads and spruces were collected from young leaflets. They were treated with 0.002 M 8-hydroxyquinoline at 4°C for 13h before they were fixed in 1:3 acetic acid: ethanol at 4°C overnight. They were washed and stored in 70% ethanol. The materials were rinsed in distilled water and then in 45% acetic acid for 10 min each at room temperature and were macerated in 45% acetic acid at 60°C for 4 min. They were placed on glass slides, removed trichomes on their epidermis and chopped into minute pieces about 0.5 mm across. In contrast, meristematic tissues of chrysanthemums were harvested from root tips pretreated with 0.002 M 8-hydroxyquinoline at 18°C for 1.5 h. Their pieces were macerated in the enzyme mixture of 3% cellulase Onozuka R-10 (Yakult) and 0.25% PectolyaseY-23 (Seishin) in distilled water (w/v) at 37°C for 20 min. They were washed in distilled water for ten min. and were squashed in 45% acetic acid on glass slides. The slides from the coverslips were removed by the dry-ice method and air-dried.

2.2 Preparation of DNA Probes

The 18S-5.8S-26S rRNA gene of wheat, pTa71, which contained spacer region, was used as a 45S rDNA probe (Gerlach and Bedbrook, 1979).

Young leaflet (2.5 g weight of each plant studied) was harvested and homogenized in frozen liquid nitrogen. This homogenized powder was transferred to the centrifugation tube which contained 10 ml washing buffer [0.1 M Tris-HCl, pH 8.0; 2% 2-mercaptoethanol (w/v), 1% polyvinylpyrrolidone X-30 (w/v), 0.05 M/L-ascorbic acid, dissolved in distilled water]. The tubes were shaken at room temperature for 10 min, and centrifuged at 10,000 rpm for 10 min. The supernatant was removed from the tube, and continued this process until supernatants became clear. In the tube with the centrifuged deposit was added: 10 ml CTAB extraction solution [2% Cetyltrimethylammonium bromide (CTAB; w/v), 1.4 M NaCl, 0.1%Tris-HCl pH 8.0 (w/v), 20 mM EDTA-Na₂, dissolved in distilled water], and treated for 90 min at 55°C. Then, 10 ml chloroform/ isoamylalcohol (25:1) was added and was slowly shaken for 10 min. This mixture was centrifuged at 10,000 rpm for 10 min, and the supernatant was transferred to another tube. The new tube which contained the supernatant was added 5 ml neutral equilibrated phenol and 5 ml chloroform/ isoamylalcohol (25:1), and was shaken for 10 min. The preparation was centrifuged at 10,000 rpm for 10 min, and its supernatant was transferred to another tube. Then, the supernatant was mixed with 10 ml chloroform/ isoamylalcohol (25:1) and was shaken slowly for 10 min. The preparation was centrifuged at 10,000 rpm for 10 min and transferred to another tube. The extraction processes with chloroform/ isoamyl alcohol were continued until any dusts appeared on the border of the supernatant layer and chloroform/ isoamyl alcohol layer. The final supernatant was transferred to a new tube and 6 ml isopropanol was added and DNA salting-out was carried by slowly shaking. The derived DNA was isolated in centrifugation tube

1.5 ml capacity, and 500 μ l of 70% ethanol was added for cleaning and then centrifuged at 15,000 rpm at 5°C for 10 min. Then, the supernatant was thrown away and evaporated from the tube in the desiccator, and the dried DNA was dissolved with 200 ml TE solution which contained 10 mM Tris-HCl and 1 mM EDTA with 0.1 mg/ml RNase A(Sigma), and treated at 37°C overnight. Then, 100 ml equilibrated phenol and 100 ml chloroform/ isoamyl alcohol (25:1) were added into the tube, stirred and centrifuged at 15,000 rpm at room temperature for 10 min. The supernatant was transferred to another tube and the same amount of chloroform/ isoamyl alcohol (25:1) was added. The tube was treated with the same process from stirring to centrifugation and its supernatant was transferred to another tube. Twenty μ l of 3 M sodium acetate and 500 μ l of 99.5% ethanol refrigerated at -20°C were added to the isolated supernatant. After shaking, the tube was refrigerated in the freezer at lower than -80°C for 10 min. The frozen tube was centrifuged at 15,000 rpm at 4°C for 10 min, and the supernatant was removed. After washing with 70% ethanol, this tube was centrifuged to remove the supernatant. The dried DNA was dissolved in TE buffer. The mixture was used as total DNA solution for the preparation of 5S rDNA probe.

The 5S rDNA probe was synthesized and amplified by the polymerase chain reaction (PCR). Two primers of 5S rDNA were selected on the basis of sequences of *Pisum sativum* of 5'-CGGTGCATTAATGCTGGTAT-3' and 5'-CCATCAGAACTCCGCAGTTA-3'. The PCR reaction mixture contained 0.2 M dNTP (TAKARA), 0.5 mM each of the primers, 0.05 μ g of total DNA extracted from the plant, and 1.25 units of *Taq* polymerase (TAKARA *Taq*; TAKARA) in 50 ml PCR buffer (TAKARA), and cycles of the PCR were as follows: The first cycle was denaturation at 95°C for 90 sec, annealing at 55°C for 30 sec and primer extension at 75°C for 30 sec; and the second to 30 cycles were denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and primer extension at 75°C for 30 sec. Amplified oligonucleotide was precipitated by ethanol and dried oligonucleotide was dissolved in TE solution.

The *Sau3A* tandem repeat DNA which is commonly present in spruces (Brown *et al.*, 1998) was also amplified by PCR using primers of P_g-r-f: CAAAAGAATAACCCGCAGC and P_g-r-r: TCTGAAGAACAGCAGTGC (Hizume *et al.*, 1999).

The *Arabidopsis* type telomere sequence repeats were synthesized and amplified from the primers of (5'-TTTAGGG-3')₅ and (5'-CCCATT-3')₅ by PCR.

Among approximately 500 plasmid clones tested one inserted with the 278 bp DNA segment was selected and named pCrT7-4 (Hizume *et al.*, 1998). The base sequence of the selected clone was determined by an autosequencer using the dye-primer method.

The pTa71, 5S rDNA, *Sau3A*, *Arabidopsis* type telomere sequence repeats, and pCrT7-4 amplified were labeled by nick translation method using Bionick labeling system (GIBCO BRL). The 45 μ l of labeling mixture contained 5 μ l dNTP solution (GIBCO BRL), 5 μ l enzyme mixture (GIBCO BRL), 1.5 μ g of any one of the pTa71, 5S rDNA, *Sau3A*, the telomere sequence repeats, and pCrT7-4 were reacted for labeling at 16°C for 75 min and were then added 5 μ l of stop buffer (GIBCO BRL) for stopping the enzyme reaction. The mixture 5 μ l of 3 M sodium acetate and 100 μ l of 99.5% ethanol refrigerated at -20°C was added. This mixture was shaken and stored at below -80°C for 15 min. Then, it was centrifuged at 15,000 rpm at 0°C for 30 min, and supernatant was removed. The labeled DNA in centrifugation tube was dried in desiccator, and dissolved with 100 μ l formamide and 100 ml 4XSSC containing 20% dextran sulfate (w/v). After denaturation at 85°C for 10 min and stored in -20°C, the mixture was used in fluorescence *in situ* hybridization as a probe mixture.

2.3 Fluorescence *in situ* Hybridization (FISH)

Fluorescence *in situ* hybridization followed Kokubugata and Kondo (1998): The preparations were

treated by 50 μ l RNase solution containing 0.1 mg/ml RNase A (Sigma) in 2XSSC at 37°C for 1 h. After washing in 2XSSC for 10 min, they were refixed in 4% paraformaldehyde dissolved in PBS at room temperature for 10 min. Then, the preparations were washed in sterilized water and treated with an ethanol series (70%, 100%). After they were air-dried, 10 μ l each of the probe mixtures of one of the predenatured 45S, 5S rDNA, *Sau3A*, *Arabidopsis*-type telomere sequence repeats or pCrT7-4 were applied on the slides, and covered with a Novix. Then, the slides were denatured at 80°C for 10 min and incubated at 37°C overnight in Omnislide (HYBAID). They were washed in 2XSSC and 4XSSC at 37°C for 10 min each. The hybridization probes were detected by avidin-Fluorescein isothiocyanate (avidin-FITC; Boehringer Mannheim) and were counterstained by 1 mg/ml propidium iodide (PI; Sigma). The slides were observed by fluorescence microscope using blue filter (B-2A; Nikon). Microphotographs were taken on Super G400 (FUJI) film. For multicolor FISH, the hybridized probes on chromosomes were detected by anti-digoxigenin antibody conjugated by 10 μ g/ml rhodamine (Boehringer Mannheim) and streptavidine conjugated by 5 μ g/ml Cy 5 (Amersham Pharmacia Biotech) in 4XSSC for 30 min. Then, the preparations were washed in 4XSSC and were mounted with 0.2% DABCO-50% glycerol-PBS containing 0.1 μ g/ml DAPI. The fluorescence images of DAPI, FITC, rhodamine and Cy-5 were detected by cooled CCD camera (Sensys 1400, Photometrics) and captured as a digital image and pseudocolor images were constructed using IP Lab software (Scanalytics) (Hizume *et al.*, 1999).

3. Results and Discussion

During our course of investigations upto present, FISH using the probes of pTa71 visualized the large property and distribution of the 45S rDNA chromosome site in the plant kingdom; in many speceis of chrysanthemums (*e.g.*, Kondo *et al.*, 1996a, b; Honda *et al.*, 1997; Khaung *et al.*, 1997) as well as *Drosera* (*e.g.*, Furuta and Kondo, 1999) in the angiosperm and cycads (Kokubugata and Kondo, 1998; Kondo *et al.*, 1996b; Tagashira and Kondo, 2000) in the gymnosperms, some ferns (Kawakami *et al.*, 1999) and some mosses (Perez and Kondo unpublished). In contrast, FISH using the probes of 5S rDNA, *Sau3A*, *Arabidopsis*-type telomere sequence repeats, and p CrT7-4 visualized their chromosome sites in some gymnosperms such as various cycads (Hizume *et al.*, 1992, 1998; Kondo and Tagashira, 1998; Tagashira and Kondo, 2000) and spruces (Hizume *et al.*, 1999). Our investigations reveal that those specific probes may hybridize certain common sites in chromosomes of almost all plants of the plant kingdom.

Among the three signals studied on plant chromosomes the pTa71 probe gives the largest signal (*e.g.*, Fig. 1). Those pTa71 hybridization signals corresponded to the positive bands of chromomysin A (CMA) and the C- bands (Matsuyama *et al.*, 1996, Galasso *et al.*, 1998). Thus, those sites indicate whether or not the heterochromatic region has large component with 45S rDNA. The pTa71 signals occurred around the centromere in certain chromosomes of *Zamia muricata* (Fig. 1A) and corresponded to the positive C-band and CMA positive band. The signals of *Z. muricata* suggested that the heterochromatin regions of chromosomes consisted of the 45S rDNA region of GC-rich sequences.

Zamia muricata had the 5S rDNA signal on the terminal region of certain median- and terminal-centromeric chromosomes (Fig.1B). The chromosomal locations of these signals implied the chromosome change, centric fission or fusion.

The *Arabidopsis*-type telomere sequence repeats are generally placed on the terminal region of both long and short arms of the chromosomes in most of the plants studied (Fuchs *et al.*, 1995). However, signals of the telomere sequence repeats sometimes appeared on regions other than terminal

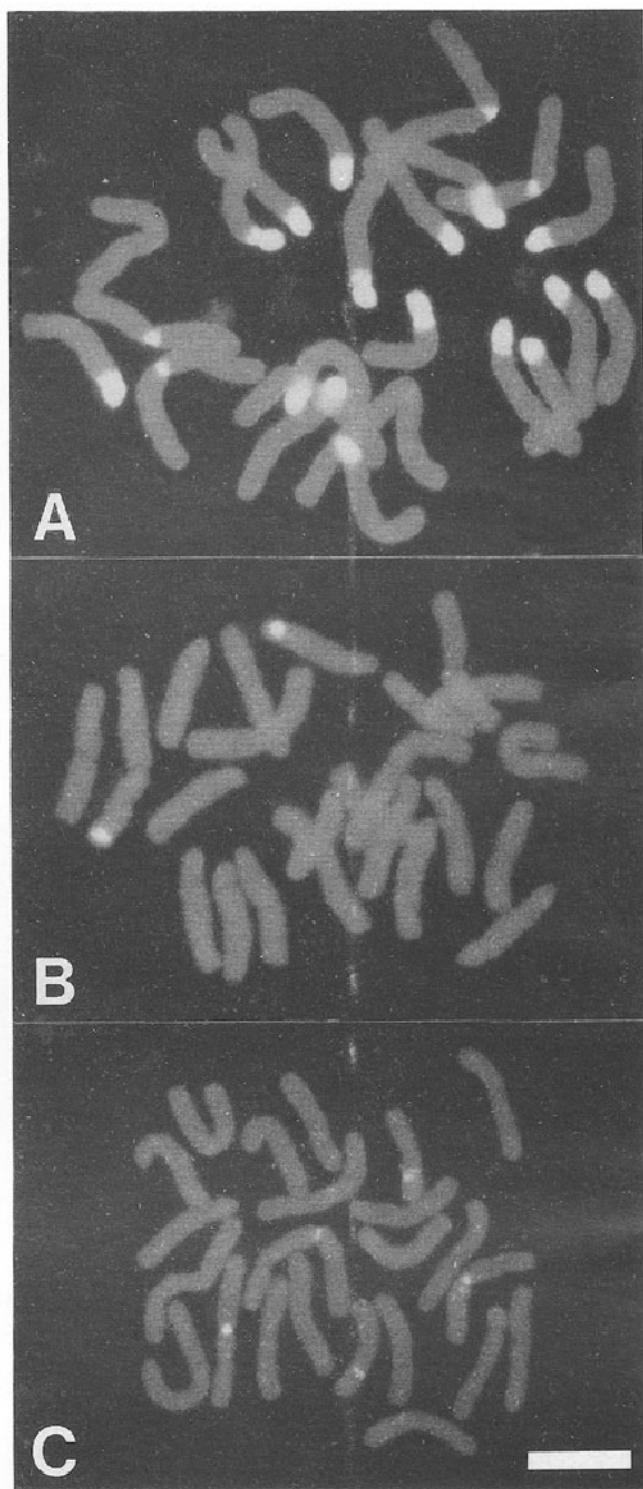


Fig. 1. Karyotypes of *Zamia muricata* male after fluorescence *in situ* hybridization with the probes of the biotinylated pTa71 rDNA, 5S rDNA and *Arabidopsis*-type telomere sequence repeats. The signals were fluoresced by avidin- FITC and the other parts of the chromosomes were counter stained by PI. (A) Large pTa71 signals appeared around the centromere in 20 chromosomes (B) 5S rDNA signals appeared in two heteromorphic chromosomes (C) Non-terminal telomere repeat signals appeared around the centromere in six chromosomes, but the signals in the terminal sites were unclear. Bar: 10 mm.

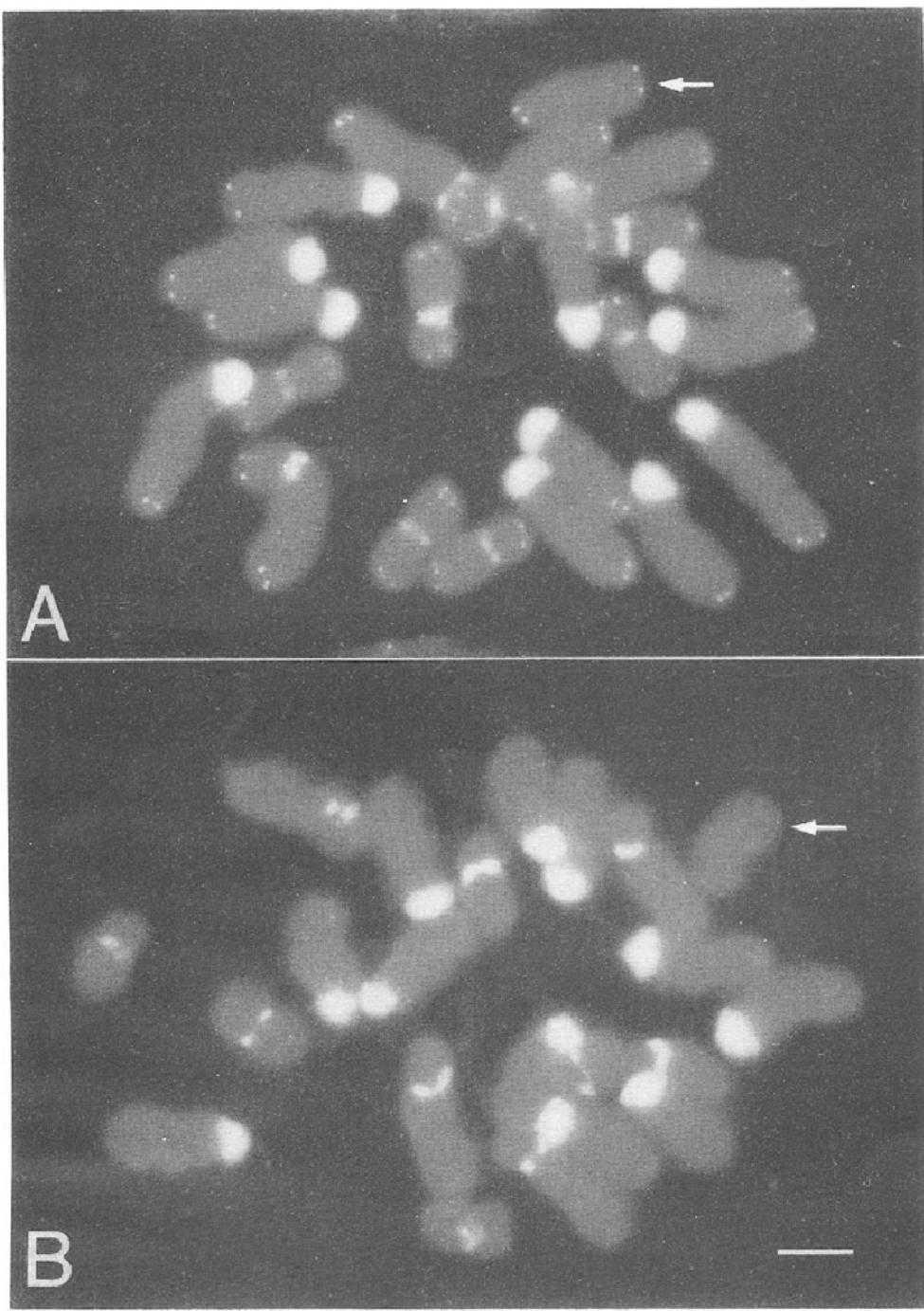


Fig. 2. The chromosome complement of *Cycas revoluta* male after fluorescence *in situ* hybridization with the probes of the *Arabidopsis*-type telomere sequence repeats (A) and pCrT7-4 (B). The signals were fluoresced by avidin-FITC and the other parts of the chromosomes were counterstained by PI, arrows point Y-chromosome. (A) The signal of the *Arabidopsis*-type telomere sequence repeats appeared at the terminal region of all the chromosomes, the proximal heterochromatin of all the terminal centromeric chromosomes, and the centromeric region of all the long, almost subterminal-centromeric and all the short median-and submedian-centromeric chromosomes. (B) The pCrT7-4 signals appeared in the proximal heterochromatin of all terminal-centromeric chromosomes with a few exceptions and at the centromeric region of all the long, almost subterminal-centromeric and all the short median-and submedian-centromeric chromosomes, but none at the terminal region of the chromosomes. Bar: 5 mm. (After Hizume et al., 1998)

regions of chromosomes in cycads; e.g., large, bright signals on non-terminal regions and minute, weak dot-signals on the terminal regions were seen in five chromosomes in *Zamia muricata* (Fig. 1B).

Furthermore, the large, bright signals appeared around the centromere of 21 chromosomes in a male plant and 22 chromosomes in a female plant in *Cycas revoluta* (Fig. 2) and small, weak dot-signals on the terminal region of all the chromosomes (Hizume *et al.*, 1998). Those sequences of the

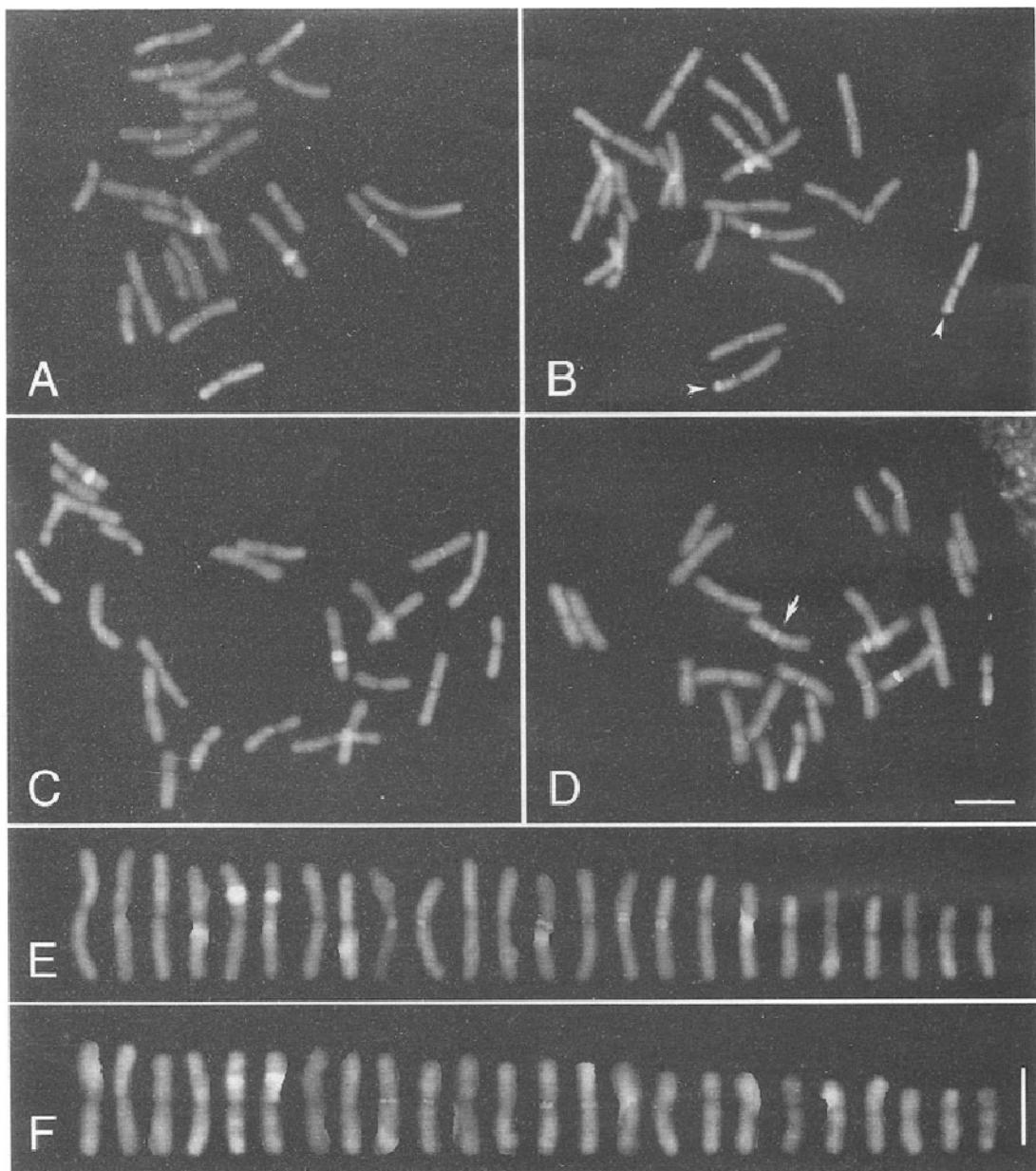


Fig. 3. The chromosome complement and the karyotype of *Picea koraiensis* (A, B, E) and *P. crassifolia* (C, D, F) after pseudomulticolored fluorescence *in situ* hybridization with the probes of 45S rDNA (red signals), 5S rDNA (yellow-green signals) and centromeric Sau3A repetitive DNA (pink signals) amplified and synthesized the fluorescence images by the CCD camera and IP Lab software. Bar: 10 mm. (After Hizume *et al.* 1999)

large, bright signals in *C. revoluta* were determined and named as the pCrT7-4 (Hizume *et al.*, 1998) and were not present at a terminal region of a chromosome in the male plant, which characterized the Y chromosome. Thus, the signal of the *Arabidopsis*-type telomere sequence repeats and the pCrT7-4 may mark and detect restructured chromosomes after chromosome aberrations and species- or individual-specific chromosomes for cytobotany and chromosome phylogeny as well as Y chromosome of *Cycas revoluta* male.

The centromeric *Sau3A* repetitive DNA is specific to spruce chromosomes (Fig. 3) and its signal number in chromosome complement is dependent on species and their frequencies in population constitutions in sympatric areas of more than two spruce species may be analyzed (Hizume *et al.*, 1999). It may also promise phylogenetic relationships among the species of the spruce.

The digital and pseudocolor images may amplify small, weak, or faint signals and synthesize coloration for visualization (Fig. 3) (Hizume *et al.*, 1999).

Thus, combined FISH using the universal and most important probes of the 45S rDNA, 5S rDNA, *Arabidopsis*-type telomere sequence repeats to the higher plants must be very useful to investigate, clarify and justify properties of species and individuals and origins of different chromosome numbers, heteromorphic chromosomes and restructured chromosomes.

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5. *Planococcus citri* (Homoptera, Coccoidea): A Paradigmatic Case of Chromosome Structure as an Epigenetic Way to Control Gene Expression

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Abstract: It is becoming increasingly clear that chromatin structure might play an essential role in regulating gene expression. The elucidation of the different ways through which this type of epigenetic control can be accomplished is therefore of apparent importance. In this context the mealybug *Planococcus citri* represents an excellent experimental model system. Males and females, in fact, start development as diploid from fertilized eggs in which both maternally and paternally derived genomes are euchromatic. However, early in the development in embryos which will develop as males, the haploid set of paternal origin undergoes heterochromatization and remains so and mostly inactive, in most of the tissues. Amongst the factors possibly involved in this event we investigated methylation at CpGs and histone H4 acetylation. Our results suggest that in *P. citri*, most probably underacetylation of histone H4 plays a role in the heterochromatization/silencing of the paternal genome rather than methylation at CpGs.

Key words: Imprinting-heterochromatin

1. Introduction

It is becoming more and more evident that in higher eukaryotes, the regulation of gene expression may be greatly influenced by the local chromosomal environment. One of the best examples is found in female mammals where the inactivation of one of the two X chromosomes required for dosage compensation implies the modification of its cytological features (*e.g.*, the level of condensation). Variations in the chromosomal environment are often described as depending on the different ways in which DNA is packaged into chromatin. In this context, expressed genes are associated to euchromatin, this term indicating a more “open” state of chromatin as compared to the “closed” inactive state known as heterochromatin. Chromatin structure can, therefore, be considered a relevant mechanism of epigenetic control of gene expression. Epigenetic modifications are often the base of the so called “cell memory”, *i.e.* the maintenance of the committed state of gene expression through cell lineages. X inactivation in female mammals, parental imprinting and maintenance of the homeotic genes determinated state, are all examples of cell memory, though it is very probable that they are accomplished in different ways.

Planococcus citri (Homoptera, Coccoidea) is a mealy bug in which both males and females start development as diploid from fertilized eggs where both maternally and paternally derived chromosome complements are euchromatic. However, early in the development in embryos which will develop as

males, the haploid set of chromosomes of paternal origin becomes heterochromatic (Brown and Nelson-Rees, 1961), and maintains the heterochromatic state throughout the cell cycle in most of the tissues. Paternally inherited chromosomes are distinguishable from those maternally inherited until metaphase when all the chromosomes reach the same degree of condensation (Fig. 1a). The heterochromatization implies inactivation: in fact when adult males are irradiated, their male progeny, in which the paternally inherited genome is heterochromatized, show a higher rate of survival as compared to that of female progeny, in which the paternally inherited genome remains euchromatic. Nevertheless, when highly irradiated, the surviving males are sterile thus indicating an incomplete genetic inertia of the heterochromatized chromosomal set (Nelson-Rees, 1962).

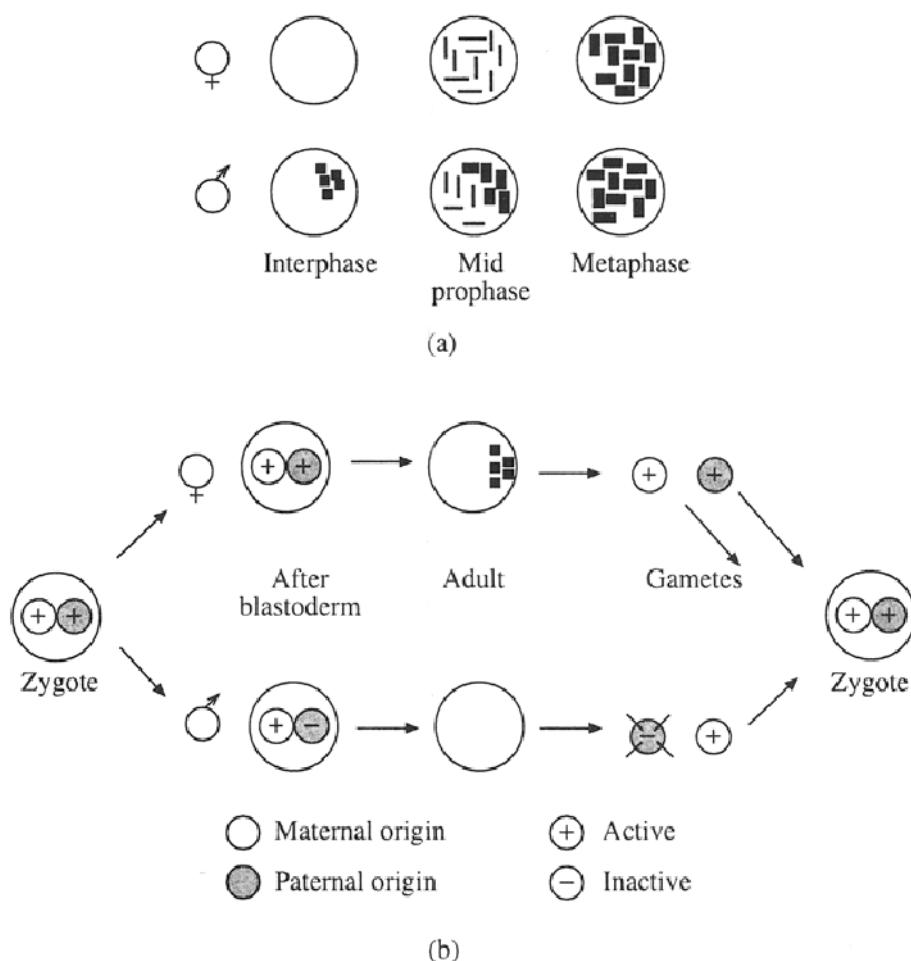


Fig. 1. (a) Different behaviour of condensation shown by maternally and paternally derived chromosomes during cell cycle in female and male cells. (b): Schematic representation of the different fate of the paternally derived chromosome set if passing through male or female germline.

The heterochromatic set does not participate in gamete formation as in males, following an inverted type of meiosis, the heterochromatic and the euchromatic sets of chromosomes segregate to opposite sides of the cell and only the euchromatic derivatives proceed to form sperm (Brown and Nur, 1964). The same haploid chromosome set can, therefore, be active in somatic cells and transmitted to the next generation or inactive and not transmitted depending on the germ line it passed through (Fig. 1b). This is, therefore, a clear example of parental imprinting. We decided to study *Planococcus citri* since, in our opinion, it represents a very good experimental model system to investigate such

fundamental biological phenomena as heterochromatization mechanisms and chromosome imprinting. In fact, in this organism the parental imprinting is mediated by a manifest modification at the chromatin level. An important feature of *Planococcus citri* is that an entire haploid chromosome set undergoes a phenomenon at the same time, which is similar to that found in a single chromosome in female mammals.

In spite of this and of the fact that mealybugs are very harmful insects which exist all over the world with more than 2000 species, they have nevertheless received little attention until recently. This article summarizes and discusses our findings at the different levels at which we have studied this organism.

2. Cytogenetic Characterization

Mealybugs have been very poorly studied by modern cytogenetic techniques possibly due to their very small and holocentric chromosomes. No chromosome banding of any species of Coccids nor cytotoxicological criteria to distinguish between closely related species has ever been done previously. A careful, extensive cytogenetic characterization has, therefore, been considered a necessary, preliminary step of our investigations on *Planococcus citri* ($2n = 10$).

Starting from ovaries removed from gravid females, which contain both male and female embryos, chromosome spreads were obtained both by air drying according to a modification of the technique described by Odierna *et al.* (1993) for *Encarsia berlesei* or by squashing. The air drying technique proved to be particularly useful since, for the first time, it was possible to obtain metaphase spreads suitable for chromosome banding. Both AT-specific (H33258, DAPI, D287/170) and GC-specific (CMA3) fluorochromes were then used to characterize DNA base composition variations along *P. citri* chromosomes. The best results were given by D287/170, a compound derived from a modification of DAPI, having affinity for AT-rich DNA as suggested by the Q-banding pattern produced on mouse chromosomes, and also sequence specificity when used at high concentration (Schneid et al., 1981). Through this fluorochrome, it was possible to get a banded karyotype which allowed the unequivocal identification of each pair of homologous chromosomes (Fig. 2) (Ferraro *et al.*, 1998).

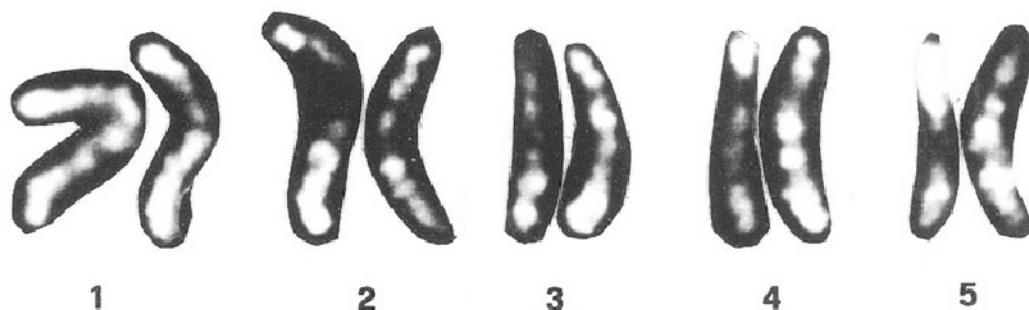


Fig. 2. Karyotype of one metaphase cell after staining with D287/170. Note that in each chromosome pair one of the two homologs shows a higher fluorescence intensity, particularly in more elongated chromosomes; the two members of each pair of homologs show different degrees of condensation.

At metaphase, in male as well as female cells, all the chromosomes reach the same degree of condensation, nevertheless a careful analysis of several karyotypes showed that homologous chromosomes consistently show different fluorescence intensities. This fact could possibly reflect a different structural organization due to differences in the level of genetic activity between the two homologs. Differences of this kind are well known as the way to achieve dosage compensation in different organisms (Latt, 1973; Kerem *et al.*, 1983).

Several authors in the past suggested, by indirect observations, the possibility of some residual activity in the inactivated chromosome set (Nelson-Rees, 1962), however, no further investigations have subsequently been attempted.

Our characterization of *P. citri* included the localization of ribosomal genes (Fig. 3) that was obtained by fluorescent *in situ* hybridization of a 28S rDNA gene of *Drosophila melanogaster* (Lohe and Roberts, 1990), which crosshybridized in Southern hybridization with *P. citri* DNA. What is more, since it is known that rDNA genes can function in a heterochromatic background, we investigated the possibility that rDNA genes of the heterochromatic set of male cells could be active. Silver staining is specific for active ribosomal genes in vertebrates, plants and several species of insects (Sumner, 1990). Using this technique we found that in male cells positive silver staining was present both on euchromatic and heterochromatic chromosomes, therefore demonstrating for the first time that in the heterochromatic set of male cells at least some genes, namely the ribosomal genes, are active (Fig. 4) (Ferraro *et al.*, 1998).

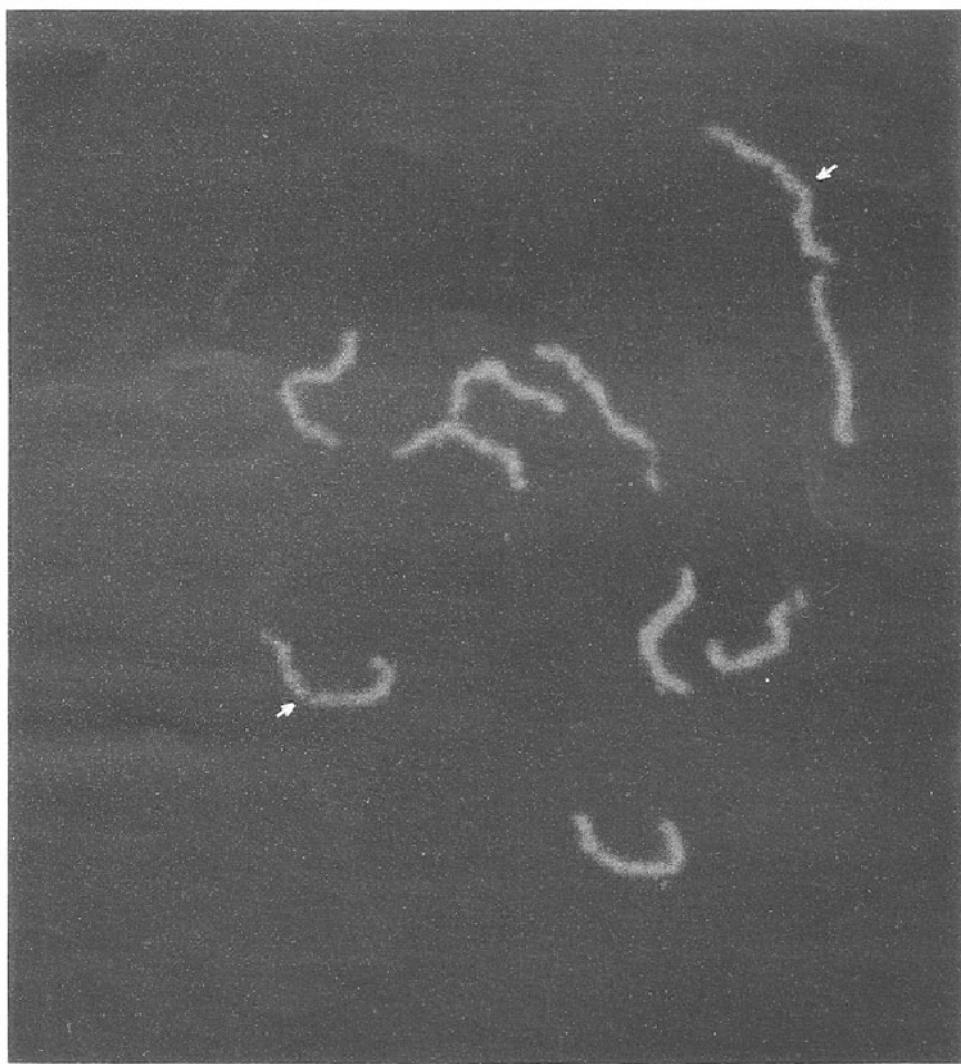


Fig. 3. Localization of 28S rDNA by fluorescence *in situ* hybridization. The two fluorescent spots at the more median position (arrows) and two of those more distally located are always present. Six hybridization spots, as seen in the figure, are only seldom visible. Note that the hybridization spots are more often seen as a single lateral signal.

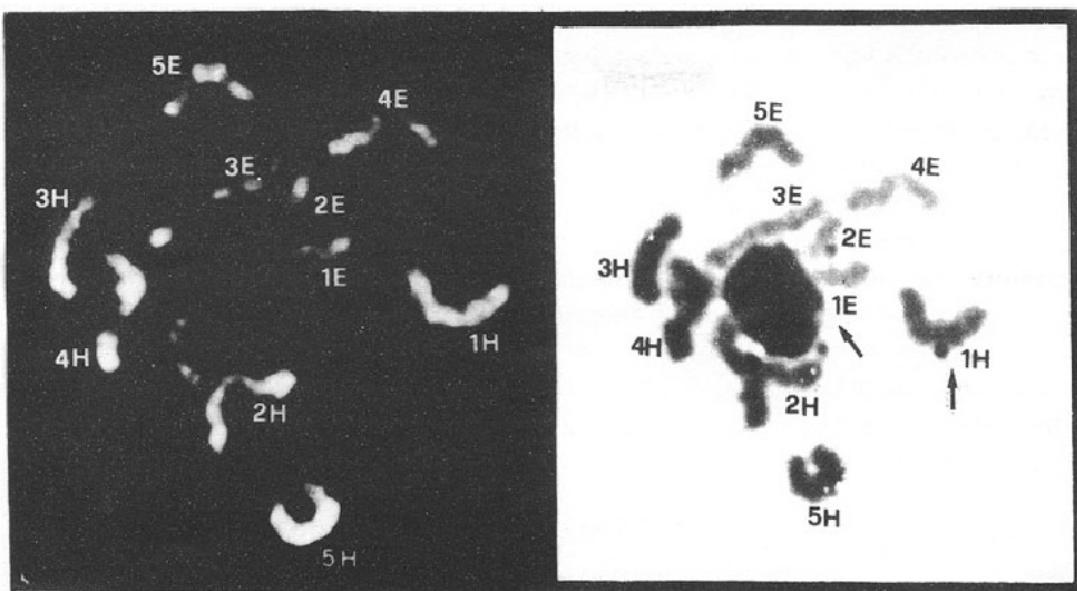


Fig. 4. Demonstration of ribosomal gene activity in the heterochromatic chromosome set. A male metaphase sequentially stained with D287/170 and silver where for each chromosome pair it is possible to distinguish between the eu-and the hetero-chromatic homologs. A silver spot is lying on one heterochromatic chromosome (1H, arrow) while its euchromatic homolog (1E), is also stained by silver and still associated with chromosome 2E.

3. Studies on CpG methylation and histone H4 acetylation as epigenetic factors involved in the maintenance of a different state of activity in the paternal and maternal chromosome sets. Heterochromatin indicates the presence of a structure determining the silencing of a particular region, but for this silencing to be considered as an epigenetic modification this same region must have the possibility to be in the active state also, *i.e.* euchromatic.

Moreover, for an epigenetic modification to be considered as involved in cell memory at least two requirements must be fulfilled, namely heritability and reversibility. This means that any modification, both at DNA or protein levels, involved in the determination of a particular functional pattern, once established, has to be maintained throughout the cell cycle. Nevertheless, the possibility of elimination of the pattern established must exist.

With regard to several features, both at cytological (*e.g.*, condensation cycle, replication time) and biochemical (*e.g.*, histone acetylation, CpG methylation) levels, the two different states of chromatin, eu- and heterochromatin show opposite patterns.

In Eukaryotes, DNA methylation at cytosine residues in the dinucleotide CpG has often been shown to be among the epigenetic mechanisms playing a role both in the establishment and/or the maintenance of that particular example of cell memory represented by parental imprinting (Razin and Cedar, 1994). This term usually indicates the parent-of-origin-dependent silencing of autosomal genes, the transcriptional repression being mediated, at least in mammals, through the interaction of methylated DNA with specific proteins (Meehan *et al.*, 1989). The methylated state at particular CpGs is maintained because of the existence of a system of enzymes acting on hemimethylated DNA, but is fully reversible by a demethylase.

Nonetheless, DNA methylation has evolved relatively recently and is not found in lower eukaryotes where an easy, alternative way to store cell memory might well be provided by the protein components themselves of chromatin. Moreover, the DNA of organisms like *Drosophila* and yeast, where the

existence of epigenetic control of gene expression has been demonstrated, lacks cytosine methylation. In these organisms it has also been shown that transcription in many genes is regulated by histone acetylation (Braunstein *et al.*, 1993; Struhl, 1998).

Histone acetylation is found throughout eukaryotes, from unicellular organisms to the most complex higher plants and animals (Jeppesen, 1997). It also meets both the requirements of being reversible and maintained through cell division, it can, therefore, be regarded as representing a good alternative epigenetic mechanism for propagating cell memory.

In addition, since these two different types of modifications mark the two different (active/inactive) states of chromatin, *P. citri* male cells, where the 10 chromosomes that make the genome 5 each are euchromatic and heterochromatic, these can be considered as particularly favourable to investigate both mechanisms. Therefore, our analysis of factors potentially involved in the imprinting of paternal chromosome set in *P. citri* males started just from the study of the methylation state at CpG dinucleotide and of the acetylation state of histone H4.

The methylation studies have been carried out in only four species of Coccids. The results obtained even for the same species were different. (Loewus and Brown, 1964; Achwal *et al.*, 1983; Scarbrough *et al.*, 1984). We investigated the possible presence of cytosine methylation in *P. citri* both at DNA and chromosome levels by using the two isoschizomeres HpaII and MspI currently used for this purpose since they have the same recognition sequence (CCGG), but HpaII however, fails to cut the sequence when the internal cytosine is methylated, whereas MspI is insensitive to its methylation state. After digestion, male and female genomic DNAs were analyzed and compared both on a standard 1% agarose gel and after end-labelling with $\alpha^{32}\text{P}$ in order to check the possible presence of a heterogeneous fraction that, when amounts to less than 5% of DNA, is not detectable by staining with ethidium bromide (Cooper *et al.*, 1983). In both cases, no differences between sexes were observed (Fig. 5) (Buglia *et al.*, 1999).

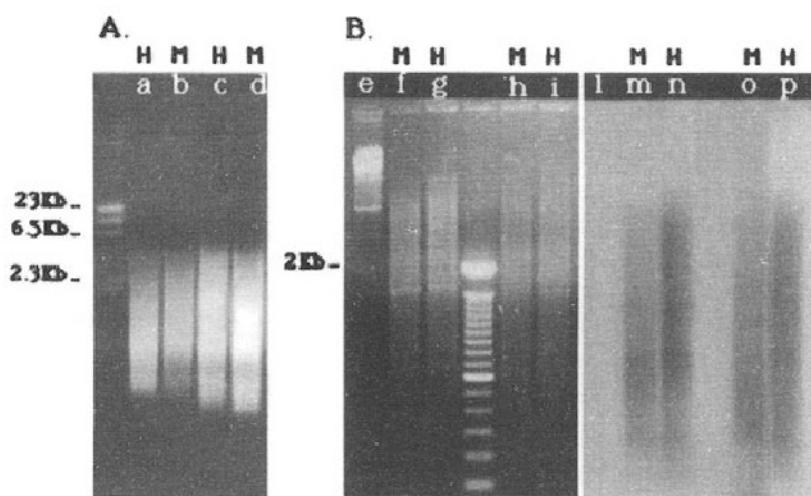


Fig. 5. Analysis of cytosine methylation level of *P. citri* genomic DNA. H = HpaII, M = MspI. **A:** ethidium bromide staining: lanes (a) and (b) = male DNA, lanes (c) and (d) = female DNA. **B:** lanes (e) to (i) = agarose gel of end-labelled DNA stained with ethidium bromide; lanes (l) to (p) = autoradiograph of the same gel. Samples (e) and (l) = undigested DNA, (f) (g) and (m) (n) = female DNA, (h) (i) and (o) (p) = male DNA.

Metaphase chromosome preparations were analyzed by fluorescence *in situ* nick-translation performed after exposing them to HpaII or MspI, fluorescence signals being indicative of the chromosome

regions that were previously nicked by the enzyme used. We chose to analyze metaphase spreads though at this cell stage the chromosomes of different parental origin can not be recognized, all of them having reached the same degree of compaction, for the following two reasons. One is the well known problem of the accessibility of endonucleases to highly condensed chromatin (Gosalves *et al.*, 1989; de la Torre *et al.*, 1991) as it could happen for the heterochromatic paternal chromosome set in interphase male cells. The second reason is the difficulty for these cells of a careful evaluation of staining intensity of chromatin regions with such a different amount of stainable material per unit length. Finally, it can be well assumed that if different cytosine methylation levels between the two chromosome sets are indeed present, they should have been present in metaphase chromosomes also when the chromosomes, having the most comparable condensation and accessibility, can be more correctly compared.

Different patterns of fluorescence (*i.e.*, nicking) have never been observed as would be expected if different overall methylation levels were present in the chromosome sets of paternal and maternal origin. All the chromosomes of a metaphase were invariably cut to a similar extent both by HpaII and MspI (Fig. 6) (Buglia *et al.*, 1999) suggesting a similar distribution of methylated/unmethylated sites along the chromosomes of paternal and maternal origin. From our data *Planococcus citri* methylation of cytosine residues does not seem to be involved in the heterochromatization through which the silencing of the genome of paternal origin is accomplished.

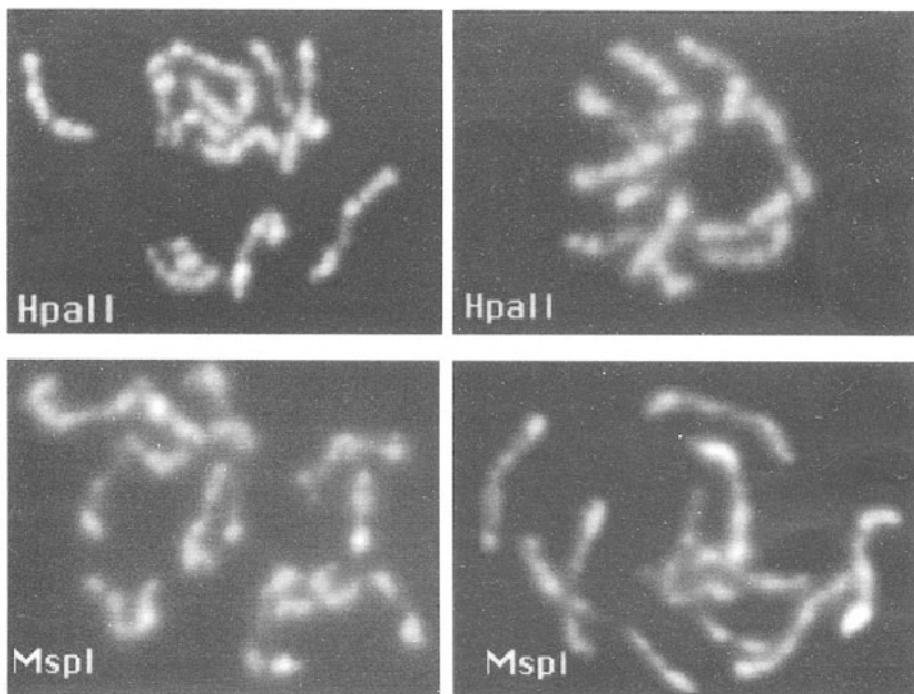


Fig. 6. **In situ** nick translation. Examples of two metaphase spreads after HpaII or MspI digestion. In both metaphases, the chromosomes are cut at a quite similar extent.

These results seem to contradict those obtained by Bongiorni *et al.* (1999).

As already mentioned, organisms exist in which an epigenetic control of gene expression has been demonstrated though their DNA lacks cytosine methylation. The way in which an epigenetic type of control is achieved seems to be, at least in some cases, a differential level of acetylation/deacetylation at the four acetylatable lysine positions (K5, K8, K12 and K16) of histone H4 in active and inactive

chromatin (Clark et al., 1993; O'Neill and Turner, 1995). Actually, histones undergo a number of post-translational modifications that might influence the higher-order chromatin packaging. Since the discovery of histone acetylation more than three decades ago (Allfrey *et al.*, 1964) numerous studies have correlatively linked histone H4 acetylation to transcriptionally active chromatin. Moreover, we considered this could well be the possible level of epigenetic control in *Planococcus citri* male cells since modification of histones by acetylation is regarded as a suitable way of modulating the activity of relatively large chromatin domains (Ashraf and Tony Ip, 1998).

As a preliminary account of this investigation, we report a few significant results to be further discussed in a forth coming paper. (Ferraro *et al.*, 2001). The analysis was carried out by us by indirect immunofluorescence using rabbit antisera to acetylated histone H4 on cell preparations obtained by standard squashing. The results very clearly show that in female cells the fluorescence signals were always evenly distributed all over the chromatin, while in male cells the heterochromatic paternal genome was in most cases devoid of fluorescence signals. This can be quite easily seen in interphase cells where the heterochromatic chromosomes are strictly associated to form a chromocenter-like structure, but is clearly visible also in more advanced cell cycle stages where the heterochromatic chromosomes are not clustered any more. On metaphase spreads where the differentially derived chromosomes are recognizable the fluorescence signals though very clear on euchromatic chromosomes are never located on heterochromatic ones. The complete analysis and the detailed results on this subject are collected in a specific paper to be published.

As a concluding remark, it may be stated that it is important to investigate the modifications of chromosome structure by which gene expression could be influenced at all different levels. To this regard our results suggest that, in *Planococcus citri*, in the silencing of the paternal genome most probably underacetylation of histone H4 plays a role rather than methylation at CpG.

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6. Cytogenetic and Molecular Analysis of Heterochromatic Areas in the Holocentric Chromosomes of Different Aphid Species

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Abstract: In this paper we review recent work concerning both cytogenetic and molecular features of constitutive heterochromatin in the holocentric chromosomes of different aphid species. Sections include an extensive survey regarding chromosomal localization and DNA base composition of C-positive areas together with a cytological and molecular analysis of nucleolar organizer regions. The possible role of mitotic crossing over on genetic variability in parthenogenetic lineages is also briefly reviewed.

Key words: Aphid, holocentric chromosomes, heterochromatin, satellite DNA, NORs, heteromorphism.

1. Introduction

Classic and molecular cytogenetics provide an integrated approach for a structural, functional and evolutionary analysis of chromosomes. This area of research ranges from karyotype analysis to molecular mapping of chromosomes. To date almost all the studies concerning chromatin structure and organization have concentrated upon those eukaryotes having monocentric chromosomes, whereas other eukaryotes possessing holocentric/ holokinetic chromosomes have been neglected. Chromosomes with diffused centromeric activity have been found in protista as well as in plant and animal species (Wrensch *et al.*, 1994). This particular type of chromatin organization has been described in almost all the eukaryotic taxa examined so far, with the exception of echinoderms and chordata. In an evolutionistic context, it has been observed that the holocentric condition is present in each principal knot of the eukaryotic phylogenetic tree. This discontinuous distribution of monocentrism and holocentrism in groups of protista, animals and plants highlights the problem of the ancestrality of the holocentric condition in respect of the condition of monocentrism (for a review see Wrensch *et al.*, 1994 and references within).

Aphids, because of the easiness with which mitotic chromosomes can be obtained from embryonic tissues, represent a useful model for a better understanding of the architecture of holocentric chromosomes, in order to work out the differences or similarities to the monocentric chromosomes. Moreover, the identification of chromosomal markers in organisms with holocentric chromosomes is extremely important, since the lack of a primary constriction, together with the difficulty in obtaining a clear-cut banding pattern, have greatly hampered cytogenetic studies in organisms possessing such a peculiar chromatin organization (Blackman 1987; Gautam *et al.*, 1993; Hales *et al.*, 1997). In this

connection, it must be emphasised that the structure of holocentric-holokinetic chromosomes allows for karyotype rearrangements, as all fragments produced by X-ray irradiation, are conserved in a chromosomal set (Khuda-Bukhsh and Datta 1981; Kuznetsova and Sapunov 1987). This may explain the high frequency of chromosomal polymorphism known for many aphid species (Blackman, 1978, 1980, 1990; Khuda-Bukhsh and Kar, 1990). Besides the above mentioned theoretical reasons, there are also economic aspects which justify studies of aphid cytogenetics. Aphids are, in fact, sap sucking insects and many species are serious crop pests, not only in view of their feeding action, but also because they can be important virus vectors in many crops. A wide knowledge of aphid genome organization could furnish information useful in the layout of strategies aimed to reduce their impact on plants of agricultural interest.

2. C-banding

The C-banding technique was first applied to aphid chromosomes by Blackman (1976, 1980, 1990) in order to develop cytogenetic markers which could be useful for the taxonomic identification of aphids, as well as for the analysis of karyotype evolution.

From the beginning of the nineties, an extensive survey, concerning both cytogenetic and molecular features of holocentric chromosomes in several aphid species, has been carried out in our laboratory (Manicardi *et al.*, 1991, 1992, 1994, 1996, 1998a,b,c; Manicardi and Gautam, 1994; Bizzaro *et al.*, 1996; Bizzaro *et al.*, 1999; Galli and Manicardi, 1998; Mandrioli *et al.*, 1999a, b, c, d). The most interesting results have been obtained with the C-banding technique which has allowed us to demonstrate that constitutive heterochromatin is not equilibrated on each chromosome, but C-positive areas are interspersed among euchromatic zones (Fig. 1a-f). These results substantially differ from those observed in monocentric chromosomes, in which the heterochromatic regions typically occupy specific zones of all chromosomes, corresponding to centromeres and sometimes telomeres (Schweizer and Loidl, 1987). C-banding has rarely been used up to now in organisms possessing holocentric chromosomes. In some cases the attempts to localize constitutive heterochromatin were unsuccessful (Collet and Westerman, 1984), whereas a telomeric localization of the C-bands has been described in different Heteropteran species (Muramoto, 1976; Solari, 1979; Camacho *et al.*, 1985; Papeschi, 1988; Kuznetsova *et al.*, 1997).

An interspersed distribution of heterochromatin amongst the euchromatin, similar to that observed in aphids, has been reported for holocentric chromosomes in plant species belonging to the genus *Luzula* (Ray and Venketeswaran, 1978; Collet and Westerman, 1984).

A preferential and in some cases exclusive localization of heterochromatin on X chromosome (Fig. 1) is another characteristic repeatedly described in the holocentric chromosomes of aphid species (Manicardi *et al.*, 1991, 1992, 1996; Kuznetsova *et al.*, 1993; Mandrioli *et al.* 1999b, d; Bizzaro *et al.* 1999). In 1987, Vig presented evidence suggesting that the size of the heterochromatic blocks may influence the order of separation of sister chromatids in different chromosomes at anaphase. This observation fits the hypothesis suggesting that the large heterochromatic blocks occurring on X chromosomes, described in different aphid species, could be implicated in the delay of X chromosomes separation occurring during the maturation of parthenogenetic oocytes, which is considered to be the basis of male sex determination (Orlando, 1974; Blackman, 1987).

In at least two species (*Megoura viciae* and *Schizaphis graminum*) C-banding pattern evidenced that the number of heterochromatic bands on each chromosome pair is not constant, but is strongly connected to the degree of chromosome condensation (Manicardi *et al.*, 1991, 1996; Mandrioli *et al.* 1999d). Decondensed pro-metaphase chromosomes showed in fact a large number of heterochromatic

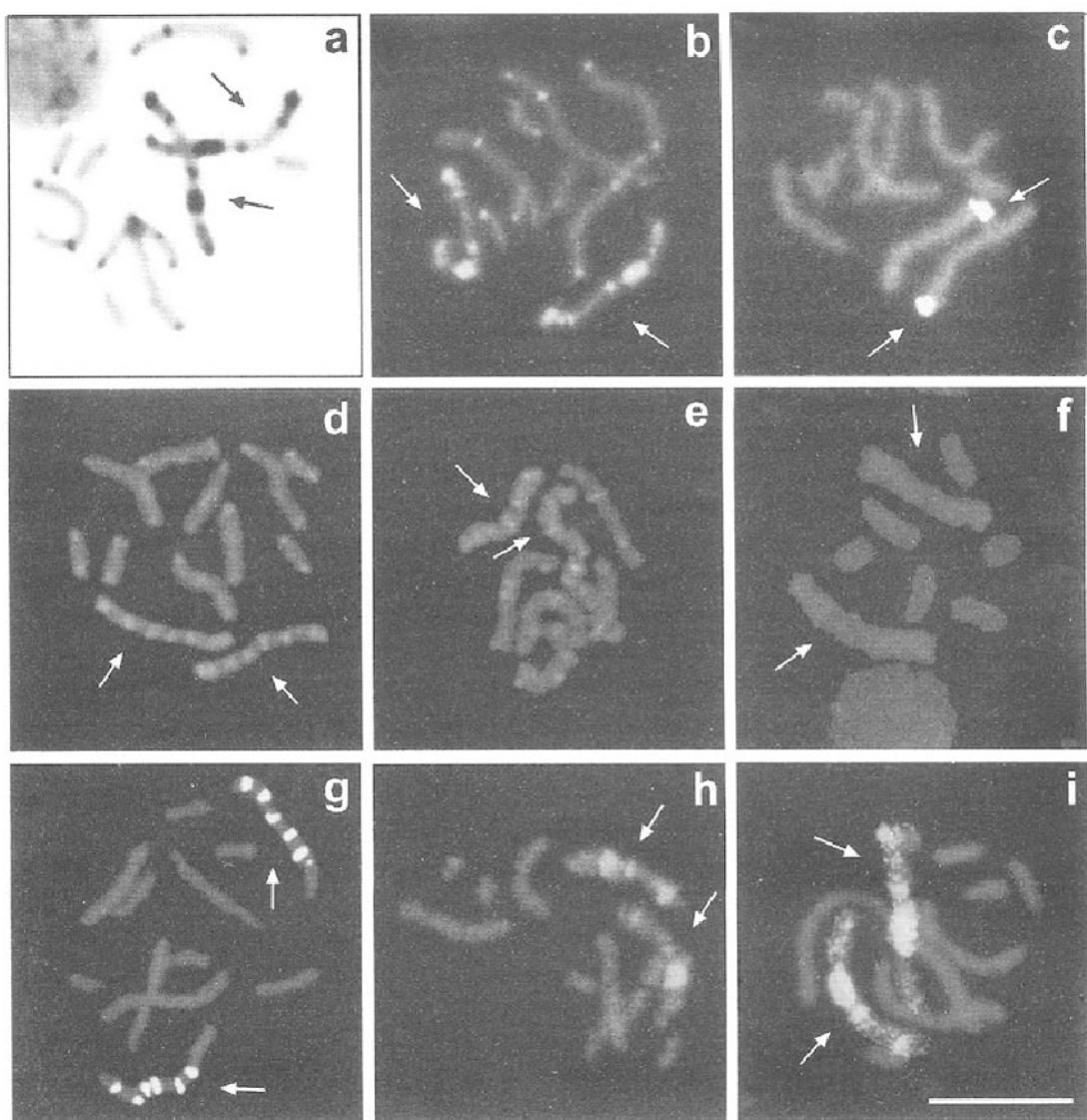


Fig. 1. Constitutive heterochromatin in the mitotic chromosomes of *M. viciae* (a-c), *M. persicae* (d), *S. graminum* (e) and *A. sambuci* (f) revealed by C-banding followed by Giemsa (a), CMA₃ (b) and DAPI staining (c-f). The localization of the EcoRI satellite on *M. viciae* X chromosomes by FISH and in situ nick translation is represented in (g) and (h) respectively whereas the localization of HindIII satellite on *M. persicae* X chromosomes is shown in (i). Note that both in *M. viciae* and in *M. persicae*, the localization of satDNAs overlaps heterochromatic blocks. Arrows indicate X chromosomes. Bar corresponds to 10 µm.

bands which fuse together during chromosome condensation and give rise to a smaller number of large C-bands at metaphase. This behaviour was particularly evident in X chromosomes since they generally have the higher heterochromatic content. This peculiar feature, already described both in animals and plants with holocentric chromosomes (Ray and Venkateswaran, 1978; Collet and Westerman, 1984), fits the hypothesis that in holocentric chromosomes, heterochromatic areas are widely scattered along the chromosomal axis, in contrast to the high heterochromatin concentration near the centromere found in monocentric chromosomes (Lagowsky *et al.*, 1973; Collet and Westerman, 1984; Goday and Pimpinelli, 1984).

C-banding and fluorochrome staining, used to analyse the DNA base composition of constitutive heterochromatin in *M. viciae* mitotic chromosomes (Fig.1b, c) have evidenced that heterochromatic blocks possess AT-rich DNA, whereas only the C-band located on one telomere of the X chromosome contains CMA₃ positive, GC rich DNA (Manicardi *et al.*, 1996). The different response to CMA₃ and DAPI staining after C-banding points out the heterogeneity of heterochromatic DNA composition which seems to be a general characteristic of aphid chromatin since it has been described in all species so far investigated cytogenetically (Gautam and Manicardi, 1994; Manicardi *et al.*, 1998b; Galli and Manicardi, 1998; Bizzaro *et al.* 1999; Mandrioli *et al.* 1999b, d).

3. Satellite DNAs

Since C-bands are generally composed of highly repeated sequences, we have extended our analysis of aphid heterochromatin by searching for satellite DNAs (sat DNAs). In this connection we have carried out enzymatic digestions and electrophoretic analyses of genomic DNA extracted from different aphid species, in order to identify highly repeated DNA fractions. This experimental approach allowed us to identify one sat DNA in *Myzus persicae* (Mandrioli *et al.*, 1999 b), two in *Megoura viciae* (Bizzaro *et al.*, 1996, 1997) and four in *Aphis sambuci* (Bizzaro *et al.*, submitted). Cloned fragments used as hybridization probes after Southern blotting revealed a regular ladder of bands composed of multimers of basic length, which are characteristic for sat DNAs for all repeated sequences isolated from aphid genome. The chromosomal localization of these satellites, investigated by fluorescent *in situ* hybridization (FISH), constantly showed fluorescent bands located on the X chromosome, all corresponding to C-positive heterochromatic areas (Fig.1g, i). Moreover, a 169bp tandem repeat DNA marker for subtelomeric heterochromatin has been described in aphids of the *M. persicae* group by Spence *et al.* (1998). These data as a whole, are in agreement with those which have been repeatedly described in monocentric chromosomes (John and Miklos, 1979; Miklos, 1985; John, 1988) and suggest that in the holocentric chromosomes of aphids also, sat DNAs are localized in heterochromatic areas and, therefore, constitutive heterochromatin is principally made of sat DNAs.

Data obtained by *in situ* nick translation experiments carried out after digestion with restriction endonucleases of aphid chromosomes (Fig. 1h) are in full accordance with FISH experiments (Bizzaro *et al.* 1996; Mandrioli *et al.* 1999 b). Moreover, nick translation experiments carried out after restriction enzyme digestion (RE/NT) demonstrate that, although the restriction enzyme targets are clustered in heterochromatic zones, they are still fully accessible to *in situ* enzyme attack. The high concentration of restriction sites in aphid heterochromatin is highlighted if one considers that RE/NT experiments showed the best results after only a few minutes of digestion. For longer times of treatment, restriction enzyme digestions overcome polymerase I reconstruction so that the DNA occurring in the main intercalary heterochromatic band is fully cut and extracted and this area is not labelled (Bizzaro *et al.*, 1996). Similar results have been reported after DNaseI/NT experiments on both human and frog chromosomes (de La Torre *et al.*, 1992; Herrero *et al.*, 1995). The high sensitivity of aphid heterochromatin to *in situ* enzyme attack has been previously evidenced by treating *M. viciae* chromosomes with different REs (Manicardi *et al.*, 1996). These results partially contradict the data obtained in monocentric chromosomes, in which it has been demonstrated that *in situ* endonuclease digestion is affected not only by the nucleotide sequence, but also by chromatin compactness that may limit the enzyme accessibility to specific targets (Burkholder, 1989; Gosalvez *et al.*, 1989; Sumner *et al.*, 1990; Mezzanotte *et al.*, 1985, 1992). In insects, for example, the heterochromatic DNA of *Tenebrio obscurus* is extensively digested *in situ* only after incubation with proteinase K, indicating that accessibility of REs to DNA is limited by DNA-protein interactions (Ugarkovic *et al.*, 1994).

Molecular analysis of aphid sat DNAs isolated till now, shows that their consensus sequences are variable in length from 160 to 650 bp (Bizzaro *et al.*, 1997; Spence *et al.*, 1998; Mandrioli *et al.*, 1999b). None of the sequenced aphid sat DNAs revealed significant direct or inverted repeats. The analysis of the similarity-plot shows that *Hind*III repeat sequences, isolated from *M. persicae* genome, are particularly conservative in the first 50 bp (Mandrioli *et al.*, 1999 b). This fact suggests that the first portion of this repeat may be functionally important. Moreover the curvature-propensity plot suggests that the region between nucleotides 60 and 80 may adopt a curved conformation. Additionally, all the sat DNAs of aphids display short stretches of adenines and thymines which, in other organisms, have been found associated with sequence-induced DNA curvature able to link peculiar heterochromatic proteins (Koo *et al.*, 1986; Martinez-Balbas *et al.*, 1990) A search for homology with other DNA sequences within GenBank and EMBL databases yielded no significant results.

Sat DNAs found in aphids display other features typical of sat DNAs isolated in organisms possessing monocentric chromosomes such as the overlapping with heterochromatic bands, a high A + T content (from 63 to 79%) and a high degree of sequence similarity between monomers (from 84 to 94%). Nucleotide substitutions are the most frequent mutations in all these satellites, whereas insertions and deletions are rare. This high sequence homology could be due either to a strong functional constriction, or to a process of "concerted evolution" determined by molecular drive (Dover *et al.*, 1982; Dover and Tautz, 1986). On the other hand, low levels of sequence similarity (constantly lower than 50%) has been found when comparing sequences belonging to different sat DNA. This difference is also present when comparing the four sat DNA sequences all isolated from *A. sambuci* genome. The species-specificity of sat DNA found in aphids supports the view of their recent origin. Probably the distinct lineages of these sat DNAs have been formed by genomic evolution after the species diverged from their common ancestor.

4. rDNA genes

18S, 5.8S and 28S rDNA genes are arranged in aphids as tandemly repeated clusters located at one telomeric region of each X chromosome (Fig. 2). Exceptions include the interstitial position of rDNA genes in *Amphorophora idaei* (Fenton *et al.*, 1994), and the autosomal location of NORs in *Schoutedenia lutea* (Hales, 1989). Ribosomal genes have been located on aphid chromosomes using various techniques such as silver staining (Blackman and Hales, 1986; Hales, 1989; Sen and Khuda-Bukhsh, 1992; Manicardi *et al.*, 1992, 1996, 1998c; Kuznetsova *et al.*, 1993; Galli and Manicardi, 1998; Mandrioli *et al.* 1999 b,d; Bizzaro *et al.* 1999), staining with fluorochrome CMA₃ (Manicardi *et al.*, 1992, 1996, 1998c; Galli and Manicardi, 1998; Mandrioli *et al.* 1999 b; Bizzaro *et al.* 1999), and *in situ* hybridization with rDNA probes (Fenton *et al.*, 1994; Blackman and Spence, 1996; Manicardi *et al.*, 1998c; Mandrioli *et al.* 1999 b; Bizzaro *et al.* 1999).

A certain amount of heterogeneity between homologous NORs has been observed with all the techniques applied (Manicardi *et al.*, 1998c; Mandrioli *et al.*, 1999a, b). The overlapping of results obtained after silver staining, which is a marker for ribosomal gene activity (Roussel and Hernandez-Verdum, 1994) together with results obtained after both CMA₃ and FISH, which are only influenced by the number of rDNA genes occurring at the NORs, allow us to suggest that the above mentioned heteromorphism is due to the occurrence of a variable number of ribosomal genes, all clustered at the two X telomeres. Different mechanisms can be invoked to explain DNA expansion and contraction, in particular unequal crossing-over and unequal sister chromatid exchanges (SCE). The presence of X chromosome association repeatedly observed in metaphase chromosome plates of somatic cells

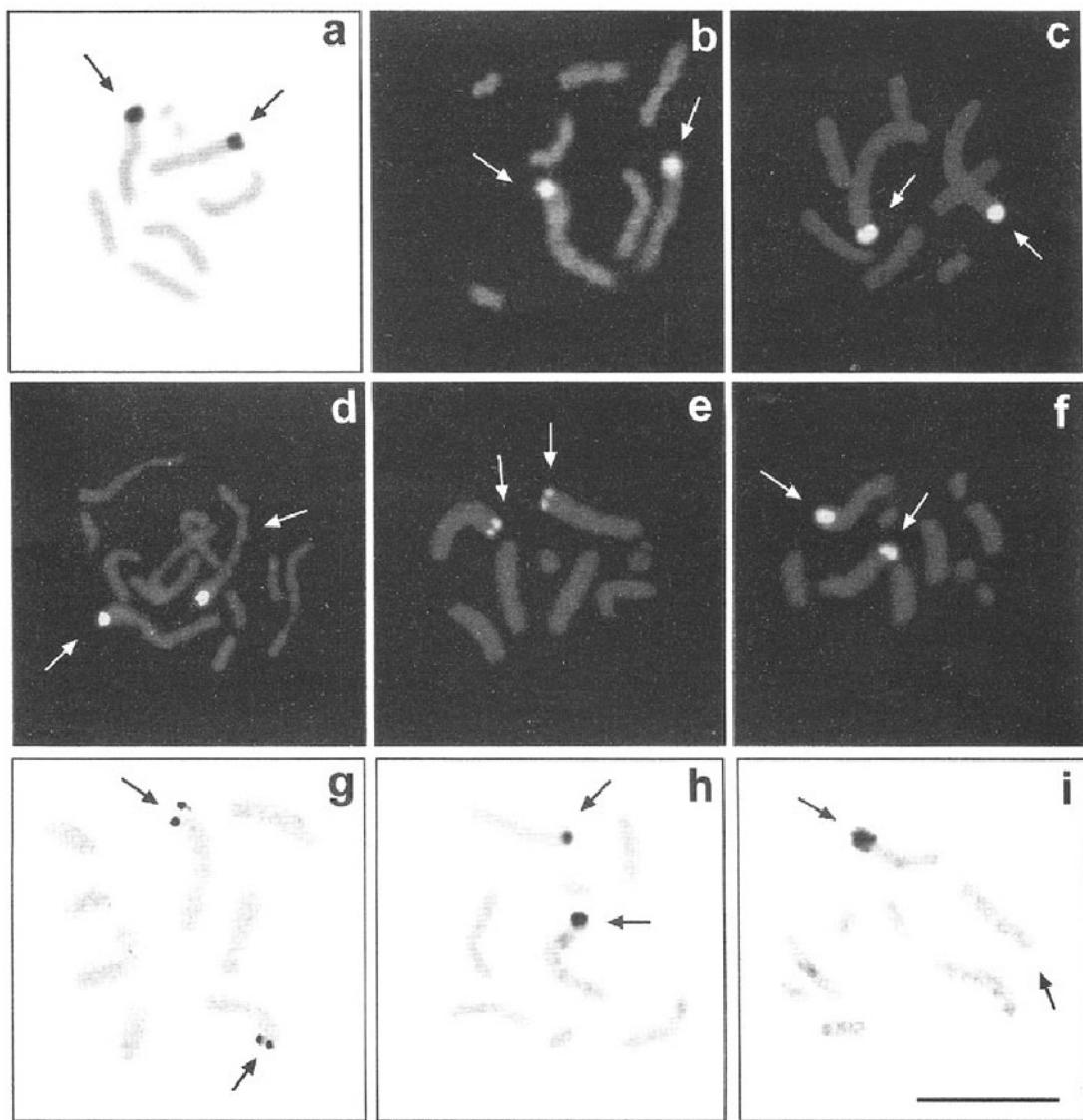


Fig. 2. Localization of rDNA genes in *Acyrthosiphon pisum* (a-c, g-i), *M. persicae* (d), *S. graminum* (e) and *M. viciae* (f) as revealed by silver nitrate (a, g-i), CMA₃ (b) and FISH (c-f). Heteromorphism between homologous NORs in *A. pisum* is shown in g-i. Arrows indicate X chromosomes. Bar corresponds to 10 μ m.

belonging to different aphid species have prompted us to investigate the cytological and molecular basis of this phenomenon.

Cytogenetic analysis carried out in a parthenogenetic lineage of the aphid *M. viciae* evidenced that more than 50% of embryo cells show heteromorphism between homologous NORs (Mandioli *et al.*, 1999b). This heteromorphism has been observed after silver staining as well as after CMA₃ and *in situ* hybridization with an rDNA probe, so that it is possible to conclude that it is due to an unequal distribution of ribosomal genes between homologous NOR regions. The total number of rDNA genes per individual aphid remained constant. Moreover, the analysis of cells from single embryos has shown that the observed heteromorphism is not only intra-clonal, but also intra-individual. These data, together with the finding of X chromosomes connected by chromatin bridges between their

NORs allow the suggestion that mitotic unequal crossing over could be the main cause of NOR heteromorphism in this taxon.

The localization of rDNA genes in a telomeric position of each X chromosome together with the "stickiness" typical of these chromatin regions, could facilitate the formation of contacts between NOR-bearing X telomeres and, therefore, cause an unequal share of ribosomal genes, when the connection is lost.

Other studies carried out in somatic cells of *Acyrtosiphon pisum* have shown that bridges connecting X chromosomes are detectable not only after silver staining, but also after CMA₃ staining (Manicardi et al., 1998c; Mandrioli et al., 1999a). This finding suggests that GC rich DNA is involved in this type of association. Molecular analysis of rDNA intergenic spacers isolated from *A. pisum* genome shows several 247 bp repeats containing short sequences having a high level of homology with the χ sequence of *Escherichia coli* and with the consensus core region of human hypervariable minisatellites (Mandrioli et al., 1999a). Moreover each 247 bp repeat presents a perfect copy of a promoter sequence for polymerase I. These aphid repeats show structural homologies with a 240 bp repeat, which is considered to be responsible for sex chromosome pairing in *Drosophila*, not only in view of their common presence within rDNA spacers but also for their length and structure (*i. e.* the presence of promoter and hotspot).

The presence of χ sequences in the IGS of *A. pisum* within an open structure of chromatin resulting from the presence of active promoters, strongly supports the hypothesis that unequal crossing over between rDNA genes, can indeed be at the basis of the NOR heteromorphism described in different aphid species.

The occurrence of somatic recombination between homologous and non homologous rDNA sites resulting in asymmetric exchanges of these tandemly repeated DNA sequences has been found to be particularly diffused in yeast (Huang and Keil, 1995). The same phenomenon has been described in other eukaryotes such as *Drosophila* (Tartof, 1974; Frankham et al., 1978; Lyckegaard and Clark, 1991), man and other primates (Arnheim et al., 1980) and also in plants like *Triticum aestivum* (Miller et al., 1980) and *Allium* (Schubert and Wobus, 1985). All these findings indicate that the genes for ribosomal RNA can operate also as hotspots of mitotic recombination (Birky, 1996).

The occurrence of NOR heteromorphism, both intra-clonal and intra-individual, in parthenogenetic lineages of aphids is particularly interesting because it suggests a high incidence of mitotic crossing over taking place, at least at certain recombination hotspots within rDNA arrays, during parthenogenetic generations, when it is generally believed that no recombination occurs (Carvalho et al., 1991).

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7. Translocation Frequencies for X and Y Chromosomes Predicted by Computer Simulations of Nuclear Structure

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Abstract. For an improved understanding of the correlation between the formation of chromosome aberrations induced *e.g.*, by ionising radiation, and the three dimensional structure of the genome, computer simulations of the human cell nucleus were applied to calculate relative translocation rates (Kreth *et al.*, 1998). In the present study an extended computer model was applied taking into account the observed different morphology of both X chromosomes in human female cell nuclei. In the modelling of nuclear genome structure, “tensegrity forces” were introduced which allowed the simulation of different compartmentalisations of a chromosome territory. With the simulation of the observed surface ratio $A_{\text{env}}(\text{Xa})/A_{\text{env}}(\text{Xi}) \sim 1.4$ (A_{env} -enveloping surface) between the two X chromosome territories, marked differences in the translocation rates of both territories were predicted. These computer simulations show that the morphology of a given chromosome territory may have a profound influence on its aberration frequency allowing to establish a quantitatively testable correlation between these observables.

Key words: Translocation frequencies, Computer simulation, Sex chromosomes.

1. Introduction

For the induction of chromosomal aberrations, a linear relationship between the aberration frequency and the DNA content of the chromosomes involved was predicted (*e.g.*, Savage and Papworth, 1973; Lucas and Sachs, 1993; Edwards *et al.*, 1994). Computer simulations of all chromosomes in the human cell nucleus have made it possible to calculate relative interchange frequencies like translocation rates (t-rates) for autosomes (Cremer *et al.*, 1996; Kreth *et al.*, 1998). These calculations indicated an exponential dependence (with an exponent of approximately 2/3) between the translocation probability of a certain chromosome and its DNA content. This is in good agreement with observed translocation rates obtained from Hiroshima A-bomb survivors (Tanaka *et al.*, 1983; Lucas *et al.*, 1992). Taking into account this result, the t-rates for the active (Xa) and inactive (Xi) chromosome should be very similar because of their equal DNA-content. This assumption, however, does not fit the experimental data on aberration frequencies described by Tanaka *et al.*, (1983) and Mühlmann-Diaz and Bedford (1994). Experimental results obtained in human amniotic fluid cell nuclei by chromosome painting and 3D-microscopy indicated that the shape of Xi-territories was rounder and their surface areas were significantly smaller as compared to the Xa-territories in these cells. The mean ratios between Xa and Xi in these cell nuclei were determined for the enveloping surface (A_{env}) to $A_{\text{env}}(\text{Xa})/A_{\text{env}}(\text{Xi}) \sim$

1.4, for the volume to Vol. (Xa)/Vol. (Xi) \sim 1.2 and for the roundness factor to RF(Xa)/RF(Xi) \sim 2.6 ($RF = 36\pi Vol^2/Aenv^3$) (Ells *et al.*, 1996).

To relate chromosome territory morphology to the induction of translocations, the hypothesis has been made that the translocation frequency is correlated primarily with the size of the territory surfaces involved (Savage and Papworth, 1973). In a semi-quantitative estimate (Cremer *et al.*, 1996) the observed X-translocation data (see above) appeared to be well compatible with this assumption. So far the morphology of X-chromosome territories in bone marrow stem cell nuclei or nuclei of cell lines containing different numbers of X chromosomes has not been analysed. Therefore, in the present study we used the surface ratio obtained from amniotic fluid cells to model Xa and Xi territories and to calculate the relative translocation rate for these territories in a strictly quantitative way.

The general goal of this approach is to clarify further relationships between the morphology and chromosomal DNA content on one side and the damage pattern on the other side. Here, we present calculations based on a simplified model of the genome architecture in the human cell nucleus, the Spherical 1Mbp-Chromatin Granule Domain model. This model was derived from a variety of experimental results and conceptual ideas (Cremer *et al.*, 1993, 1996, 2000; Zirbel *et al.*, 1993; Zink and Cremer, 1998; Lamond and Eamshaw, 1998; Münkel and Langowski, 1998; Münkel *et al.*, 1999).

2. Spherical 1 Mbp-Chromatin Granule Domain Model

Experimental basis

The Spherical 1Mbp-Chromatin Granule Domain model used here is based on the following experimental observations:

1. Existence of individual, mutually exclusive chromosome territories, and chromosome arm territories in human cell nuclei (for review see Cremer *et al.*, 1993, 1996, 2000).
2. Existence of mutually exclusive replication foci (Zink *et al.*, 1999): Pulse labelling of replication foci in living human cells (Zink and Cremer, 1998; Zink *et al.*, 1999) made a segregation of replication labelled individual chromosome territories possible when the cells were allowed to proceed for a number of cell cycles before fixation. These results supported the view that chromosome territories and chromosome arm territories are compartmentalised into replication foci/subchromosomal foci which form relatively stable subunits during the cell cycle. The DNA content and size of such foci was estimated to be 0.25–1.5 Mbp with a diameter of 400–800 nm (Zink and Cremer, 1998; for review see Cremer *et al.*, 2000). The distribution of both early and late replicating foci/subchromosomal foci was found to be organised variably within a territory; a low overlap volume between the two foci types of a few percent only was measured (Zink *et al.*, 1999).

Computer simulation

To be able to distinguish conceptually the experimental observations (replication foci/subchromosomal foci) from the theoretical ideas, the model equivalent of these foci was called “chromatin granule”. This term was already used in 1905 by E. Strasburger to denote the small granular substructures of which in his concept the chromosome territories were composed of (for a recent reprint of his model figure see Cremer *et al.*, 1993).

The Multi Loop Subcompartment (MLS) model (Münkel and Langowski, 1998; Münkel *et al.*, 1999) described the chromatin granule structure by 120kbp loops forming rosettes. The granules are linked by chromatin fiber segments with a basepair content similar to the loops. The MLS computer model is completely dynamical, taking into account the stretching, bending and the excluded volume

interaction between the segments approximating the chromatin fibers. Furthermore, it has already been shown to be compatible with a variety of quantitative data on nuclear structure, without assuming any further constraints (Münkel and Langowski, 1998; Münkel *et al.*, 1999). For example, the MLS model may be used to predict mean distances between the intensity gravity centers of fluorescent signals from closely neighbouring DNA sequences located in the same chromosome territory region and to compare the results quantitatively with experimental observations (Knoch, 1998; Münkel *et al.*, 1999). Measurements of overlap volumes of chromosome arm territories applying this model are also in good agreement with experimental results (Münkel *et al.*, 1999).

For our present intention to calculate relative translocation frequencies, it is not necessary to know the structure of the granules themselves. In a previous report (Kreth *et al.*, 1998) we showed that in the model calculations, the relative translocation frequencies depend only on the chromosomal cross sections of the chromosome territories involved, independent of their internal structure. Therefore, on the basis of the MLS-model and the Spherical Subdomain model (Cremer *et al.*, 1996), we developed a statistical model which allows significant differences in the chromosome territory morphology parameters without making further assumptions about the granule structure itself: the Spherical 1 Mbp-Chromatin Granule Domain (SCGD) model.

Here, on the basis of the experimental findings (see above), each of the chromatin granule domains was assumed to contain 1 Mbp of DNA. For simplicity, the chromatin granules were modelled by spheres with a diameter of 500 nm. This value corresponds to the mean value observed experimentally for sub-chromosomal foci in live human cell nuclei following replication labelling (Zink *et al.*, 1998), and quantitative 4D-(3D + time) analysis (Bornfleth *et al.*, 1999). A chromosome territory was then modelled by the folding of a chromatin-granule-chain. To account for the observed dynamic variability in the distances between the granules, zig-zag random walk linkers (*e.g.*, 4 linker segments of 300 nm length each, see Knoch, 1998) were used. The territory compaction was modelled by the assumption of an elastic "tensegrity force" F_a acting on the chromatin granules and directed towards the bary center of the entire territory. Tensegrity forces (Ingber, 1993; Maniotis, *et al.*, 1997) in chromosome territories might be attributed to a variety of physical causes such as the combined effects of chromatin fiber elasticity (Houchmandzadeh *et al.*, 1997) and loop-proteins, or various kinds of connecting non-histone protein chains (Herrmann *et al.*, 1996). If so, changes in tensegrity might be brought about by relatively small changes on the molecular level. Between spatially interacting granules, a hard sphere potential was assumed, allowing a minimal distance of a half granule radius.

According to the hypothesis that the interacting surface of the chromosome territories is important for the formation of translocations (see above), a different frequency distribution of morphological parameters like enveloping surfaces and volumes, observed *e.g.*, for the Xa and Xi chromosome in female amniotic fluid cells (Rinke *et al.*, 1995; Eils *et al.*, 1996), has to be taken into account to model the induction of such chromosome aberrations. One possibility to model different frequency distributions of territory morphology in the frame work of the MLS-model would be to assume different loop sizes and linker lengths for the two homologous territories. For example, one might assume that all loop sizes and linker lengths in one of the homologous territories have values a, b and that all loop sizes and linker lengths in the other have values a*, b* (Knoch, 1998 and unpublished results). Corresponding to the SCGD model, we changed the morphological parameters of two homologous chromosomes by the application of tensegrity forces different for both chromosomes.

A start configuration of the SCGD model was obtained by growing in a random way all chromatin-granule-chains, simultaneously taking into account the elastic tensegrity force and the excluded volume interaction of the hard sphere potentials. When a new position calculated for a chromatin granule did not satisfy the hard sphere potentials of other granules or the tensegrity force condition,

then only this granule position including the four linker segments was rejected and a newly computed random position including the four linker segments was tested again. This method did not satisfy, however, the polymer physical restrictions of selfavoiding random walks; it had the consequence of a higher compaction of the territories in comparison to territories simulated according to the selfavoiding random walk method. The compaction, however, is diminished by a common factor for all chromosome territories. According to the previous simulation data that relative translocation frequencies should depend only on the relative chromosomal cross section of the chromosomes involved (Kreth *et al.*, 1998), the method used here appeared to be sufficient for the calculation of translocation rates.

The number of chromatin granules was chosen to be proportional to the specific DNA contents of the chromosome territories modelled. The start points of the chromatin-granule-chains were placed at the centers of 46 "territory" spheres (corresponding to the diploid chromosome number of the human genome) which were distributed randomly in a spherical "nuclear" volume. To account for the different total DNA contents of the simultaneously growing chromatin-granule-chains, the size of the "territory" spheres was chosen to account for the corresponding number of chromatin granules. However, the growing chromatin granule-chains were not restricted to these inertial territory volumes. To get a reasonable acceptance rate for this procedure, the volumes of the "territory" spheres were appropriately diminished by a common factor (similar to the simulation procedure of the "Spherical Subdomain" model; Cremer *et al.*, 1996). In this way, statistically independent configurations (concerning the distribution of the territories in the nucleus) of whole nuclei were obtained.

Applying this algorithm for the calculation of start configurations, simulated "cell nuclei" (diameter: 10 μm) with chromosome "territories" 1–24 (including X, Y) for the human male cell nucleus were obtained (Fig. 1a). For the simulation of female cell nuclei, the chromosome "territories" 1–22 and Xa were calculated analogously, while for the Xi chromosome a higher elastic force $|F_i| = \alpha |F_a|$ was applied. The parameter α ($= 1.52$) was adapted to approximate the experimentally determined surface ratio $A_{\text{env}}(\text{Xa})/A_{\text{env}}(\text{Xi}) \sim 1.4$ (A_{env} -enveloping surface) (Rinke *et al.*, 1995; Eils *et al.*, 1996) (Fig. 1b) and the observed translocation frequencies for Xa and Xi (Tanaka *et al.*, 1983). This model assumption reflects only the experimentally found difference in the morphology of both X chromosomes. The molecular reason for a rounder shape and a apparently "smoother" surface structure of the Xi chromosome has not been elucidated so far (Walker *et al.*, 1991; Eils *et al.*, 1996; Dietzel *et al.*, 1998). The determination of enveloping

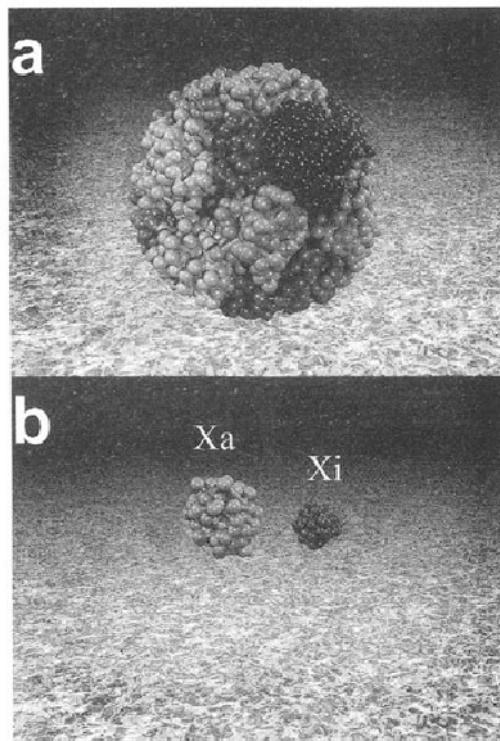


Fig. 1. Spherical human cell nuclei simulated according to the Spherical 1 Mbp-Chromatin Granule Domain model (SCGD). (a) All simulated chromosome territories of a simulated male cell nucleus are visualised using 24 different pseudo colours; (b) only the active and the inactive X chromosome territory of a simulated female cell nucleus are visualised (Visualisations were done using Persistence of VisionTM Ray-Tracer (Pov-RayTM)).

surfaces and volumes of simulated chromosome territories was obtained by virtual microscopy techniques (Münkel and Langowski, 1998; Münkel *et al.*, 1999; see below).

3. Simulation of “Virtual” Confocal Microscope Images

A “virtual” confocal microscope image stack of a simulated chromosome territory was obtained by digitising (with a grid of $80 \times 80 \times 250$ nm spacing) the spheres with diameters of 500 nm, which represented the individual chromatin granules. The chromatin granule positions were determined by the simulation procedure described above. Each data stack consisted of $256 \times 256 \times 32$ voxels (volume elements) with the voxel size mentioned above. This image stack was convoluted with a measured confocal “Point Spread Function (PSF)”. The “Full Width at Half Maximum (FWHM)” in the three directions of the measured TRITC (Tetra Methyl Rhodamine Isothiocyanate) PSF was: $\text{FWHM}_x = 279$ nm, $\text{FWHM}_y = 254$ nm and $\text{FWHM}_z = 642$ nm (for the measurement of the experimental PSF see Bornfleth *et al.*, 1998). After the convolution procedure, the maximal grey value of all 46 convoluted territory image stacks was determined and each territory image stack was normalised on this grey value. Assuming a maximum number of detected photons per voxel of 120 for the entire nucleus, an appropriate Poisson noise was added to each territory image stack. After the recalculation of the territory image stacks into grey values (sampling procedure), the image stacks were analysed by a standard image analysis procedure. For noise reduction, each section of a “virtual microscopy” data stack was filtered with a 3×3 median filter. The objects of interest (the simulated “chromosome territories”) in these stacks were segmented applying a global threshold and the 26-neighbourhood relationship. The “virtual microscopy” volumes (Vol_{virt}) and the enveloping “virtual microscopy” surfaces ($\text{Aenv}_{\text{virt}}$) of the objects were determined by the segmentation of a series of thresholds (10%–13% of the maximal intensity of the “nucleus”) and averaging of the calculated volumes and surfaces.

The volume V of a segmented object was obtained applying the Cavalieri-Estimator (for the exact definition see Gundersen and Jensen, 1987):

$$V = N_{\text{voxel}} \times V_{\text{voxel}} = N_{\text{voxel}} \times (D_{xy}^2 \times D_z).$$

N_{voxel} represents the number of voxels with the voxel volume V_{voxel} determined for the object and D_{xy} , D_z represent the lateral and axial voxel lengths, respectively.

The enveloping surface was obtained by calculating the entire voxel surface F_{voxel}

$$F_{\text{voxel}} = N_{\text{voxel}} \times (4 \times D_{xy} \times D_z + 2 \times D_{xy}^2)$$

and subtracting from this calculated voxel surface F_{voxel} the voxel surfaces situated in the interior of the object. The enveloping surface Aenv was then obtained by the equation:

$$\text{Aenv} = N_{\text{voxel}} \times (4 \times D_{xy} \times D_z + 2 \times D_{xy}^2) - N_1 \times (D_{xy} \times D_z) - N_2 \times (D_{xy} \times D_{xy})$$

N_1, N_2 represent the number of voxel surfaces in the interior of the object parallel, perpendicular to the optical (z-) axis respectively.

For the calculation of the “true” volumes (Vol_{true}) and the “true” enveloping surfaces ($\text{Aenv}_{\text{true}}$) of the territories, the territories were segmented directly after the digitalisation applying again the Cavalieri estimator (see above), using a threshold of 0.4 % of the maximal intensity of the nucleus. For this purpose, no convolution with the confocal PSF was applied. For both “virtual microscopy” and “true” images, the dependence of mean volume to chromosomal DNA content and mean enveloping surface to chromosomal DNA content was determined for 13 simulated female cell nuclei for all

chromosome territories (see Fig. 2a, b). For the autosomes and the Xa chromosome, a linear dependence between mean volume of the territories and chromosomal DNA content was found (Fig. 2a), reflecting the proportionality of the number of chromatin granules and the specific DNA content of the chromosomes simulated. The mean enveloping surfaces showed the expected exponential dependence ($\sim 2/3$) from the chromosomal DNA content (according to the relation of surface \sim volume $^{2/3}$) (Fig. 2b). The mean volume ratio and the mean surface ratio of Xa and Xi after virtual microscopy were determined to be $\text{Vol}_{\text{virt}}(\text{Xa})/\text{Vol}_{\text{virt}}(\text{Xi}) = 1.16$ and to $\text{Aenv}_{\text{virt}}(\text{Xa})/\text{Aenv}_{\text{virt}}(\text{Xi}) = 1.21$. Under these conditions, however, the “true” ratios were determined to be $\text{Vol}_{\text{true}}(\text{Xa})/\text{Vol}_{\text{true}}(\text{Xi}) = 1.67$ and $\text{Aenv}_{\text{true}}(\text{Xa})/\text{Aenv}_{\text{true}}(\text{Xi}) = 1.77$. This small but statistically clear difference between the “virtual” and the “true” ratios results from the different elastic forces used for the Xa and the autosomes on the one side and the Xi chromosome on the other side. The higher “elastic force” used for the Xi chromosome model has the consequence of a higher chromatin granule density and thus also of higher intensity values for it in comparison to the models of the Xa and the autosomes. The intensity distributions of the maximal intensity projections (on the lateral plane) show a sharp slope for the “Xi chromosome”, while the distribution for the “Xa chromosome” is widely expanded (see Fig. 3). Therefore, after convolution and segmentation of the modelled territories with the same threshold, the volume and surface of the Xi chromosome territory model will be better conserved than the volumes and surfaces of the Xa territory model and the autosomes.

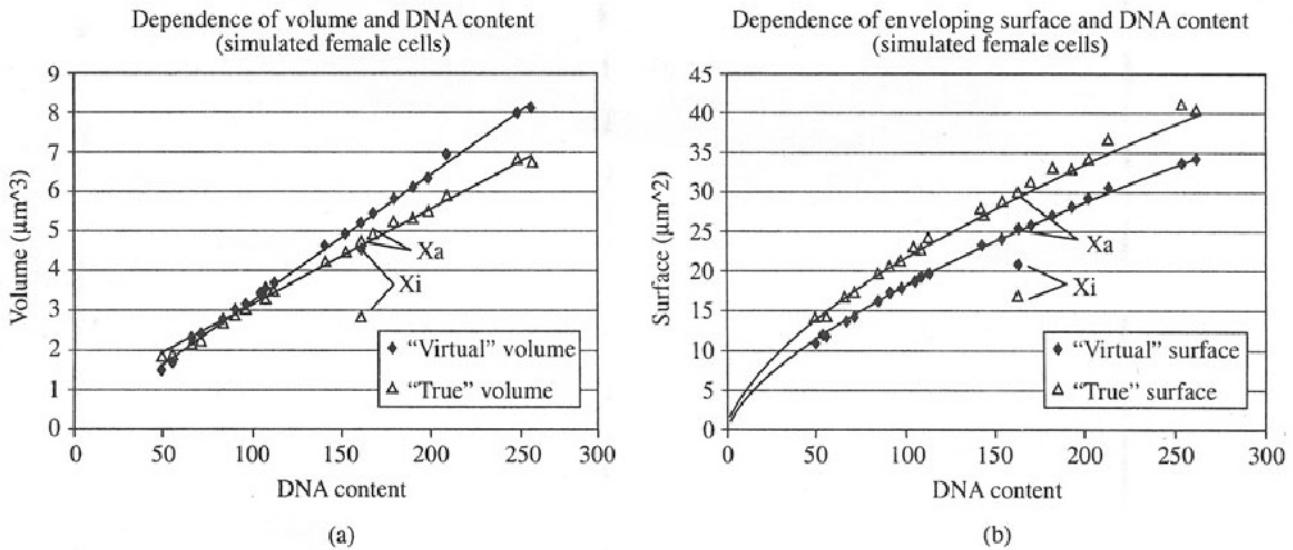


Fig. 2. The calculated relation between (a) mean territory model volume and territory model DNA content (in Mbp) and (b) mean enveloping territory surface to territory DNA content (in Mbp). The dependence is based on 13 simulated female cell configurations according to the SCGD model. The “virtual microscopy” volumes and enveloping surfaces were obtained after the convolution and the segmentation with a global threshold of the digitised territory image stacks. The “true” volumes and enveloping surfaces were obtained directly after the digitalisation (for further details see text).

4. Calculation of Translocation Break Frequencies

In the present study, the model calculation of relative translocation rates for sex chromosomes was executed in the same way as for autosomes (described in Kreth *et al.*, 1998). For a better understanding, we summarise briefly the most important steps of the method.

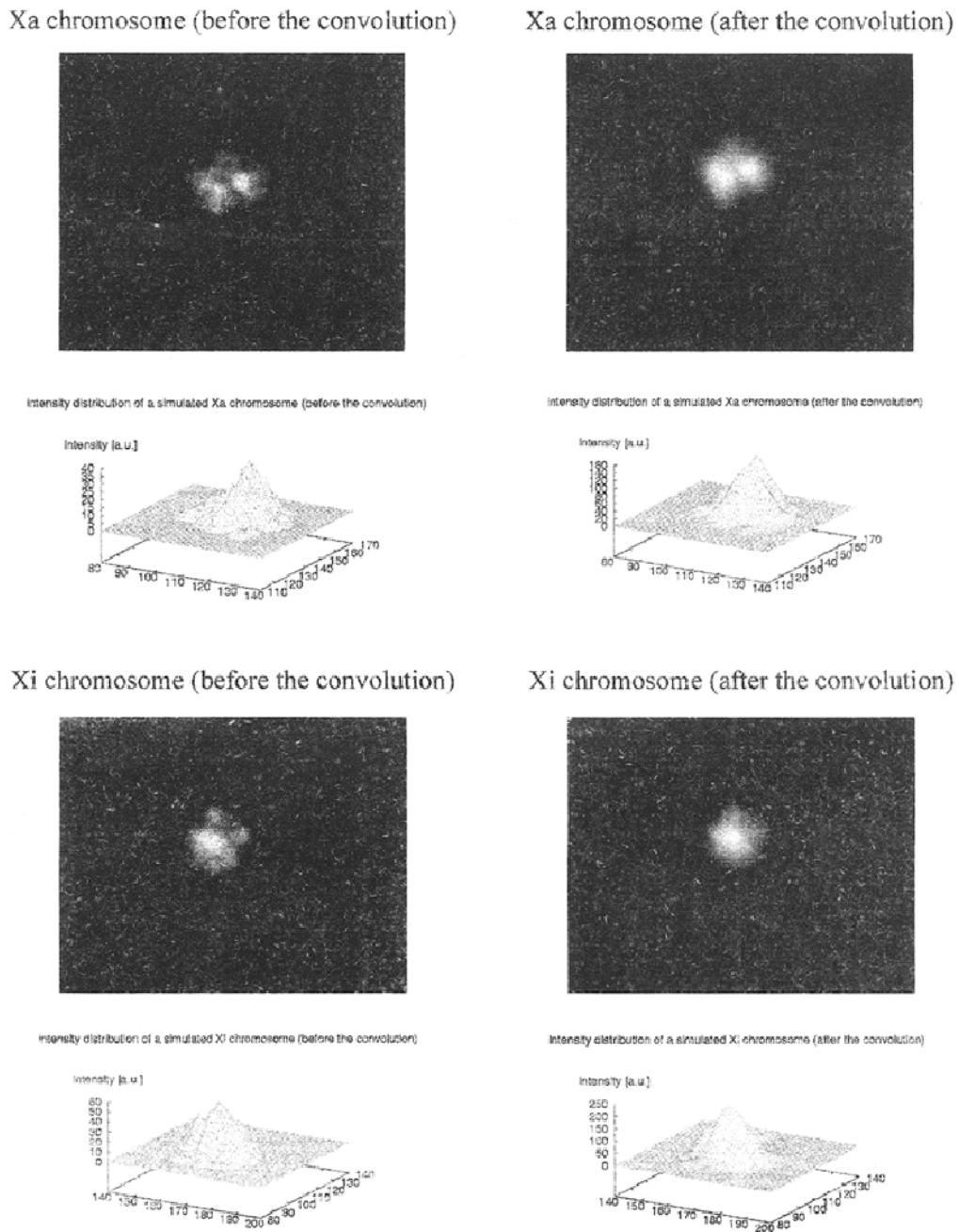


Fig. 3. Maximal intensity projections of simulated digitised Xa and Xi chromosome territories according to the SCGD model. Shown are axial projections before and after the convolution with a measured point spread function. Additionally, the intensity distributions in the projection areas in arbitrary units for each case are plotted. The relative differences of the intensity values between the Xi and Xa chromosome territories are maintained.

To determine the induction of an exchange event by a photon ray, in the frame work of the SCGD model, we used the assumption that simple interchanges like reciprocal translocations are the consequence of two “specific events” (Double Strand Breaks, DSBs) in two neighboured chromosomal elements. The photon ray was considered to be represented as one straight line which traverses randomly from all sites of the nucleus. To obtain the exchange probability, all “chromosome” pairs affected (by the simulated ray) were determined, whose nearest elements (chromatin granules) had a distance of one element radius r or less from each other (Fig. 4a). This implies that no other “chromatin granule” material is situated in between. Figs. 5a, b show the mean contact frequencies of two neighboured chromosome “territories” determined for 400 male and 400 female simulated cell nuclei.

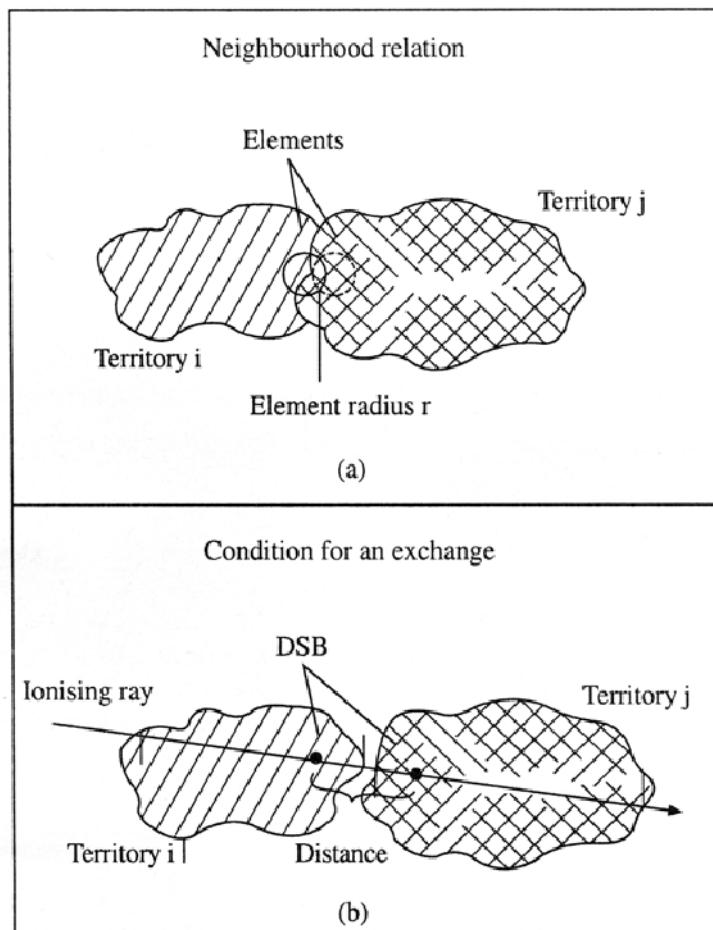


Fig. 4. Schematic drawing of the two basic conditions which were assumed for the modelling of an exchange process according to the simulation algorithm. a) the neighbourhood relationship between two territories is fulfilled when two elements of the territories have a maximal distance of one element radius r ; (b) Condition for the formation of an exchange: In each affected chromosome territory, a point on the ray representing the double strand break (DSB) was chosen at random. The distance between these two DSBs was weighted with a experimentally found distance probability function and compared with a unit distribution according to the Metropolis Monte Carlo method. When the condition according to this method is accepted, an exchange between the two neighboured chromosome territories is counted (for further details see text).

Table 1 shows the contact frequencies of some “chromosome” pairs including the frequencies determined for the “sex chromosomes”. It is obvious that the contact frequencies of the “Xa chromosome” to other “chromosomes” is higher than for the “Xi chromosome”. This is the result of the different mean size of the simulated enveloping surfaces.

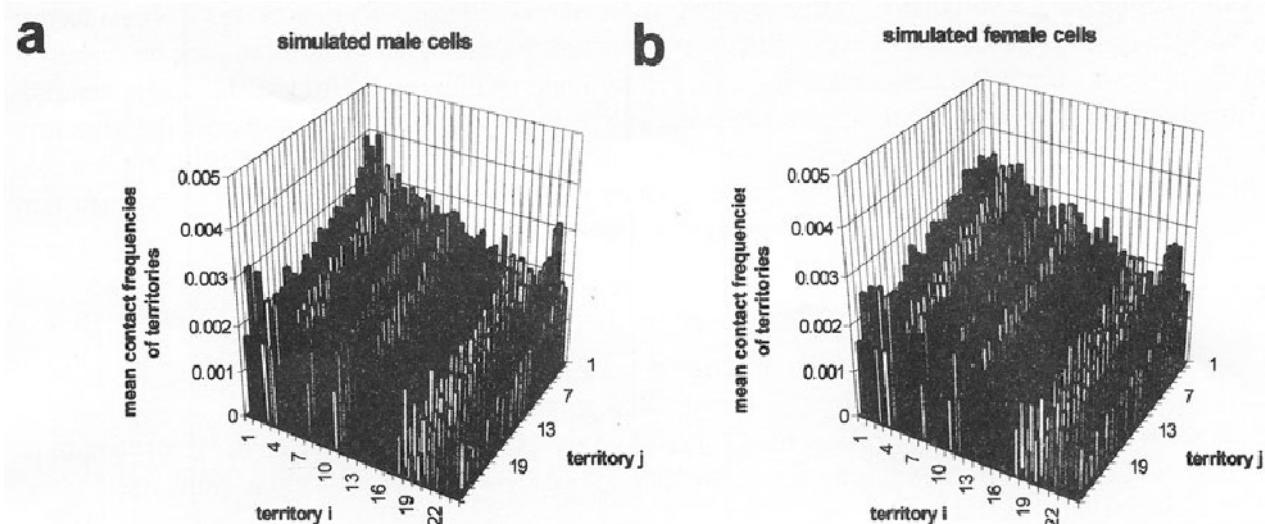


Fig. 5 Mean contact frequencies of two chromosome territories calculated for 400 simulated cell configurations applying the SCGD model for a) male cells (#23 corresponds chromosome X and # 24 chromosome Y) and b) female cells (#23 corresponds chromosome Xa and # 24 chromosome Xi).

Table 1 Mean contact frequencies of two chromosome territories determined by 400 simulated cell configurations. Upper row shows the frequencies for human male cells and the lower row for human female cells. The contact frequency describes the relative frequency f with which the nearest elements of any two neighbouring chromosome territories have a maximal distance of one granule radius. The relative frequencies for all chromosome territory pairs (i, j) were

$$\text{normalised to one } \sum_{i=1}^{24} \sum_{j=1}^{24} f_{ij} = 1.$$

#	1	5	9	15	20	X	Y
X	0.0031	0.0025	0.0019	0.0017	0.0012	0	0.001
Y	0.0017	0.0014	0.0013	0.00098	0.0006	0.001	0
#	1	5	9	15	20	Xa	Xi
Xa	0.0025	0.0024	0.0023	0.0017	0.0017	0	0.00094
Xi	0.0016	0.0014	0.0013	0.0011	0.00091	0.00094	0

After this, in each affected “chromosome” of this pair, a point (representing a Double Strand Break, DSB) on the ray was chosen at random (Fig. 4b). The normalised probability function $P(d)$ for an exchange in dependence of the distance d of the two DSBs was assumed to be given by $P(d) = r^{1.4}$

$l/d^{1.4}$. The exponent 1.4 was taken as an experimentally derived fitting parameter (Edwards *et al.*, 1994). Corresponding to the Metropolis Monte Carlo process, an exchange event for this affected chromosome pair was counted when a random number of the unit distribution $[0; 1] \leq P(d)$. In the next step, when for an affected “chromosome” pair an exchange event was counted, each “chromosome” of this pair was assigned to a translocation event. Then the calculated translocation rate t_i for a given “chromosome” i was calculated by dividing the whole number of translocation events T observed for n nuclear configurations and m rays/nucleus by n, m and the multiplicity h_i of the “chromosome” i in the nucleus ($h_i = 2$ for autosomes, $h_{23} = h_{24} = 1$ for male and female cells). On the mean, about 4 chromosome “territories” were affected by each ray transversing the spherical model nucleus: the mean number of affected “territories” was 3.72 ± 0.06 . This was obtained from 400 different nuclear configurations; each configuration was transversed by $m = 1000$ rays. Since each “territory” affected was assumed to produce 1 DSB, this would correspond to a total of about 4 DSBs per nucleus/per ray.

5. Results

The calculated t -rates obtained from simulated male and female cell nuclei (400 cell nuclear configurations and 1000 rays/configuration each), applying the SCGD model, were compared with an observed translocation rate of 39 Hiroshima A-bomb survivors using conventional cytogenetic banding procedures (Tanaka *et al.*, 1983). For the comparison the observed t -rates t_1^* and the simulated t -rates t_j were normalised in such a way that

$$\sum_{i=1}^{24} t_1^* = \sum_{j=1}^{24} t_j = 1$$

With the assumption that the t -rate for the male X-chromosome Xm and the female active X-chromosome Xa should be equal (because of the same genetic activity), by the determination of the whole t -rate t_{xa+xi} and the t -rate t_{xm} the t -rate t_{xi} results in $t_{xi} = t_{xa+xi} - t_{xm}$ (Tanaka *et al.*, 1983).

The t -rates were fitted by a least square curve applying the assumption that the t -rate increases with (DNA content) p . The t -rates obtained from fitting the observed and the simulated translocation frequencies for male and female cell nuclei are shown in Fig. 6a (male) and Fig. 6b (female),

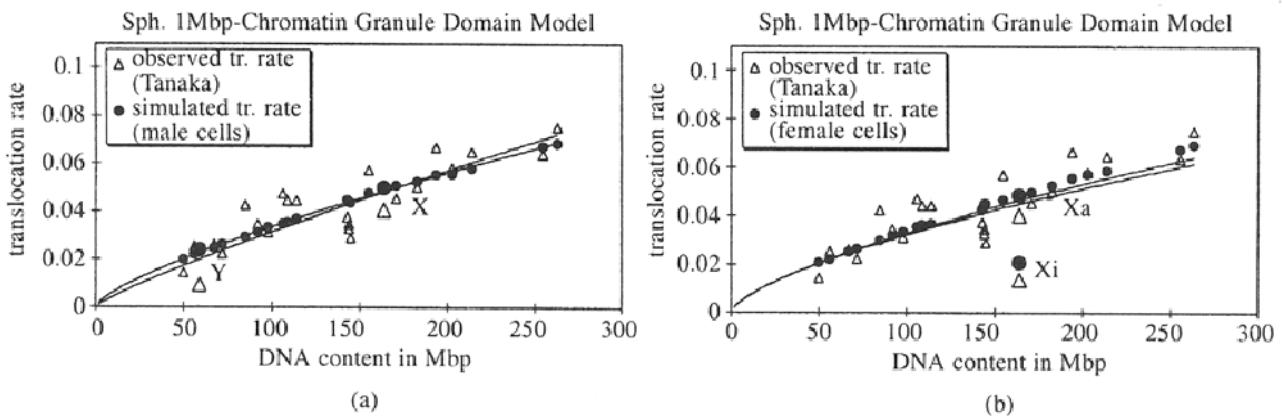


Fig. 6 The observed translocation rate (t -rate) (Tanaka *et al.*, 1983) was compared with the simulated translocation rate (calculated for 400 simulated configurations each, 1000 rays per configuration) for a) male cells and b) female cells. For the comparison the sums of both rates were normalised to one.

respectively. The observed *t*-rate (obtained from Hiroshima A-bomb survivors) was given for male and female cells together (Tanaka *et al.*, 1983); thus, no difference in the regression curve calculation was made. Table 2 shows the parameters of the regression curves determined for both simulated and the observed *t*-rate with and without the sex chromosomes. For the simulated *t*-rates, the regression curves fitted for the autosomes (of male and female cells) showed a weak difference only.

Table 2 Isotropic irradiation: the fitted regression curves for the simulated and the observed translocation rates (human cells). The total number of translocation events for all chromosomes was normalised to one. R^2 -correlation coefficient between simulated values and exponential fitting curve. The correlation level was determined with the double sided Kolmogorov-Smirnov test. A higher level is a sign for a better agreement of observed and simulated *t*-rate. The observed rate was determined for male and female cells together, while the simulated *t*-rates were determined separately for male and female cells. The regression curves are given for autosomes and together with sex chromosomes

Observed (Tanaka <i>et al.</i> 1983)	Simulated (SCGD model)	Correlation level
autosomes:	autosomes (male cells) [1]: $t\text{-rate} = 0.0011 (\text{DNA content})^{0.7409}$ $R^2 = 0.9986$	observed: simulated: [1] [2]
$t\text{-rate} = 0.0012 (\text{DNA content})^{0.7414}$ $R^2 = 0.7365$	autosomes (female cells) [2]: $t\text{-rate} = 0.002 (\text{DNA content})^{0.7314}$ $R^2 = 0.9989$	autosomes 0.97 0.99 [3]
with X, Y:	male cells [3]: $t\text{-rate} = 0.0011 (\text{DNA content})^{0.7351}$ $R^2 = 0.9975$	with X, Y 0.98 [4]
$t\text{-rate} = 0.0006 (\text{DNA content})^{0.8566}$ $R^2 = 0.7033$	female cells [4]: $t\text{-rate} = 0.0014 (\text{DNA content})^{0.6882}$ $R^2 = 0.7658$	with Xa, Xi 0.98
with Xa, Xi:		
$t\text{-rate} = 0.0015 (\text{DNA content})^{0.6684}$ $R^2 = 0.4676$		

In agreement with the *t*-rates determined in Kreth *et al.* (1998), the dependence of both simulated and observed *t*-rates showed approximately a 2/3 behaviour (see Table 2). The significance level for the agreement ("correlation level") of simulated and observed *t*-rates was determined in both cases (male and female cells) to be 0.98 according to the double sided Kolmogorov-Smirnov test.

Figure 7 shows the dependence of the translocation frequencies obtained from female cells on the enveloping mean surfaces of the respective chromosome territories. The *t*-rate was plotted as a function a) of the "true" surfaces and b) of the "virtual" surfaces. In both cases, the linear relationship between the *t*-rate and the enveloping surface was confirmed for the autosomes plus the Xa chromosome. In addition, the linear dependence was also confirmed for the Xi chromosome, if the "true" surface dependence was used. For the dependence of the *t*-rates on the "virtual" mean enveloping surface, the "virtual" Xi-value was about a factor 2 below the linear regression line value. However, a good fitting of *t*-rates by a linear regression curve was obtained for all "chromosomes" (autosomes, Xi and Xa) if the "true" surface dependence was used.

6. Discussion

In the present contribution, for the first time a quantitative computer simulation approach is presented

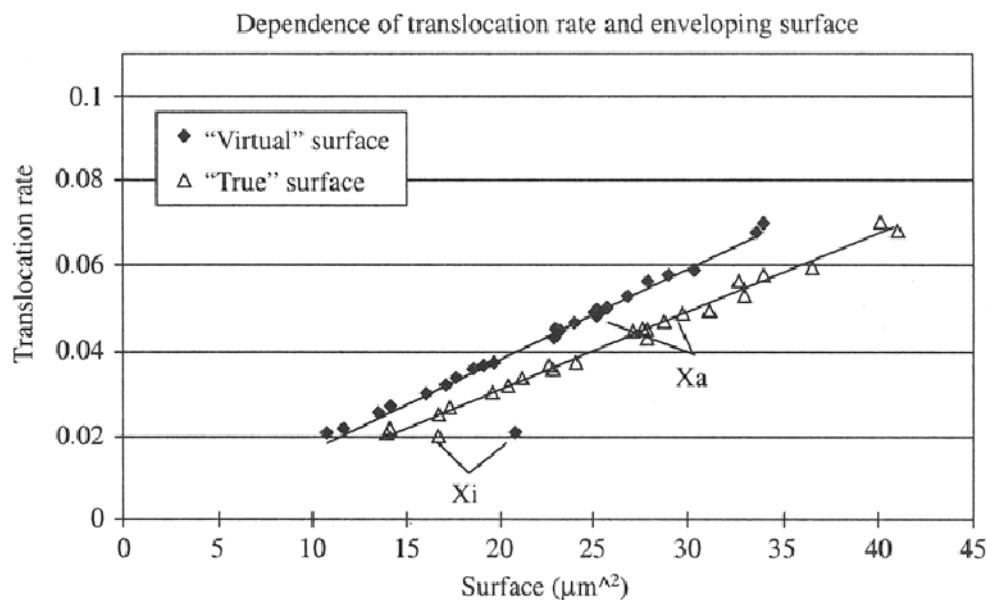


Fig. 7. The t -rate (calculated for 400 simulated female nuclear configurations) plotted as a function of the mean “true” and the “virtual” enveloping surface (determined by 13 simulated female configurations each) for all chromosome territories. For the meaning of “true” and “virtual” (see Figure 2 and text).

to predict relative translocation rates for all human chromosomes, taking into account not only chromosomal DNA content and nuclear distribution of chromosome territories, but also morphological features of chromosome territories. Such an analysis requires to establish a sufficiently “realistic” but at the same time sufficiently simple numerical modelling of nuclear architecture. Here, the calculations were based on the Spherical 1Mbp-Chromatin Granule Domain (SCGD) model which was developed in view of a large amount of experimental data on the architecture of the human cell nucleus (see introduction). As an example for the use of morphological features of chromosome territories in computer simulations of relative translocation frequencies, the X-chromosome was chosen. For this chromosome, marked morphological differences have been observed in human cell nuclei as well as marked differences in translocation rates following ionising radiation.

In the present study we used statistically independent start configurations of the SCGD model with respect to the distribution of the territories in the model nucleus. A start configuration of this model was obtained by growing in a random way all chromatin-granule-chains simultaneously taking into account: (a) an elastic “tensegrity force” directed towards the bary center of the “territory” and (b) the excluded volume interaction of hard sphere potentials. When a new position calculated for a chromatin granule did not satisfy the hard sphere potentials of other granules or the “tensegrity force” condition, then only this granule position including the four linker segments was rejected and a new computed random position including the four linker segments was tested again. This method did not satisfy the polymer physical restrictions of selfavoiding random walks; it appeared to be, however, sufficient, for the calculation of translocation rates (see results). To obtain physically correct configurations from the point of view of polymer physics, these start configurations have to be relaxed by Monte Carlo steps to satisfy the restrictions of selfavoiding random walks. Such relaxation calculations, however, are very demanding in terms of computing time, whereas the calculations performed here were executed on a standard PC-system (Intel Pentium Pro TM system with 200 MHz).

The modelling of morphological differences of chromosome territories was achieved by changing

the elastic “tensegrity force”; thus it was possible to simulate different compartmentalisation levels of homologous territories. For the Xi chromosome, a higher elastic force was chosen. After virtual microscopy, this resulted in a mean enveloping surface ratio $A_{env,virt}(Xa)/A_{env,virt}(Xi) \sim 1.21$, close to the ratios obtained from 3D-microscopical observations (Rinke *et al.*, 1995; Eils *et al.*, 1996). With these model assumptions it was possible to simulate translocation frequencies also for the Xa, Xi and the Y chromosome territories in good agreement to the observed frequencies obtained from Hiroshima A-bomb survivors (Tanka *et al.*, 1983). The present simulations are compatible with previous modelling results (Kreth *et al.*, 1998) which indicated that relative translocation frequencies depend on the chromosomal cross sections of the chromosomes involved only, rather than on the distance of the damaged chromosomal “elements” and not on the structure of the chromosomal “elements” themselves.

However, our simulations indicate that the mean “virtual” volumes and enveloping surfaces determined after the virtual microscopy differ to some extent from the “true” volumes and enveloping surfaces. The simulations showed that the mean “true” volume ratio $Vol_{true}(Xa)/Vol_{true}(Xi)$ and the mean “true” enveloping surface ratio $A_{env,true}(Xa)/A_{env,true}(Xi)$ are somewhat higher than the “virtual” ratios adopted from previous experimental estimates. This suggests that the translocation rates observed (being close to those predicted here) may require somewhat higher morphological differences than the experimental estimates obtained by using the same segmentation threshold for both chromosome territories in a given nucleus (Rinke *et al.*, 1995; Eils *et al.*, 1996). This segmentation procedure, however, seemed to be necessary to avoid errors by interactively choosing different thresholds for homologous territories in the same nucleus. Taking into account the “true” volume ratio $Vol_{true}(Xa)/Vol_{true}(Xi) = 1.67$ and the “true” enveloping surface ratio $A_{env,true}(Xa)/A_{env,true}(Xi) = 1.77$ for the X chromosomes in a female cell, it was possible to calculate the translocation frequencies for Xa and Xi in good agreement with the observed frequencies. With these “true” ratios, the predicted linear dependence of translocation frequencies and size of interphase contact surfaces of the chromosome territories involved (Savage and Papworth, 1973; Cremer *et al.*, 1996) was compatible also for the X chromosomes.

On the whole, our simulations confirmed the predictions (Cremer *et al.*, 1996) that volume and enveloping surface ratios are sufficient to explain the observed difference in the translocation frequencies of the Xa and the Xi chromosome.

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8. Evolution of DNA Repair Mechanisms

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Abstract: Repair mechanisms for alkylation damage are described. Similarities and differences of repair enzymes in different groups of organisms are discussed with respect to the evolution of these proteins. Some aspects of DNA repair in plants are described.

Key words: DNA repair mechanisms, alkylation damage, adaptive response, evolution.

1. Introduction

DNA repair is essential for the survival of organisms. Even if exposures to exogenic and endogenic mutagenic agents are not taken into account, DNA repair is indispensable with respect to inherent instability of DNA. Many different DNA lesions are induced spontaneously or by exogenous DNA damaging agents which interfere with transcription, DNA replication and DNA integrity. The various DNA repair systems, which are summarized in Table 1, guarantee the survival of cells and organisms. The mechanisms of DNA repair are bound to the highly conserved structure of DNA which is basically the same in viruses, bacteria, plants and mammals. Only regulatory proteins (*e.g.*, transcription factors) and structural proteins are different in pro-and eukaryotes. It can, therefore, be expected that

Table 1. Mechanism of DNA repair and tolerance of DNA damage

Repair of DNA lesions

1. Reversion of DNA lesions
 - 1.1. Enzymatic photoreactivation
 - 1.2. Ligation of DNA strand breaks
 - 1.3. Dealkylation of O⁶-alkylguanine by O⁶-methylguanine-DNA methyltransferase
2. Excision of DNA lesions
 - 2.1. Base excision repair
excision mediated by DNA glycosylases (with or without apurinic endonuclease activity)
 - 2.2. Nucleotide excision repair
 - 2.3. Repair of DNA crosslinks
 - 2.4. Repair of DNA mismatches

Tolerance of DNA lesions

1. Replication with gap formation (post-replication repair)
 2. Translesional DNA synthesis
-

DNA repair enzymes are structurally and functionally conserved. In the following we will show that this is indeed the case.

With the exception of damage reversion, DNA repair is a very complex mechanism. More than 25 different proteins are involved in nucleotide excision repair in mammalian cells (for review see Friedberg *et al.*, 1995). The complexity is especially impressive for inducible cellular repair mechanisms, such as the adaptive response and SOS repair, which are error free (adaptive response) or error prone (SOS repair) respectively. It would be interesting to know whether these complex mechanisms contain conservative elements in a wide number of different organisms. In this review we discuss mainly two repair enzymes, namely, O⁶-alkylguanine-DNA alkyltransferase (MGMT) and N-methylpurine-DNA glycosylase (MPG). We want to compare the sequences of the genes in pro- and eukaryotes and discuss the possible importance of conserved and divergent structures (for genes see Table 2). At the end of the review we will discuss DNA repair mechanisms in plants where still many aspects, as compared to mammalian cells, are unknown.

2. O⁶-Methylguanine-DNA Methyltransferases

Mutagenic and carcinogenic activities of alkylating agents result mainly from alkylation of the O⁶ position of guanine and O⁴ of thymine in DNA (Loveless 1969; Lawley 1984). O⁶-alkylguanine is about 10 times more frequent than O⁴-alkylthymine (Beranek 1990). Both types of alkylation comprise less than 10 % of all alkylation types in DNA. O⁶-guanine and O⁴-thymine mispair during DNA replication with thymine and guanine, respectively, and this leads to GC to AT or AT to GC transition mutations (Singer 1985). All organisms analyzed so far have a repair system protecting them from mutagenic and toxic activities of O⁶-alkylguanine and O⁴-alkylthymine. The repair of these alkylated bases is mediated by O⁶-alkylguanine-DNA alkyltransferase which transfers the alkyl group from the base to an own cysteine residue (Demple *et al.*, 1985). The alkylation of the cysteine is irreversible and, therefore, it inactivates the protein (suicide enzyme).

O⁶-Alkylguanine-DNA alkyltransferase was first described in *Escherichia coli*. It was shown that pretreatment of bacteria with methylating agents makes cells more resistant to the toxic and mutagenic activity of a second treatment with the same agent (Samson and Cairns, 1977). This resistance was shown to result from a more effective repair of methylations in the DNA of bacteria. The main enzyme of this repair system is the Ada protein (see Table 2) which is an inducible O⁶-alkylguanine-DNA alkyltransferase (Lindahl *et al.*, 1988). The Ada protein has two active centers with reactive cysteine residues at positions 69 and 321 respectively (Sedgwick, 1983; Demple *et al.*, 1985; Tano *et al.*, 1989). Cysteine 321 binds alkyl groups from O⁶-alkylguanine and O⁴-thymine, whereas cysteine 69, which is resided in the second functional center, binds alkyl groups from alkylated phosphotriesters. Alkylation of cysteine 69 leads to a conformational change of the Ada protein (Teo *et al.*, 1986). Upon this conformational alteration, Ada is able to bind to promoter regions of genes involved in the adaptive response (such as Alk A) and activates their transcription (Teo *et al.*, 1984; Lindahl *et al.*, 1988).

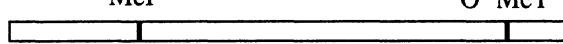
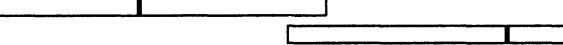
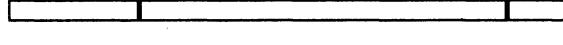
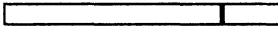
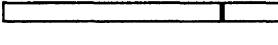
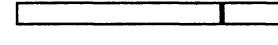
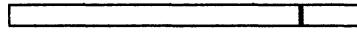
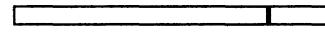
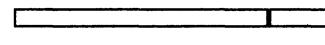
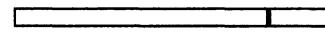
An active center which transfers alkyl groups of alkyl phosphotriesters to a cysteine moiety has also been shown in *Salmonella typhimurium* and *Bacillus subtilis* (Samson, 1992). The two activities of the Ada protein of *E. coli* and *S. typhimurium*, which were described above, are catalysed by two different proteins in *B. subtilis*, namely, Ada A and Ada B (Fig.1, Table 2). As in the Ada protein of *E. coli*, alkylation of Ada A changes the enzyme to a transcription factor which stimulates transcription of adaA-adaB operons in *B. subtilis*. Such an activation of the alkyltransferase was not found in *S. typhimurium* (Samson, 1992).

Table 2. Some cloned genes from bacteria, yeast and mammalian cells involved in the repair of DNA alkylation damage

Gene	Organism	Enzymatic activity	Inducibility
<i>ada</i>	<i>Escherichia coli</i>	O ⁶ -methylguanine-DNA methyltransferase and alkylphosphotriester alkyltransferase; activator of transcription of genes involved in the "adaptive response"	yes
<i>ogg</i>	<i>Escherichia coli</i>	O ⁶ -methylguanine-DNA methyltransferase	no
<i>alk A</i>	<i>Escherichia coli</i>	N-alkylpurine-DNA glycosylase	yes
<i>tag</i>	<i>Escherichia coli</i>	N3-alkyladenine-DNA glycosylase	no
<i>alkB</i>	<i>Escherichia coli</i>	?	yes
<i>aidB</i>	<i>Escherichia coli</i>	?	yes
<i>ada</i>	<i>Salmonella typhimurium</i>	O ⁶ -methylguanine-DNA methyltransferase and alkylphosphotriester alkyltransferase; activator of transcription of genes involved in the "adaptive response"	yes
<i>ogg</i>	<i>Salmonella typhimurium</i>	O ⁶ -methylguanine-DNA methyltransferase	no
<i>ada A</i>	<i>Bacillus subtilis</i>	O ⁶ -methylguanine-DNA methyltransferase	yes
<i>ada B</i>	<i>Bacillus subtilis</i>	alkylphosphotriester alkyltransferase; activator transcription for the genes involved in the "adaptive response"	yes
<i>alk A</i>	<i>Bacillus subtilis</i>	N-alkylpurine-DNA glycosylase	yes
<i>mgt1</i>	<i>Saccharomyces cerevisiae</i>	O ⁶ -methylguanine-DNA methyltransferase	no
<i>mag</i>	<i>Saccharomyces cerevisiae</i>	N-alkylpurine-DNA glycosylase	yes
<i>mgmt</i>	<i>Rat, man</i>	O ⁶ -methylguanine-DNA methyltransferase	yes
<i>mpg</i>	<i>Rat, man</i>	N-alkylpurine-DNA glycosylase	?
<i>pol β</i>	Human	DNA-polymerase for "short patch" repair-synthesis	yes
<i>ape/refl/</i>	Human	apurinic/apyrimidinic endonuclease	yes

E. coli not exposed to alkylating agents display low activity of O⁶-alkylguanine-DNA alkyltransferase. This is due to the activity of another alkyltransferase, the so-called Ogt protein (Potter *et al.*, 1987). The Ogt protein is constitutively expressed at low level giving rise to about 30 molecules per cell. The expression of Ogt is not influenced by alkylating agents, indicating that the *ogg* gene is not inducible. The Ogt protein has only one active center which mediates transfer of alkyl groups from the O⁶-position of guanine and O⁴ of thymine on an internal cysteine 85. The structure of this active center is functionally similar to the active center of the Ada proteins in the area of cysteine in position 321 (see Fig.1).

All organisms analyzed so far (for plants, see later) possess at least one O⁶-alkylguanine-DNA alkyltransferase (Table 2). The alkyltransferases of mammals were designated as MGMT (MGMT = O⁶-methylguanine-DNA methyltransferase; E.C.2.1.1.63). Like the Ogt protein of *E. coli*, MGMT has only one active center. The MGMT protein removes alkyl groups very effectively from the O⁶-position of guanine and very much less from the O⁴-position of thymine (Koike *et al.*, 1990; Zak *et al.*, 1994; Kawate *et al.*, 1995). The alkyltransferase of mammalian cells is comparable to the ones of other organisms in that the transfer of alkyl groups leads to its inactivation (suicidal protein).

Alkyltransferase	Species/Protein	Inducibility ^a
MeP	O^6 MeG O^4 MeT	
	<i>E. coli</i> Ada	+
	<i>B. subtilis</i> AdaA, AdaB	+
	<i>S. typhimurium</i> Ada	+
	<i>S. typhimurium</i> OGT	+
	<i>E. coli</i> OGT	-
	<i>B. subtilis</i> DAT1	-
	<i>S. cerevisiae</i> MGMT	-
	Rat MGMT	+
	Chinese hamster MGMT	?
	Human MGMT	+
*	PCHR	Consensus sequence
FRPCKRC		

^aInduction data by Fritz *et al.*, 1991; Samson, 1992; Lefebvre *et al.*, 1993; Grombacher *et al.*, 1996.

Fig. 8.1 Schematic comparison of the structure of alkyltransferases in different organisms.

Enhanced expression of MGMT in cells (upon transfection of cDNA) or transgenic mice leads to significant increase of resistance of cells and individuals to the toxic, mutagenic, clastogenic and carcinogenic activities of simple alkylating agents (Kaina *et al.*, 1993; Becker *et al.*, 1996 and further references herein). This clearly shows the importance of these repair proteins for protecting cells from alkylation damage.

3. Structural Comparison of Different O^6 -Alkylguanine-DNA Alkyltransferases

All known alkyltransferases have extended homologies at the level of amino acid sequence and secondary structure (see Figs. 1 and 2) (Moore *et al.*, 1994; Pegg *et al.*, 1995). Especially impressive are the helix-turn-helix motives of *Ada* which are typical for DNA binding proteins (for amino sequences see Fig. 2). The high extent of conservation of the structure of alkyltransferase proteins points to a highly conserved mechanism of enzyme action. In order to analyse this in more detail,

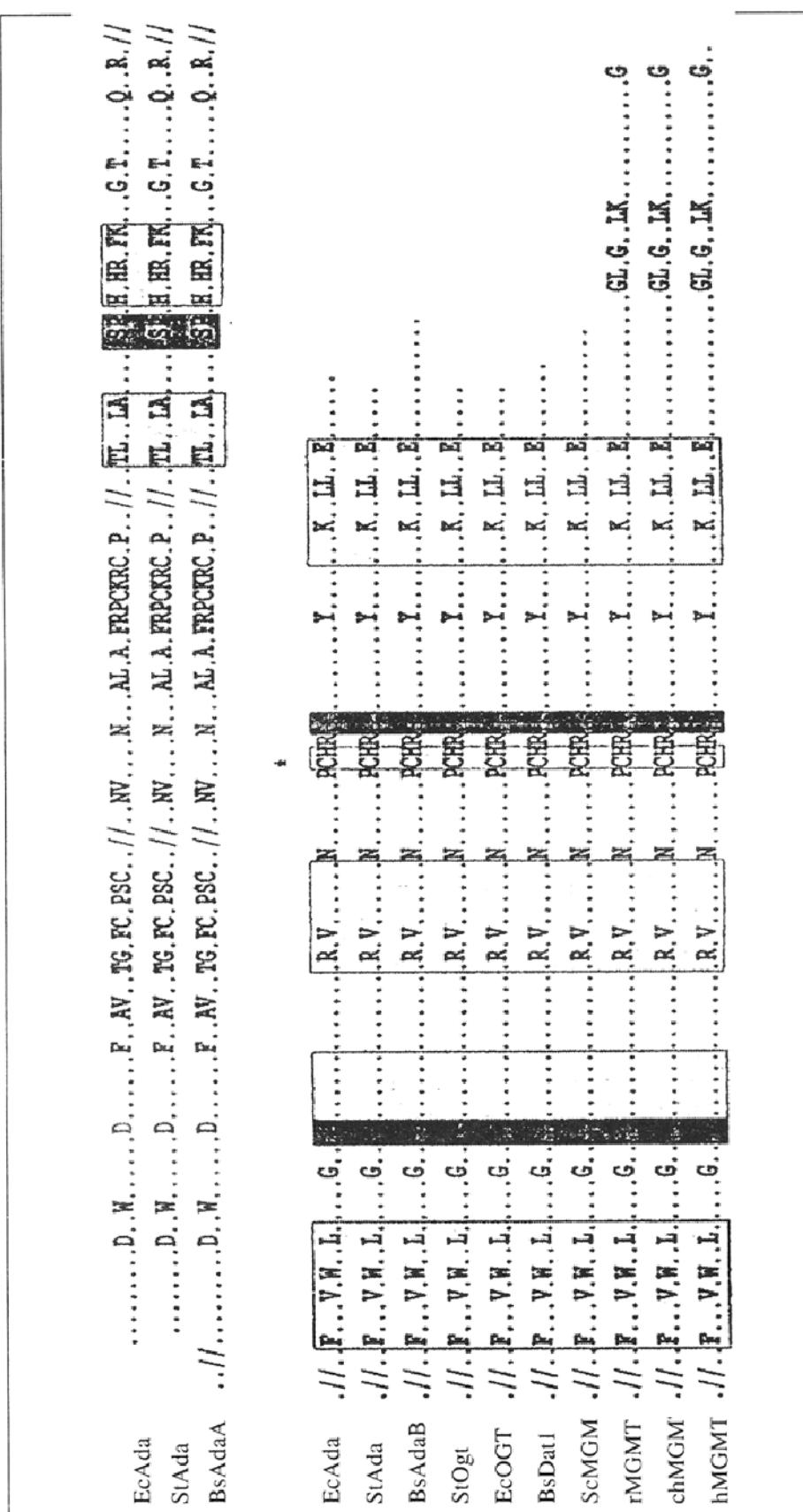


Fig. 2. Comparison of the amino acid sequences of O^6 -alkylguanine-DNA alkyltransferases from different organisms. Bold printed letters indicate amino acids. The homologous areas of the proteins of *E. coli* (*Ec*), *S. cerevisiae* (*Sc*), Rat (*r*), Chinese hamster (*Ch*) and human are given. The Two cysteines in the active center of the proteins are indicated by asterisks. Homologous secondary structures are shown as shaded areas. The lighter shading indicates alpha-helical structures, the darker indicates H^*P -pleated sheet structures. Data are taken from the gene bank of the National Institute of Health. Data concerning comparisons of sequences and secondary structures are from Samson, 1992; Moore *et al.*, 1994 and Pegg *et al.*, 1995.

single amino acids were exchanged to find out the association between structure and function. As expected, replacement of cysteine for other amino acids in the active center of alkyltransferases leads to a total loss of function (Tano *et al.*, 1989). Only very few exchanges which substituted proline, cysteine, histidine and arginine led to active proteins (Ling-Ling *et al.*, 1992). Even these exchanges led to a clearly lower activity of the enzyme (*e.g.*, change of histidine 146 for alanine or arginine 147 to alanine) and to a lower stability of the protein in *E. coli*. Not only amino acids in the active center, but also other amino acids have been shown to be important for the activity of the proteins. For example, an exchange of glutamine acid 172 for glutamine results in a complete loss of activity of human MGMT (Pegg *et al.*, 1995). These experiments show that these enzymes have been fine-tuned in structure and function during evolution.

In this respect transformation experiments with *E. coli* should be noted (Christians and Loeb, 1996). Human MGMT cDNAs with mutations in the area of the active center were used for transformation of bacteria which were unable to express their own alkyltransferase (*ada-*, *ogt-*) and tested for gain or loss of function by high-dose selection with an alkylating agent. The surviving bacteria expressed a functionally active, but nevertheless mutated human alkyltransferase whose amino acid sequence was determined. None of the analyzed alkyltransferases showed an exchange in the conserved area of the amino acid sequence. Interestingly enough, most of the selected alkyltransferases did not have the amino acid sequences of wild-type proteins. Of the isolated alkyltransferases, 70 % had an amino acid exchange at valine 139 to phenylalanine. Human alkyltransferase modified in this way gave rise to a higher survival in *E. coli* and to reduced frequency of mutations as compared to the wild-type alkyltransferase. Why evolution did not favour this amino acid sequence is unclear. One reason could be that interaction of alkyltransferase protein, which was mutated in this way (valine 139 to phenylalanine), with other proteins could impair its proper function. Therefore, this seemingly positive mutation could impose negative influence on other cellular activities. Alternatively it may be speculated that the stability of the mutated protein or the mode of degradation after the repair reaction is affected.

O⁶-Alkylguanine-DNA alkyltransferase can also accept ethyl groups from the O⁶-position of guanine. As compared to methyl groups, there are clear differences in the efficiency of this activity. Ada proteins from *E. coli* and alkyltransferase from rat accept methyl and ethyl groups with a similar efficiency, whereas human proteins are much less active in transferring ethyl groups when compared to methyl groups (Liem *et al.*, 1994). Since the alkyltransferases of rat and man have a high extent of homology (69 % identity on amino acid level, see Fig. 2), the different efficiencies with respect to repair of methyl and ethyl groups are likely explainable by few differences of sequences of amino acids in the active center area. In contrast to bacteria, eukaryotes have no alkyltransferases with associated alkylphosphotriester repair function. It seems that removal of alkyl groups from alkylphosphotriesters in the DNA is not absolutely necessary for the survival of cells following DNA alkylation.

4. Adaptive Response

Transfer of alkyl groups on cysteine 69 of the *E. coli* Ada proteins as well as respective alkylations of the Ada protein of *Salmonella typhimurium* and *Bacillus subtilis* are not a prerequisite for increasing the survival of cells upon alkylation. Instead they are sensitive intracellular sensors for the exposure of cells to alkylating agents. The alkylation signal induces an adaptive response in bacteria which protects cells from damage of further exposures. The alkyltransferase of fungi (Baker *et al.*, 1992) and mammalian cells, especially those of liver cells of rats, are also inducible. However, unlike the situation in *E. coli*, MGMT can be induced not only upon exposure to alkylating agents, but also by

other genotoxins, including ionizing radiation and UV-light (Laval, 1990; Fritz *et al.*, 1991; Fritz and Kaina, 1992; Grombacher and Kaina, 1996, see Fig. 1). In mammalian cells, DNA breaks are likely the signals for the induction of MGMT (Fritz and Kaina, 1992). The intracellular signal chain involved in MGMT induction in eukaryotes is not fully understood. It appears to be different, however, from the adaptive response in bacteria, because MGMT itself does not act as transcriptional activator (as the Ada protein does). Also, DNA alkylation is not required for eliciting the response. The adaptive response in bacteria appears to be a very specific inducible system elicited by and directed against DNA methylation (and ethylation) damage, whereas the response involving MGMT in mammalian cells seems to be related to a more general damage defense reaction of the cells, likely including many more genes and defense functions than in bacteria. Thus, *p53* appears to be required (Rafferty *et al.*, 1996; Grombacher *et al.*, 1998). Also, *Fos* and *Jun* may be involved in the adaptive response in mammalian cells (Boldogh *et al.*, 1998). These proteins are known to regulate various other genes, such as those involved in cell cycle checkpoint control, thus influencing the status of drug resistance. It is interesting that liver cells have the highest expression of MGMT when compared to other cell types. There is a close correlation between the extent of differentiation of liver cells and the expression of MGMT. This appears not to be correlated with the induction of MGMT by genotoxic stress (Grombacher and Kaina, 1995).

5. Evolution of Alkyltransferases

As already discussed, there are relatively extended homologies in the amino acid sequences of alkyltransferases in different organisms. The highest extent of homology can be seen in the active centers of the proteins (Fig. 2). Apart from this, there are many conserved amino acids outside of the active center which are mostly situated in the areas of the alkyltransferase which are responsible for their overall structure (Fig. 2). Outside of these functionally important areas there are variations in amino acids which can be correlated with the evolutionary distance of the organisms in which this has been analyzed. The extent of homologies between alkyltransferases of man and rats, mice and Chinese hamsters is 64 to 68% (Fig. 2). The homology between alkyltransferases of human and yeast (*Saccharomyces cerevisiae*) and *E. coli* is only 19 to 25%. There is 75% homology between *E. coli* and *S. typhimurium*. These findings show that, in bacteria, the alkyltransferases have an optimal sequence of amino acids which has not been changed considerably during further evolution. In areas of the enzyme which are not centrally important for their activity as repair proteins, a relatively high variability of amino acid sequences has been found. These changes may be responsible for the slight differences in the alkyltransferase activities in different organisms, such as different substrate affinities.

6. N-Alkylpurine-DNA Glycosylase

N-Alkylpurines (such as N7-alkylguanine, N3-alkyladenine, N3-alkylguanine) are formed following exposure of cells to alkylating agents. N-alkylpurines do not lead to mispairing and, therefore, they are not direct premutagenic DNA damages. They may be responsible, however, for the toxic activity of alkylating agents. The main mechanism of action seems to lie in a block of DNA replication caused by N3-alkyladenine and N3-alkylguanine as well as apurinic sites which are formed because of labilization of N-glycosylic bonds of alkylpurines in DNA.

All organisms analyzed so far have an effective system for repairing N-alkylpurines. The repair of N-alkylpurines occurs by base excision (see Table 1) and is mediated, in the first step, by N-alkylpurine-DNA glycosylase (also called N-methylpurine-DNA glycosylases, MPG). The damaged base is excized by enzymatic hydrolysis of the glycosidic bond between base and deoxyribose. The original DNA

sequence is reformed by consecutive activity of apurinic endonuclease, DNA polymerase and DNA ligase (for review see Friedberg *et al.*, 1995). N-alkylpurine-DNA glycosylases were first described in *E. coli*. The N-alkylpurine DNA-glycosylase AlkA of *E. coli* is part of the adaptive response of bacteria which, as outlined above, is regulated by the Ada protein (Lindahl *et al.*, 1988). Following exposure to alkylating agents, the synthesis of the AlkA protein is enhanced (Karran *et al.*, 1982). Comparable to the Ogt alkyltransferase, which is constitutively expressed in *E. coli*, bacteria have another constitutively expressed N-alkylpurine-DNA glycosylase, the Tag protein (see Table 2). As compared to the Tag glycosylase, AlkA has a broader substrate specificity. It leads to repair of N3-alkylguanine, O²-alkylpyrimidine, N7-alkylpurine, hypoxanthine and 1,N⁶-ethanoguanine (Lindahl *et al.*, 1988; Saparbaev and Laval, 1994; Saparbaev *et al.*, 1995). From these different substrate specificities, it can be concluded that the mechanism of action of the two enzymes is different. The N-alkylpurine-DNA glycosylase of eukaryotes with its broad substrate specificity is similar to the AlkA enzyme of *E. coli*. Both enzymes repair N3-alkyladenine and N7-alkylguanine (O'Conner and Laval, 1990; Chakravarti *et al.*, 1991) as well as 8-hydroxyguanine, hypoxanthine and 1,N⁶-ethanoadenine (Bessho *et al.*, 1993; Saparbaev and Laval, 1994; Saparbaev *et al.*, 1995).

7. Comparison of Amino Acid Sequences of N-Alkylpurine-DNA Glycosylases

Other than the O⁶-alkylguanine-DNA alkyltransferases Ogt and Ada of *E. coli*, there is no homology in the sequence of amino acids of the N-alkylpurine-DNA glycosylases AlkA and Tag (Fig. 3) irrespective of the fact that both enzymes catalyze the release of N3-alkyladenine. There are homologies between the inducible N-alkylpurine-DNA glycosylases Alk A of *E. coli* and *B. subtilis* (Morohoshi *et al.*, 1993). Also the MAG protein of yeast, *Saccharomyces cerevisiae*, shows partial homology to these bacterial glycosylases (Berdal *et al.*, 1990; Chen *et al.*, 1990; Morohoshi *et al.*, 1993).

There is extensive homology in the amino acid sequences of mammalian N-alkylpurine-DNA glycosylases (Fig. 3). In contrast, there is no homology of the mammalian enzymes to N alkylpurine-DNA glycosylases of *S. cerevisiae* or bacteria. Since the molecular mechanism of the activity of these enzymes is not very well known (at the time of writing the review), it is not yet possible to give an explanation for the function of conserved and not-conserved amino acid sequences in the different N-alkylpurine-DNA glycosylases. In mammalian cells, these enzymes are highly conserved in their amino acid sequences and, therefore, it can be assumed that the molecular mechanism of enzyme action is the same or at least very similar. Whether this is also true for the AlkA protein of *E. coli* and *B. subtilis* and MAG of *S. cerevisiae* is not yet clear.

8. Comparison of the Promoter Region of N-Alkylpurine-DNA Glycosylase of Mouse and Rat

The N-alkylpurine-DNA glycosylases of mammals are not only highly conserved in their amino acid sequences (Fig. 3); they are also conserved with respect to their regulation. This can be concluded from a comparison of promoters of MPG coding genes of rat (Grombacher and Kaina, 1996) and mouse (Tatsuka *et al.*, 1995). The promoters of the genes in both species display high sequence homology (Fig. 4). Since promoters are not coding for proteins, the selection pressure for these structures can be expected to be not as high as the one for coding genes. The extensive homology in the DNA sequence of both promoters, therefore, indicates a conserved regulation of these repair genes. Further cloning of promoters of other DNA repair genes will lead to distinction between essential and non-essential regulatory sequences. Research on the evolution of regulatory sequences of genes is still in its beginning.

ECTAG	MERCQWWSQDPLF ^a TAYHDNEWGVPETDSKKLFEMICLEGQQAGLSSWITVLICKRKENYRACFHQFDPPVCAAMQEEDVER
ECALKA	MYTLINWOPPYDWMLGFLAARAVSSVETVADSYYYARSLSLAGEYRGVVTAAIPDIAHTLHINLSSLAGLEPV
BSALKA	MTWHEVNDVIVITLPEITFDMNNANLGYLTREKNECMYEIENNITTIVKVIAGETRSLV . QVSUTINNKOMIVQFLINDSRPV
SCMAG	MKLKREYDELIAKDAVKEIAKELGSRPLEV
rmMPG	MPARGGSARPGRGSLKPKVSUTLLPDTEQPPFLGRARRPGNARAGSLVTGGYHEVGOMPAKPLSRKIGQRKQRLADSEQQ
mUMPG	MUTPALQMKKPKQFCRRMGQKKQRPARAGQPHSSSDAAQAPAEQPHSSSDAAQAPCP
hMPG	
ECTAG	LVDAGTIRHRGKIQATIGNARAYLQMEQNGEPPFDWNSFVNHQPOVQTQATTLESIPTSTSASDALSKALKKGKF
ECALKA	A . . . AECLAKMSRLPFDLQCN PQLVNGALGRLGAAR . PGRLLPGCVDAPEQGVRAILGQLVSVAMAAKL . TAR
BSALKA	EOWKREKIVKYTHEWDLNDLDTPLPFYEMAKADPLLKMPARKFYGLVIGIDPLDVFIRLASTLSSQAAESSTKARV
SCMAG	ALPEKTYARHEEKFMACNEFTLKDPSLFPILKNEFTYLKETQVPTNLEDVFIRLASTLSSQAAESSTKARV
rmMPG	TPKEKLSSTPGLLRSIXYPSSEPDPRARLFLGQVPLVRLRADGTTELGRGRIVETEAXLGPEDEAAH
mUMPG	TPKEKLLSTPGLLRSIXYPSSEPDHDHSRGRLGPFFDQPAUTLARAFLGLGQVPLVRLRADGTTELGRGRIVETEAXLGPEDEAAH
hMPG	ERCLGPPPTPGPYRSIXYPSSEPDQPAVFLARAFLGLFEDQPAVFLVRLPNGTTELGRGRIVETEAXLGPEDEAAH
ECTAG	VGTTCYSPFMQACGLLVNLDHUVGCCYPGNK.P
ECALKA	VAOLYGERLDDFPEYXICPPPTPORLAADPOALKAALGMLPLKRAEALTHLANAALLEGTLPMТИPQGDVEQAMXKTLOTFFP .
BSALKA	VEAFGDSIIEWNGKXWUWPPYERIARLPTDADIKMTVKKSKEYIIGIARLMASGELSREKLMKMNFKDAEKNLKIKR
SCMAG	VSLYGGCAFPPDKYKILFEDPFDKPARCAELIAKCGLSSKRKMITYLESLAVYFTEKYKDIEKLFGQKDNDEEVIESLVTNTVKG
rmMPG	SRGGRQTPRNRCGMPPKPGTLYVXLYLXGMYFCLNVSSQGAGACULRALEPLEGLETMRKOLRNSLRKSTVGRSILKDREL
mUMPG	SRGGRQTPRNRCGMPPKPGTLYVXLYLXGMYFCLNVSSQGAGACULRALEPLEGLETMRKOLRNSLRKSTVGRSILKDREL
hMPG	SRGGRQTPRNRCGMPPKPGTLYVXLYLXGMYFCLNVSSQGAGACULRALEPLEGLETMRHURSTLRKGTSASRVLKDRREL
ECTAG	GIGRTANTYPLMPCTARPTAAPPIDDVGLIHSIKILRNMRKPTKDEIISYPMKEWOSXATFYLMRVLX
ECALKA	GIGRTANTYPLMPCTARPTAAPPIDDVGLIHSIKILRNMRKPTKDEIISYPMKEWOSXATFYLMRVLX
BSALKA	G . . PWSAKMFLISGKRMIDVAPEDLGIAARGFSKYLSDKPELEKELMRERKVKKSKIKHKKYNWKIYDDIMEKCSE
SCMAG	CNGPSKLCQALARSKSFDQRDLAQDEAVCGWMSMALW . SPAASCGGSSRRYWPGRGMDETEALALLCPCGQPMGQCGGR
rmMPG	CSGGPSKLCQALATIDSFDQRDLAQDEAVWLEHGPLESSSPAVUAAAARIIGIGHAGNEWTOKPLRFYVOGSPPWVSVVDRV
mUMPG	CSGGPSKLCQALAINCSFDQRDLAQDEAVWLERGLEPLEPSEPA . UAAAARVGVGHAGNEWTOKPLRFYVOGSPPWVSVVDRV
hMPG	
SCMAG	TPSPYRSVFMFILWRLASTNTDAMMAEENFVKS
mUMPG	AEQMDQPOQQTACSEGLLIVQE
hMPG	AEQDTQA

Fig. 3. Comparison of amino acid sequences of N-alkylpurine-DNA glycosylases. Gold printed letters indicate identical amino acids. The homologous areas of the AlkA proteins of *E. coli* (Ec), *B. subtilis* (Bs), MAG proteins of *S. cerevisiae* (Sc) as well as of rat (r), mouse (mu) and human are given. Both groups of MAG proteins have no homologies. The Tag protein of *E. coli* has no recognizable homology to the other N-methylpurine-DNA glycosylases. The sequences were taken from the gene bank of the National Institute of Health.

Fig. 4. Base sequence of the promoters of the MGMT genes of rat and mouse. Identical bases are connected by vertical dashes. Sequence homologies in areas where transcription factors bind and the ATG start codon for the translation of both genes are indicated in bold printed letters. The starting point of transcription for both genes is indicated by pointers. The promoter of rat has an insertion of 289 base pairs which is missing in the promoter of the mouse gene. Sequence data are from Tatsuka *et al.*, (1995) and Grumbacher and Kaina, (1996).

9. Some Aspects of DNA Repair in Plants

In view of different endogenic factors as well as chemical and physical agents in the environment plants are exposed to, such as pesticides, microbial toxins, UV irradiation and ionizing radiation, it is clear that effective DNA repair functions have to be evolved in plants. Since repair processes in such different organisms as bacteria and human are remarkably conserved, it can be expected that DNA repair mechanisms in plants should also contain conserved elements with respect to damage specify, enzymatic mechanisms and regulation. However, in contrast to the well known repair mechanisms in bacteria and mammals very little information is available on these aspects of DNA repair in plants. Various DNA repair functions analyzed in plants are summarized in Table 3.

10. Repair of UV-Induced DNA Damage in Plants

One should expect that plants, which are dependent on exposure to light and which are, therefore, permanently exposed to it, should have very efficient systems for repairing UV-induced DNA damage. Basically, UV-induced pyrimidine dimers can be repaired by two mechanisms, namely by reversion of the damage with photolyases and by nucleotide excision (Table 1). Photolyases were found in plants (Table 3). It would be interesting to analyze whether the activity of photolyases from plants are comparable to those found in bacteria and various animals such as the fruit fly *Drosophila melanogaster* and the marsupialian *Potorous didactylis*. What wave-lengths are activating the chromophore and, in general, what importance photoreactivation has in plants? In mammals photoreactivation very likely does not play a role in the repair of pyrimidine dimers at all.

Like in other organisms, in plants UV-induced lesions and DNA adducts induced by chemicals are repaired by nucleotide excision. In mammals, at least 25 different proteins functioning as enzymes and regulatory proteins are involved in nucleotide excision repair. Some enzymes and nucleotide excision repair genes have also been characterized in plants up to now (Table 3). Analyses concerning homologies between plant and bacteria or mammalian cells have not yet been done.

11. Repair of DNA Alkylation Damage in Plants

Apart from endogenous processes (*e.g.*, caused by S-adenosylmethionine) leading to spontaneous DNA methylation, alkylating agents (*e.g.*, nitrosamines) in air and soil may lead to N-alkylpurines and O⁶-alkylguanines notably in plants. Also in plants these lesions are premutagenic in that they lead to GC to AT base substitutions (Dolferus *et al.*, 1990; Orozco *et al.*, 1993). Strangely enough, it was not possible up to now to prove unambiguously the existence of alkyltransferases in plants like the ones found in bacteria, fungi and mammalian cells. Investigations conducted with the unicellular green alga *Chlamydomonas* showed that methylguanine lesions induced by N-methyl-N'-nitro-N-nitrosoguanidine persist in *Chlamydomonas* for at least 30 hours without any measurable repair of the damage (Frost and Small, 1987). Mammalian cell lines and knock out mice that are deficient in MGMT are unable to repair O⁶-methylguanine (Day *et al.*, 1980; Tsuzuki *et al.*, 1996). Nevertheless the accumulation of this DNA lesion apparently does not interfere with the survival of these cells and organisms, at least under laboratory conditions. In view of the high mutagenic and carcinogenic potential of O⁶-alkylguanine, it seems to be highly improbable that under natural conditions higher organisms exist which do not have the MGMT repair function. Recently an enzyme from *Arabidopsis thaliana* which is involved in base excision repair (3-methyladenine-DNA glycosylase) was cloned (Santerre and Britt, 1994). The cDNA of this enzyme shows sequence homology in few areas to the MPG protein of rat (see Fig. 5) and man, but there is no sequence homology to the respective

Table 3. DNA repair in plants

DNA repair mechanism	Plant	Protein	Gene	Substrate/Activity	Reference
1. Damage reversal					
Photoreactivation	<i>Arabidopsis</i>	—	HY-4 (Homology to microbial pyrimidine dimer photolyases)	Blue-light receptor; no photoreactivating function	Nakgitra et al., 1995
White mustard	—	SA-phrl	Blue-light receptor	Bartschauer, 1993	
<i>Arabidopsis</i>	—	constitutively expressed	Repair of 6-4 photoproduct in vivo	Chen et al., 1994	
<i>Arabidopsis</i>	no homology to bacterial photolyases	DRT 103 (cDNA)	Complementation of <i>phrl</i> mutation in <i>E. coli</i>	Pang et al., 1993	
O ⁶ -Methylguanine-DNA methyltransferase	<i>Chlamydomonas</i>	—	—	—	Frost and Small, 1987
2. Excision of DNA damage					
3-Methyladenine-DNA glycosylase	<i>Arabidopsis</i>	28.4 kDa	ATMAG	Removal of 3-methyladenine from DNA	Santerre and Britt, 1994
Uracil-DNA glycosylase	different species; e.g. potatoe	Immunological cross-reactivity with mammalian glycosylases	—	Removal of UMP from calf thymus DNA in vitro	Bones, 1993; Talpaert-Borle, 1987
UV endonuclease	Spinach	—	—	Single-strand endonuclease; specific for 6-4 photoproducts	Strickland et al., 1991
<i>Arabidopsis</i>	—	UVRI (not yet cloned)	Excision of 6-4 photoproduct	Britt et al., 1993	
<i>Arabidopsis</i>	no homology to other repair proteins found	DRT 101, 102 (cDNA's)	Complementation of <i>E. coli</i> UvrB ⁻ and UvrC ⁻ mutation (UV-endonuclease activity)	Pang et al., 1993	
<i>Lucerne</i>	—	—	Excision of pyrimidine dimers	Quaite et al., 1994	

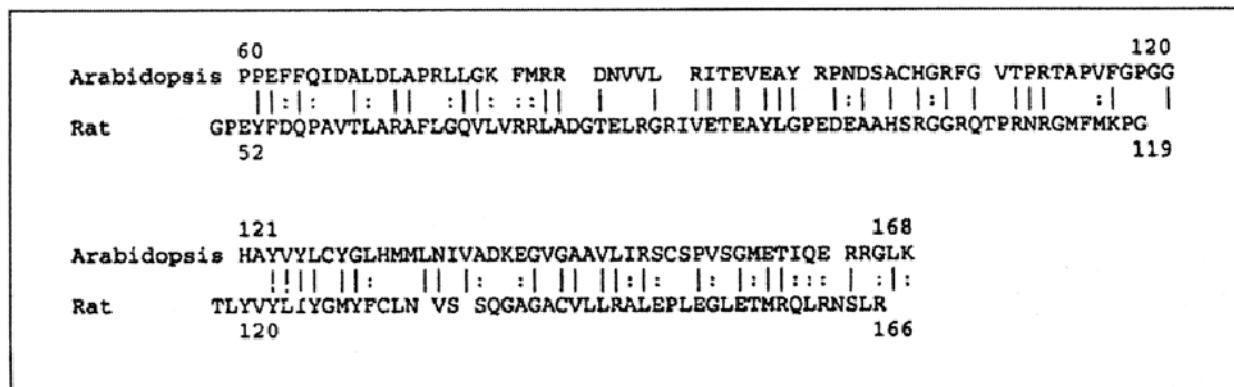


Fig. 5. Partial comparison of the sequence of N-alkylpurine-DNA glycosylase of *Arabidopsis thaliana* and rat. Identical bases are connected by a vertical solid line. Similar bases are connected by dotted lines. The sequence data are from Santerre and Britt (1994).

glycosylases AlkA and Tag from *E. coli*. The evolutionary diversification of N-methylpurine-DNA glycosylases from bacteria and eukaryotes can be explained by assuming that, because of the instability of the N-glycosidic binding of 3-methyladenine, the enzymatic removal of the alkylated base does not need a highly conserved enzyme structure. Another possibility is that the sequences known up to now belong to a larger family of N-glycosylases whose function is not exclusively bound to the removal of N-methylpurines (Seeberg *et al.*, 1995).

12. Repair of Base Deaminations

Another relevant DNA damage is deamination of cytosine and methylcytosine which can be induced by spontaneous or chemical hydrolytic deamination of the respective bases. Deamination of cytosine leads to uracil which is recognized as wrong base and enzymatically removed. The uracil-DNA glycosylases are important and highly specific enzymes which have been analyzed so far. These enzymes excise exclusively uracil and hydroxy-uracil in DNA, but not in RNA. The enzymes are highly conserved with 66 % identity of amino acid sequences between bacteria and human (Seeberg *et al.*, 1995). The existence of uracil-DNA glycosylase in plants was proven by the activity of the enzyme (Table 3) (Britt, 1996). The respective gene was not isolated up to now.

Plants are also supposed to protect themselves against the mutagenic potential of deaminations of 5-methylcytosine. Deamination of 5-methylcytosine leads to thymine and hence to T-G mispairs which is an important premutagenic lesion leading to C to T transitions. In bacteria as well as in mammalian cells, G-T mispairings are corrected by mismatch repair. For this type of DNA repair, at least four different proteins are required, among them are *Mut H* and *Mut S* (Kolodner, 1996). Deficiency in mismatch repair leads to genomic instability and to a higher frequency of tumours, which was observed both in man and mouse (Eshleman and Markowitz, 1996). Plant genomes, as compared to human DNA, have an up to ten-times higher amount of 5-methylcytosine. Therefore, it could be expected that the frequency of deaminations leading to C to T changes would be quite high in the genome of plants, if there would not be an efficient mechanism of repair of these lesions. It is interesting to speculate that in consequence of the enormous size of some plant genomes, mutations in non-coding areas (such as introns and repetitive DNA) as well as in repetitive genes might be induced with high frequencies in plants. This could be an important aspect of plant evolution.

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9. Cytogenetics of Lepidopteran Insects

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Abstract: Lepidoptera is one of the largest orders of class Insecta having approximately 100,000 known species. The cytogenetic work has been carried out only on about 2000 species so far, may be, due to technical difficulties in handling lepidopteran chromosomes. This group shows some interesting features regarding high chromosome number, their small size, female heterogamety and sticky nature of chromosomes. There exists a controversy regarding the centromeric organization. While EM studies and experimental data show the presence of holocentricity, some cytological observations tend to prove monocentricity. There are very few reports on the banding pattern analysis of lepidopteran chromosomes. The distribution of chromosome numbers in various groups of this order show wide ranges but there are clear cut concentrations around a modal number in each case. The sex chromosomes have also been identified only in a very few species. The most common sex-chromosome mechanism is ZZ: ZW although ZZ/ZO, XY1Y2: XX as well as A Z: ZZ have also been reported. The females of this group exhibit achiasmatic meiosis and non-homologous telomeric association between the bivalents.

Key words: Chromosomes, sex chromosomes, evolution, lepidoptera.

1. Introduction

The order Lepidoptera including butterflies and moths is one of the largest orders of class Insecta. There are approximately 100,000 species known taxonomically (Imms, 1977). In larval stages, majority of species are of great importance. A large number of injurious species devour the foliage and shoots of trees and crops, some bore into stem or attack underground parts and many are harmful to timber. Still others attack manufactured goods like carpets, clothings, and stored products like grain and flour. Some of the species are predacious to lac insect, while others live in beehives. However, saturnids and *Bombyx mori* confer a direct benefit upon man from the fact that they yield silk of commercial value. Lastly they are efficient pollinators.

This order has been intensively studied taxonomically, but its cytogenetic knowledge has remained at its lowest due to certain technical difficulties. This group has high chromosome number and small size of chromosomes, generally dot-like isodiametric bodies. Moreover, the chromosomes have tendency to clump together at metaphase.

The karyology of Lepidoptera has many other interesting features in addition to high chromosome number and their smaller size. The nature of centromere in these insects has remained controversial from holokinetic to monokinetic, the females are heterogametic and meiosis in them is achiasmatic.

In Lepidoptera, cytogenetic findings have usually been referred as haploid chromosome numbers from male meiotic stages. The work in this field was started by Henking (1890) and since then, the chromosome complement of a large number of species has been reported by various workers. After

more than hundred years of the beginning of lepidopteran cytology, the data available is only for about 2000 species that form only 2% of the available fauna. The chromosome banding pattern studies are still scarce (Bigger, 1975, 1976; Rishi and Rishi, 1990) and the heterochromatin pattern or C-band studies are only by Maeki, (1981a); Nilsson *et al.*, (1988) and Rishi and Rishi, (1990). There is single reference available for NOR-Ag staining by Bedo (1983).

2. Chromosome Number and Evolution

The studies on chromosome number of Lepidoptera initiated by Henking (1890) were followed by many workers and in the mid 20th century, about 245 species were known as reported by White 1954. Later on many workers added data to this list (Bigger, 1960, 1961, 1975, 1976; de Lesse 1952, 1953a, b, c, d, 1954, 1955a, b, c, d, 1958, 1959a, b, c, 1960, 1961, 1964, 1966, 1967, 1968, 1970; Maeki, 1953a, b, 1957a, b, c, 1958 a, b, c, 1959, 1961, 1980a, b, 1981a, b, 1982, 1986, Maeki and Ae, 1966, 1968a, b, 1970; Maeki and Makino, 1953; Maeki and Ogata, 1970; Maeki *et al.*, 1965; Maeki and Remington, 1959, 1960a, b, c; Saitoh, 1959a, b, 1960, 1966, 1968, 1988, 1989; Saitoh and Abe A, 1969, 1970a, b, 1994; Saitoh *et al.*, 1969, 1973, 1989, 1981; Saitoh and Kudoh 1968, 1971, 1972a, b; Saitoh and Kumagai, 1973; Suomalainen, 1965, 1969a, b, 1971; Suomalainen *et al.*, 1971, 1973; Larson, 1975; Ennis, 1976; Trentini and Marini 1986; Kawazoe, 1992; Brown *et al.*, 1993). These contributions were mainly from European fauna. The Indian fauna was exposed for cytogenetic studies by Srivastava and Gupta, 1961; Gupta, 1964; Gupta and Narang, 1980; Rishi, 1973, 1975; Rishi and Rishi, 1977, 1978, 1979, 1981a, b, 1985, 1990, Rishi *et al.*, 1992; Mohanty and Nayak, 1983; Sharma and Bajwa, 1992, 1995 a, b. With all these studies, the number of species known cytologically has gone up to approximately 2000. The examination of chromosome number in different species of Lepidoptera shows the most common haploid number to be $n = 31$ (Robinson, 1971; Rishi, 1973; White, 1973; Nayak, 1975; Werner, 1975; Ennis, 1976; Kaur, 1988; Sharma *et al.*, 1993, Belyakova and Lucktanov, 1994. Sharma and Bajwa, 1992, 1995a, b, Sharma 1999, 2000 a, b). However, the range of variations in chromosome number of Lepidoptera are very vast i.e., from $n = 8$ in *Padotricha euchroia* to $n = 141$ in *Lysandra nivisens* (de Lesse, 1967, 1960). There is a wide variation in chromosome number in closely related forms, while the widely distant families and genera have the same haploid number which results in tremendous difficulty in determining relationship of lepidopterans on cytological basis.

According to White (1954), 31 is the most frequent number, but 29, 30 and 31 are so nearly equally common that it does not seem legitimate to consider any one of them as the type number in preference to others. On the other hand, Beliajeff (1930) considered $n = 30$ as the ancestral number common to Lepidoptera and Trichoptera. However, it can be interpreted on the basis of available data that 31 is the commonest haploid number and there is a marked modes at 29 and 31. Nevertheless it is quite difficult to establish an ancestral number as there are families or subfamilies without any member with $n = 31$ (as in Nymphalidae) or $n = 30$ (as Limenitini and Papilionidae) (White, 1973). As a whole in Lepidoptera remarkable stability in chromosome number is clear although variations do occur in many genera and species. These variations can be considered to be due to simple fusion and fragmentation of chromosomes.

Lorkovic (1941, 1949) was of the opinion that the species with higher number of chromosomes were of polyploid origin on the basis of cytological data on three genera *Lycaena*, *Leptidea* and *Erebia*. But the evidence advanced by various workers (Stebbins, 1950; White, 1954; Golysheva, 1961; Gupta, 1964; Suomalainen, 1979) go against the view of polyploid evolution in Lepidoptera.

The studies on different species of Genus *Vanessa* with $n = 31$ (Beliajeff, 1930; Federley, 1938;

Lorkovic, 1941; Maeki, 1961; de Lesse, 1967 and Kaur 1988) and *Vanessa indica* having $n = 15$ in Indian individuals (Gupta, 1964) and $n = 31$ in Japanese form (Maeki and Makino, 1953; Maeki, 1961) point towards the role of fusion of chromosomes in the two geographically different forms of the same species. The assumption of diffused kinetochore provides a basis for this view which is in accordance with fusion and fragmentation hypothesis put forth by various investigators (Seiler, 1914, 1922; Harrison and Doncaster, 1914; Malan, 1918; Seiler and Haniel, 1921; Crestschmer, 1928; Beliajeff, 1930; Federley, 1938; Dederer, 1940; White, 1946, 1954, 1957; Suomalainen, 1953, 1958, 1963; de Lesse, 1954, 1955d, 1959a; Golysheva, 1961; Bauer, 1967; Murakami and Imai, 1974; Ennis, 1976).

Harrison and Doncaster (1914) reported $n = 14$ and 56 for two species of genus *Biston*. Beliajeff (1930) ruled out the possibility of role of polyploidy on the basis of comparisons with another species *B. pomonaria* with $n = 51$ (Malan, 1918) which does not fall in the series of polyploidy. Similarly, role of polyploidy in this group of insects has further been ruled out by the studies on different species of genera *Dasychira*, *Erebia* and *Lysandra* with different haploid numbers. So it can be assumed that aberrant chromosome numbers in Lepidoptera did not arise abruptly and they probably represent the end products of evolutionary process, each step of which was an increase or decrease in the number by one element. The frequency of number of species with extreme aberrant chromosome number is comparatively less.

Thus, the fragmentation and fusion hypothesis is a more plausible explanation for the distribution of chromosome numbers in Lepidoptera. The species with higher numbers are comparatively fewer than those with lower numbers, if 31 is taken as the ancestral number. Thus, the trend of evolution is more towards the lower side of the scale than the higher number.

3. Chromosome Banding in Lepidoptera

It is very difficult to get banding patterns in lepidopteran chromosomes as they are highly condensed at metaphase. G-banding procedures have not been much successful in producing linear differentiation of lepidopteran chromosomes. Replication banding which differentiates between early and late replicating portions of chromosomes and restriction enzyme banding also have not been applied to the chromosomes of this group. Bedo (1983) applied C-banding and fluorescent staining with quinacrine and Hoechst 33258 to chromosomes of *Phthorimaea operculella*, but did not succeed. However, he could highlight nucleoli in the interphase and early to mid prophase cells.

G-banding has been applied to a few species by Bigger (1975, 1976) and Rishi and Rishi (1990) on early metaphase chromosomes that are quite elongated. A large number of bands were observed throughout the length of chromosomes. The bands are constant and specific for each homologous pair (Maeki *et al.*, 1990).

C-banding was initiated by Venkatachalaiah (1988) in *Bombyx mori* and then in *Pieris brassicae* by Rishi and Rishi (1990). According to Gassner and Klemetsen (1974) and Traut (1986) mitotic chromosomes of some Lepidoptera have very large single kinetochore. Goodpasture (1976) employed heterochromatin staining procedure and observed a distinct chromomere pattern at pachytene. Traut and Rathjens (1973) also described distinct chromomere and interchromomere patterns on pachytene autosomal bivalents of *Ephestia kuhniella*.

C-banding can also help in detecting sex-chromosomes in Lepidoptera (Traut and Rathjens, 1973; Maeki, 1981b; Nilsson *et al.*, 1988). It has been opined by workers that primary step in differentiation of sex-chromosomes is the concentration of highly repetitive DNA sequences i.e., heterochromatization in W/Y which precedes morphological differentiation of heteromorphic sex-chromosomes.

4. Nature of Centromere

Both types of centromeric organization, holokinetic as well as monokinetic, have been claimed for lepidopteran chromosomes (Bigger, 1975, 1976; Traut, 1986). Holokinetic nature was deduced from the missing primary constriction and a parallel separation in mitotic anaphase (Murakami and Imai, 1974), chromosome fragmentation and fusion in evolution giving rise to highly different chromosome numbers in related species (Suomalainen, 1953), high doses of X-rays necessary to induce sterility, (North and Holt, 1968) variability of chromosome fragments (Maeki, 1981a) and high rate of viability of X-ray induced translocations (Bauer, 1967). Electron microscopic studies further supplement the evidence for the holokinetic structure of lepidopteran chromosomes as in M-1 chromosomes of *Eumeta variegata*, the microtubules were located all along the length of the chromosomes (Maeki, 1981a).

Earlier workers were unanimous about diffused nature of the lepidopteran centromere (White, 1957; Darlington, 1958; Smith, 1960). Similarly many workers have favoured the presence of diffused centromere (Virkki, 1963; Guthrie et al., 1965; Jolly et al., 1970; Puttaraju and Nagaraju, 1985; Padhy, 1986; Padhy and Nayak, 1986; and Sharma et al., 1993). The karyotypes prepared by Narang and Gupta (1979c) from spermatogonial prometaphase chromosomes of *Philosamia ricini* revealed much elongated chromosomes lacking primary constriction. Even colchicine treated mitotic metaphase chromosomes of *Charaxes jasus* revealed clearly the parallel splitting of chromatids without any detectable primary constriction (Trentini and Marini, 1986).

Bigger (1975, 1976) reported variations in centromeric structure at mitosis and meiosis. According to him, the primary spermatocyte and oocyte plates revealed holocentric meiotic chromosomes, but monocentric chromosomes were found during early stages in some butterflies. Various studies on nature of centromere in Lepidoptera during meiosis suggest that there are no constrictions present in the meiotic chromosomes.

In contrast to the above findings, a monokinetic organisation of mitotic chromosomes in Lepidoptera has been derived from electron microscopic studies of *Ephestia* and *Trichoplusia* chromosomes (Gassner and Klemetson, 1974). A monokinetic structure of mitotic chromosomes is further deduced from observations of primary constriction in *Pieris brassicae*, *Polyommatus icarus*, *Pyronia tithonus*, *Pieris napens*, *Pieris rapae*, *Trabala vishnu* and *Danaus limniaceae* (Bigger, 1975, 1976; Rishi and Rishi, 1978, 1979, 1981a,b, 1985, 1990). The G banded karyotypes and diagrammatic representations by Bigger (1975, 1976) clearly reveal chromosomes of different centric types. The C-banding protocol applied successfully to somatic metaphase chromosomes of *P. brassicae* by Rishi and Rishi (1990) revealed conspicuously large heterochromatin blocks located pericentrically pointing out the centromeric position.

The presence of localized centromere in early mitotic stages is quite interesting in relation to theories proposed to explain karyotypic evolution in Lepidoptera. Because in such a case reappraisal of fusion and fragmentation hypothesis becomes necessary. Suomalainen (1969a) pointed out that the chromosome numbers in Lepidoptera are less variable as the diffused centromere would theoretically allow. So the role of structural rearrangements becomes more implicated under the present situation.

Therefore, both types of kinetic organisations have been claimed for lepidopteran chromosomes. Traut (1986) also interpreted that his results on *Ephestia* did not fit in with either typical holokinetic or a typical monokinetic structure and, therefore, the dual nature of kinetochore organization.

According to Bauer (1967), holokinetic chromosomes have evolved from eukinetic ones and so perhaps this primary centromere organization is a restrict phenomenon. The primary centromere may not have any function at all other than holding chromatids together at prometaphase, but the disappearance

of constriction early in metaphase may simply reflect the evolutionary change from monokinetic to holokinetic chromosomes.

5. Sex-Chromosomes

In earlier cytogenetic studies, the heterogametic sex could not be predicted on the basis of chromosomes (Beliajeff, 1930; Federley, 1945; Deodikar *et al.*, 1962; Gupta, 1964; Saitoh and Abe, 1969). Other workers reported female heterogamety mainly on the basis of genetical experiments. (Doncaster and Raynor, 1906; Goldschmidt, 1934, and Tanaka, 1953). The cytological identification (evidence) for the heteromorphic sex chromosomes is lacking as about 100 species from a total of about 2000 cytologically worked out forms, have been described to possess sex-chromosomes with female heterogamety (Traut and Mosbacher, 1968; Suomalainen, 1969b, 1971; Suomalainen *et al.*, 1973; Traut and Rathjens, 1973; Bigger, 1976; Ennis, 1976; Fontana, 1976; Weith and Traut, 1980; Nilsson *et al.*, 1988; Kawazoe, 1992). Almost all types of sex-chromosome mechanisms have been described in Lepidoptera with female heterogamety. The ZW: ZZ/XY: XX, ZO: ZZ/XO: XX, XY₁ Y₂: XX as well as AWAZ: ZZ sex-chromosome systems appear to have evolved several times in these insects.

Suomalainen (1971) found unequal sex chromosomes in *Lozotaenia forsterana*, but he stated that unequal sex chromosomes are rare in butterflies and usually sex-chromosomes are similar in size. Later studies have shown varying degrees of heteromorphism of sex-chromosomes. Maeki and Miyawaki (1987) described karyotype of *Pieris rapae crucivora* from mitotic chromosomes of embryonic cells and revealed heteromorphic sex-chromosomes with a largest Z and smallest W. Maeki (1981b), Maeki *et al.* (1990) and Kawazoe (1992) showed W to be the largest in female karyotypes of *Graphiae sarpendon* and *Pachyligia dolosa*. W chromosome in 16 species of Lepidoptera was shown to be the largest and Z chromosome was still longer than the largest autosome in 8 species.

As male is homogametic, an analysis of both sexes becomes necessary for designating any heteromorphic chromosome pair as the sex - chromosome pair. The differential staining techniques of G and C-banding can prove quite helpful in confirming sex-chromosomes, however, at present there are very few reports in Lepidoptera.

Several lepidopteran species lack W chromosome and thus females are ZO types (Seiler, 1922; Traut and Mosbacher, 1968; Rishi and Rishi, 1985). Gupta and Narang (1980) reported an interspecific chromosomal polymorphism in *Philosamia cynthia ricini*. The variation is interpopulational and in female sex only. Titabar population has $2n = 28$ and Borduar-Dhenubhang population has $2n = 27$, the lost chromosome in the latter is the Y(W) of XY (ZW) sex chromosome mechanism.

In most Lepidoptera, the sex is determined very likely by the sex-genes, but they do not seem to have any chromosome differentiated as sex chromosome (Tanaka, 1953; Tazima, 1964; Traut, 1976; Doira 1986; Barlett and Fosse, 1991).

Studies on heteropycnotic sex-bodies in female interphase nucleus have revealed that there is present a distinct sex - chromatin in interphase of somatic nuclei in many lepidopteran species (Traut and Mosbacher, 1968; Bigger, 1975). Presence of female specific sex-chromatin body interpreted as W has further been supported by observations of Suomalainen (1969) on *Bactra lactaena* where two such bodies of unequal size were visible, corresponding to the two W-chromosomes of sex-chromosome trivalent. Ennis (1976) found that out of 103 species examined, 78 had sex chromatin positive females and negative males.

Crossing over does not take place in the heterogametic female sex. Females of *Ephestia kuehniella* are typically the heterogametic sex (Marec and Traut, 1994) like other lepidopterans. The sex-chromosomes, W and Z appear to be completely non-homologous (Weith and Traut, 1986), but are

paired in pachytene stage of M-I and form a synaptonemal complex (SC) (Rasmussen, 1976, 77; Weith and Traut, 1980). However, sex-chromosome pairing in contrast to that of autosomes is frequently incomplete and the W and Z chromosome axes of SC often display length differences (Weith and Traut, 1986; Marec and Traut, 1993). Pairing behaviours in both cases is different. Marec and Traut (1993) and Hogan *et al.* (1992) have put forth two phases of W: Z pairing. In 1994, Marec and Traut described two more phases in pairing of Z and W. All the four phases of sex-chromosome pairing can be observed in pachytene when all the autosomes are fully applied (Hogan *et al.*, 1992; Wang *et al.*, 1993).

Two basic paths can be postulated for the origin and development of sex-chromosomes in Lepidoptera. In the first, the differentiation of sex-chromosomes may primarily be related to structural rearrangements, resulting in a process for prevention of meiotic exchange. In the second case, the differentiation between Z and W chromosomes might have resulted from an initial process of heterochromatization of the latter.

The multiple sex-chromosomes described in some species of Lepidoptera show that structural modifications are the primary factors of the differentiation of these chromosomes. C-banding of AA WZ : ZZ sex chromosome system show the occurrence of constitutive heterochromatin associated with the corresponding W-chromosome (Nilsson *et al.*, 1988). The general C-band pattern in these cases of special sex-chromosome mechanisms seems to refer to pre-existing heterochromatin portions involved in the differentiation of these systems. Heterochromatization involving any element of trivalent at later stage might interfere with its meiotic equilibrium. However, C-banding data of sex chromosomes in Lepidoptera is scarce and these considerations are not concrete.

6. Female Meiosis in Lepidoptera

The absence of chiasma in the oogenesis of Lepidoptera was first noted by Bauer (1933). Maeda (1939) observed distinct chiasmata in spermatogenesis of silk worm, but proposed that chiasmata are not in the females. Bauer concluded from the photographs of oogenesis of *Erebia medusa polaris* (Federley, 1938) that no chiasmata are formed in oogenesis of butterflies. Suomalainen *et al.* (1973) while studying oogenesis in Heliconine butterflies described the bivalent to be parallelly oriented, well separated from one another during late diplotene, diakinesis and first metaphase. The achiasmatic meiosis in female lepidopterans has been confirmed by many workers (Murakami and Imai, 1974; Fontana, 1976; Narang and Gupta, 1979; Trentini and Marini, 1986; Nokkala, 1987; Nilsson *et al.*, 1988; Barlett and Fosse, 1991; Kawamura and Niino, 1991; Marec and Traut, 1993).

The achiasmatic meiosis in females has been established in members of the primitive as well as advanced families of Lepidoptera, indicating it as a common feature of this group.

The lack of chiasmata in female Lepidoptera is accompanied by the lack of crossing over. This implies that in all breeding experiments where recombination is being studied, test crosses should be set up using both sexes, the females to establish linkage groups and the males to establish recombination fractions. The absence of recombination of linked loci has been observed in females of *B. mori* (Sturtevant, 1915; Tazima, 1964), *Galleria mellonella* (Smith, 1938) *Helioconius* sps. (Turner and Sheppard, 1975) and *Ephestia kuehniella* (Traut, 1977).

In females, a non-homologous telomeric association between bivalents has also been observed by Nilsson *et al.* (1988) in six species of small ermine moths and Nokkala (1987) in *Sphinx ligustri*, resulting in formation of long chains.

Conclusions

The review of available data on lepidopteran cytogenetics shows that there are many questions still unanswered with regard to the nature and behaviour of centromere, sex-chromosome mechanisms and sex-determination and non-homologous telomeric associations during M-1. Such problems can be solved in this group of insects by molecular studies.

So the molecular studies are warranted in this group of insects in order to unfold the mysteries of chromosome structure and behaviour.

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10. Chromosome Structure in Fishes

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Abstract: Cytogenetic studies have shown that several characteristics of fish chromosomes, such as nucleolus organizer regions, satellite and telomeric sequences are similar (and even have the same nucleotide sequence in the case of telomeres) to those observed in the chromosomes of other eukaryotes. The chromosomes of warm-blooded vertebrates display patterns of longitudinal bands resembling G-and R- bands plus some heterochromatin when stained with fluorochromes which are associated with A/T-or G/C-rich DNA. In contrast, the staining of fish chromosomes with the same fluorochromes does not produce longitudinal bands suggesting that differences exist between the chromosomes of warm-and cold-blooded vertebrates, particularly A/T-and G/C-rich chromosomal segments. In the present paper we discuss the hypothesis that the occurrence and distribution of retroelements, such SINEs and LINEs, may play an important role for the origin of A/T- and G/C-rich euchromatic bands in fish chromosomes.

Key words: Chromosome structure, retroelements, SINE, LINE, fish.

The first studies concerning the morphology of fish chromosomes began at the end of the last century. Only recently, with the development of new techniques, a large number of fish species have been the focus of cytogenetic investigation. The karyotypes of about 3,000 fish species are currently known (Klinkhardt *et al.*, 1995). A significant fraction of fish cytogenetic studies have been conducted with systematic and/or taxonomic objectives. More recently, however several chromosome banding techniques, mainly developed to study mammalian chromosomes, have been employed in studies of fish chromosome structure, including the use of fluorescent *in situ* hybridization (FISH).

Cytogenetic analysis has shown that several characteristics of fish chromosomes, such as nucleolus organizer regions, satellite and telomeric sequences are similar (and even have the same nucleotide sequence in the case of telomeres) to those observed in the chromosomes of other eukaryotes (Almeida-Toledo, 1996). One of the most intriguing questions regarding fish chromosomes, however, is why it is so difficult to obtain a clear, reproducible pattern of euchromatic bands in their chromosomes? Although some authors have suggested that the answer to this question is related to technical factors (Gold *et al.*, 1990), the large number of species studied over several years suggests that the chromosomes of fishes differ in certain structural components compared to the chromosomes of warm-blooded vertebrates.

Historically, the development of human and mammalian cytogenetics experienced a significant advance in the 1970s with the development of G-and R-banding techniques which display patterns of longitudinal bands in the euchromatin of chromosomes (Sumner, 1990). "G-" and "R-band"-like patterns can be generated in fish chromosomes (Wiberg, 1983, Delany and Bloom, 1984, Almeida-Toledo *et al.*, 1988, Gold *et al.*, 1990, Sánchez *et al.*, 1993; Bertollo *et al.*, 1997; Maistro *et al.*, 1999).

The “G-bands” can be obtained with different trypsin treatments, as in mammalian chromosomes. However, the production of “R-bands” with hot buffers, as usually done for mammalian chromosomes, does not work for fish chromosomes. Alternatively, “R-band”-like patterns have been obtained in fish chromosomes with the use of 5-bromodeoxyuridine incorporation during the S phase of interphase (Delany and Bloom, 1984). The detection of “replication-bands” is the result of DNA synthesis during interphase, a phenomenon which occurs in almost all eukaryotes. Now, chromosome cleavage with restriction endonucleases has been used to produce euchromatic longitudinal bands in fish chromosomes (Sánchez *et al.*, 1993, Abuín *et al.*, 1995, Oliveira and Wright, 1998, Maistro *et al.*, 1999). Although almost all reports show clear banding patterns in the chromosomes, the resolution is not as well defined as that observed for mammalian chromosomes (Sumner, 1990).

The differences between the chromosomes of cold- and warm-blooded vertebrates are more evident when they are stained with reagents which show strong fluorescence when associated with A/T-or G/C-rich DNA segments (fluorochromes). The chromosomes of warm-blooded vertebrates display patterns of longitudinal bands resembling G- and R-bands plus some heterochromatin when stained with fluorochromes which are associated with A/T- or G/C-rich DNA, such as DAPI and chromomycin A₃, respectively (Sumner, 1990). In contrast, the staining of fish chromosomes with these fluorochromes does not produce longitudinal bands and, sometimes, only some heterochromatin or the NOR regions remain discerned by this method (Gold *et al.*, 1990, Almeida-Toledo, 1996). These results strongly suggest that the main difference between the chromosomes of warm-blooded vertebrates and fishes is the presence of A/T- and G/C-rich chromosomal segments in warm-blooded vertebrates.

Molecular studies have shown that while the genome of warm-blooded vertebrates (mammals and birds) seems to be highly compartmentalized, that of cold-blooded vertebrates either lack or is depauperate in G/C-rich segments (Thiery *et al.*, 1976, Cuny *et al.*, 1981; Bernardi, 1995). DNA segments of fairly homogeneous composition are known as isochores (Bernardi, 1995), which were originally identified as long (>300 Kb) DNA segments homogeneous in base composition. The isochores can be divided into several families characterized by their different base compositions, as initially demonstrated by density gradient fractionation of DNA molecules (Bernardi, 1995). In the human genome, which is typical for most mammals, “light”, G/C-poor (L1 and L2) isochores families represent 62% of the genome, whereas “heavy”, G/C-rich (H1 and H2) families correspond to 22.9% and very G/C-rich (H3) isochores family to 3–4 % of the genome (Bernardi, 1995). The remaining 3–4 % of the genome consists of satellite and ribosomal DNAs which can also be viewed as isochores because of their homogeneous base composition (Bernardi, 1995). The gene concentration is low in G/C-poor isochores, increases with increasing G/C in isochores families H1 and H2, and reaches a maximum in isochores family H3, which exhibits up to 20-fold higher gene concentration compared to G/C-poor isochores (Bernardi, 1995).

The compositional DNA patterns of cold-blooded vertebrates are substantially different from those of warm-blooded vertebrates (Bernardi and Bernardi, 1990; Aissani and Bernardi, 1991; Bernardi 1995; Bernardi *et al.*, 1997). Indeed, the former are characterized by lower intermolecular compositional heterogeneities and CsCl band asymmetries, as well as by the fact that their buoyant densities are lower than G/C-rich fractions found in warm-blooded vertebrate genomes (Bernardi, 1995). According to Bernardi *et al.* (1997), the vertebrate genome underwent two major compositional transitions between therapsids and mammals, and between dinosaurs and birds.

From a cytogenetic point of view, the human G-bands are formed almost exclusively by G/C-poor isochores, with only a minor contribution of G/C-rich isochores from the H1 family (Saccone *et al.*, 1993; Bernardi, 1995). T-bands are formed mainly by isochores of the H2 and H3 families and R'-bands, defined as R-bands exclusive of T-bands, comprise both G/C-rich isochores (mainly of the H1

family) and G/C-poor isochores in almost equal amounts (Saccone *et al.*, 1993; Bernardi, 1995). The distribution of four isochore families in mouse chromosomes, as investigated by *in situ* hybridization of single-copy sequences from compositional DNA fractions, showed that G/C-poor isochores are distributed over all G-bands and most R-bands (Saccone *et al.*, 1997). Hybridization of these segments with DNA from the mouse H1 isochore family produced a pattern largely corresponding to R-banding and hybridization with DNA from mouse H2 isochores was restricted to a subset of R-bands called T and T', the former containing a higher concentration of H2 isochores than the latter (Saccone *et al.*, 1997).

For mammals, therefore, there is a very good correlation between the presence of chromosome bands, mainly those obtained with base-specific fluorochromes, and the presence or absence of G/C-rich isochores. On the other hand, excellent G-banding can be produced in lower vertebrates such as *Xenopus laevis* and reptiles, in which there is no large scale variation in base composition (Sumner, 1990). Additionally, other eukaryotes, such as the monocotyledonous plants, which also have G/C-rich isochores, are also considered to lack euchromatic bands (Sumner, 1990).

In situ hybridization studies with different isochore families were not performed in fishes, but an investigation by Medrano *et al.*, (1988) showed that while the fish species *Anguilla anguilla*, which has a high compositional heterogeneity and a strong asymmetry, exhibits A/T-and G/C-rich chromosome segments identified by specific fluorochromes, the fish species *Epinephelus guttatus*, which has a very low compositional heterogeneity and a very weak asymmetry, does not exhibit A/T-or G/C-rich chromosome segments. Although Medrano *et al.* (1988) described the bright chromosome segments of *A. anguilla* as "chromosome bands", their microphotographs clearly show that there are entire chromosome arms or large chromosome segments which are A/T-or G/C-rich, but lack euchromatic longitudinal bands similar to those observed in mammalian chromosomes.

The data discussed above show that the isochores have an important role in the development of euchromatic chromosome bands, but other factors need to be considered for a good explanation of the absence of euchromatic bands in fish chromosomes.

Vertebrate genomes are composed of about 2–10% single copy sequences, the remainder of the genome being composed of repetitive sequences (Franck *et al.*, 1991; Wagner *et al.*, 1993; Bernardi, 1995). Repetitive DNA includes the tandemly-arrayed satellite, minisatellite and microsatellite sequences, and dispersed repeats such as transposons and retrotransposons (Charlesworth *et al.*, 1994). The tandemly-arrayed sequences are mainly found in centromeres and telomeres and the dispersed elements are found as isolated units or as clusters distributed throughout chromosomes.

Among the most extensively studied dispersed repetitive elements are the short interspersed nuclear elements (SINEs) and the long interspersed nuclear elements (LINEs) which are found in eukaryote genomes of organisms as diverse as plants and mammals (reviewed in Singer, 1982; Okada, 1991a, b; Martin, 1991; Charlesworth *et al.*, 1994; Smit, 1996; Okada *et al.*, 1997). All SINE elements described, except for the primate *Alu* and the rodent *B1* families, are derived from tRNAs (Okada *et al.*, 1997). Okada *et al.* (1997), proposed that in the tRNA-derived SINEs, the 3' end of these repetitive elements is derived from the 3' end of LINE elements and that this organization may be related to the acquisition of retropositional activity of SINE elements. Okada *et al.* (1997) also proposed that there are two different types of LINEs, one group of LINEs whose specificity for recognition of the 3' end sequence of SINEs tRNAs is absolute, and another group of LINEs whose specificity for recognition is more relaxed. The existence of this 'relaxed' type of LINE, the L1 family in mammals, might account for the large number of retroelements present in mammalian genomes (Okada *et al.*, 1997).

Both *in situ* hybridization (Manuelidis and Ward, 1984; Korenberg and Rykowski, 1988; Chen and

Manuelidis, 1989; Boyle *et al.*, 1990; Baker and Kass, 1994) and studies of chromosomal DNA replication (Holmquist, 1988) have shown that LINEs are concentrated in late-replicating DNA, or positive G-band segments, while SINEs occur predominantly in the early-replicating, negative G-band segments of DNA. Since mammalian SINEs and LINEs are G/C- and A/T-rich, respectively, it was suggested that their bias in base composition might be responsible for the transposition of these elements into DNA segments that show the same bias in base composition (Sumner, 1990; Bailey and Shen, 1993). Additionally, their bias in base composition could also be related to the base-specific fluorochrome banding of mammalian chromosomes (Korenberg and Rykowski, 1988). This last argument is reinforced by the fact that SINE elements with different sequences such as the human *Alu* and the rodent *B1* families are distributed in the same chromosome bands in both species (Boyle *et al.*, 1990).

A comparative analysis of the base composition of fish SINEs shows that these elements are usually G/C-poor, with values below 51% (Kido *et al.*, 1991; Izsvák *et al.*, 1996; Bryden *et al.*, 1998), contrasting with the G/C-rich *Alu* family of humans which are 56% G/C (Korenberg and Rykowski, 1988). Thus far, no complete LINE element has been described for fishes. The 3' ends of the partially known elements are A/T-rich, with values ranging from 57% in *Danio rerio* (Okada *et al.* 1997) to 63% in *Anguilla japonica* (Ohshima *et al.*, 1996). These values are similar to those observed for the human L1 elements which are about 58% A/T (Korenberg and Rykowski, 1988). Since it is known that the 3' end of LINEs is usually A/T-rich, the base composition of the LINEs in fishes will only be well understood when the sequences of complete elements are known.

Studies of SINE and LINE distribution in fish chromosomes are just starting but some preliminary results suggest that these elements may be related to the absence of base-specific fluorochrome bands in fish chromosomes. For example, FISH experiments with a partial LINE element of tilapia, *Oreochromis niloticus* (Oliveira *et al.*, 1999), showed that all chromosomes exhibited very small signals distributed more or less randomly over the chromatids, but strikingly enriched along the terminal two-thirds of the long arm of chromosome 1. Moreover, FISH experiments with the SINE element, ROn-1 (Bryden *et al.*, 1998), showed that a large chromosome segment rich in this sequence occurs in an interstitial position on the long arm of chromosome 1 of tilapia. Small signals dispersed over all chromosomes were, however, also observed under different experimental conditions (Oliveira, unpublished data). In the two FISH experiments described above, no similarity was observed between the distribution of SINEs and LINEs in the tilapia chromosomes and the distribution of these elements in mammalian chromosomes.

Considering that the chromosomes of tilapia do not display longitudinal bands when stained with the fluorochromes chromomycin A₃ or DAPI (unpublished data), and the chromosomal distribution of SINE and LINE elements described above, we suggest that the absence of longitudinal euchromatic bands in tilapia chromosomes is due to the absence of clusters of large number of A/T-rich LINE and G/C-rich SINE elements in this species. Since many SINE and LINE elements described for several cold-blooded vertebrates have similar characteristics to those found in tilapia (Okada *et al.*, 1997), further analysis of the structure and distribution of these elements in the chromosomes of other fish species, and also in the chromosomes of other cold-blooded vertebrates, may lead to a better understanding of the role of retroelements and other dispersed repetitive sequences in the origin of A/T-and G/C-rich euchromatic bands.

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11. A Review of Chromosome Banding in Fishes

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Abstract: Karyotypic studies are invaluable means in solving systematic and evolutionary problems in fishes. Their significance becomes more viable by applying different types of chromosome banding techniques which give further impetus to the accurate karyotyping of chromosomes. Though a limited success has been achieved in case of chromosome banding in fishes but still enough progress has been made in this field. The present paper reviews the studies carried out on C-, G-and NOR- banding patterns in fishes so far the world over. Reasonably good results in fish chromosomes particularly with respect to C-and NOR-banding have been obtained, whereas not much success has been achieved with respect to G-bands. Most reports on C-banding in fishes, revealed the distribution of C-heterochromatin to be of multisite type. NOR-banding pattern is known for well over 200 fishes. Most species of fish show only small NORs on a single pair of chromosomes. This condition has been considered as fundamental and original in fishes.

Key words: Chromosome banding, Fishes.

1. Introduction

From a consensus of estimates, there are approximately 20,000 species of fishes living in the world today. This is almost half the existing vertebrates with amphibians numbering about 2,500, reptiles 6,000, birds 8,600 and mammals 4,500 (Lagler *et al.*, 1962). There are many more fishes that are undescribed and have caused some estimates to range as high as 40,000. Considering the depths of the oceans and remote areas of the world that have never been investigated, such estimates are justified.

The study of chromosomes have been the subject of interest to not only the Ichthyologists, but also the Geneticists for several reasons. In fishes the use of chromosomal information as tool in taxonomy has even an added importance, since classification is beset with many difficulties. Moreover since this group is the originator of all the vertebrates, the karyotypic data in them can help in the understanding of the vertebrate evolutionary pathways. Many fishes have been found to have extensive chromosomal polymorphism (Roberts, 1967). Their chromosomal analysis can, therefore, be useful in understanding the process of speciation. The use of cytogenetics in fish-breeding and fish-culture can be of immense help. In experimental hybridization, fish karyology can be instrumental in selecting compatible genomes of related species and in distinguishing intraspecific races, otherwise not easily recognizable by morphological criteria. In hybridization experiments, chromosome analysis can demonstrate whether hybridization in real terms has taken place or not. The role of chromosomal anomalies is well known for many pathological conditions in man. A similar study would be significant in genesis of fish pathology.

On the Indian scene, most of the work has been done by Prasad and Manna (1971, 1974), Khuda-Bukhsh (1975, 1979a, b; 1980a, b; 1982, 1984), Manna and Khuda-Bukhsh (1977a, b), Khuda-

Bukhsh and Nayak (1982), Khuda-Bukhsh *et al.*, (1986), Khuda-Bukhsh and Barat (1984, 1987), Rishi (1971, 1973a, b, 1975, 1976a, b, 1978a, b, 1979, 1981), Rishi and Singh (1982), Rishi and Haobam (1987, 1990a, b), Nayak and Khuda-Bukhsh (1987), Sharma and Agarwal (1978, 1980, 1981a, b), Sharma and Tripathi (1981a, b, c, 1982a, b, 1984a, b, 1986, 1988), Tripathi and Sharma (1987), Sharma *et al.*, (1990, 1992, 1995a, b, 1998), Sharma and Sharma (1995, 1998a, b).

Differential banding of metaphase chromosomes has provided a powerful tool for the study of systematic and phylogenetic relationship among a variety of vertebrates. Although much of the work in this area has been focussed on mammals, considerable chromosome banding data also have been published on birds, turtles, snakes and amphibians (Baker *et al.*, 1982, 1983; Stock and Bunch, 1982; Schmid, 1980; Stock and Mengden, 1975; Bickham and Baker, 1976; Mengden and Stock, 1980). Comparatively very little work on chromosome banding in fishes has been done. The reason for this largely being the difficulties in obtaining sufficient number of quality well spread metaphases in fishes and most fish complements contain relatively a large number of comparatively small chromosomes (Gold, 1979). However, of late reports have started pouring in with reasonably good results in the fish chromosomes particularly with respect to C-banding and NOR-banding, whereas not much success has been achieved with respect to bands producing linear differentiation. For example, the reports of G-R-and Q-banding have given a confusing picture of chromosomes in several cases. However, some other types of linear and fluctuant differential banding like early and late replication banding and restriction enzyme banding have produced quite satisfactory results.

2. Serial Banding (G-bands)

Serial or Fluctuant bands (commonly known as G-or R-bands) are lateral striations or transverse bands along the arms of the chromosomes. Serial bands produced by protein or heat denaturation treatment have been reported in only a few fishes (Rishi, 1979; Weiberg, 1983; Sola *et al.*, 1984; Hartley and Horne, 1982; Almeida Toledo *et al.*, 1988). However, in most of the cases, the resolution of individual bands was poor and did not permit to preclude definitive karyotyping.

G-banding has become a common method to identify accurately the homologous pair of chromosomes in case of human and mammals (Sumner *et al.*, 1971). G-banding has been attempted on fish chromosomes too, but variable results have been reported by different workers. The occurrence of positive G-bands in fish chromosomes have been reported by Abe and Muramoto (1974) in *Salvelinus leucomaenis* and *S. malma*, Rishi (1978b, 1979) in *Channa punctatus*, *Colisa fasciata*, *Mystus tengara*, *Puntius sophore* and *Labio calbasu*, Passakas(1981) in *Anguilla anguilla*, Weiberg(1983) in *A. anguilla* and Sola *et al.*, (1984) in *A. rostrata*. The G-bands obtained in *A. anguilla* by application of GAC and GTG technique of Sumner *et al.*, (1971) by Weiberg (1983) are of good quality. Ojima *et al.*, (1986) obtained G-like bands with the application of a new method developed by Takayama and Tachibana (1981). Infact, their technique was of early replication banding. Zhou *et al.*, (1989) reported good G-banding with BrdU-BSG method in silver carp. Liu and Xuecong (1986) reported the use of BrdU culture method to gain high resolution G-banding in rice eel (*Monopterus albus*) and cat fish (*Silurus asotus*). Gold *et al.*, (1990 b) produced distinct G-bands in a Cyprinid fish, *Opsopoeodus emiliae* using trypsin G-banding method. Lakra and Krishna (1994) reported G-banded karyotypes of 3 species. of Indian major carps i.e. *Catla catla*, *Labeo rohita*, *Cirrhinus mrigala*. By using trypsin G-banding technique of Gold *et al.* (1990b),good G-bands were obtained in *Danio rerio*, *Amblyceps mangois*, *Trypophysa microps*, *Ptychobarbus conirostris*, *Xenentodon cancila* and *Puntius ticto* (Sharma and Sharma, 1998b).

The difficulty in obtaining good quality structural banding patterns on the fish chromosomes and

other cold blooded vertebrates seems to be related to their chromosome structure. It is generally believed that the genomes of the warm blooded vertebrates can be divided into GC-rich and GC-poor compartments, whereas cold blooded vertebrates either lack or show weak compartmentalization of their genomes by base composition (Bernardi, 1989). This absence of compartmentalization in fish genomes can be the reason of failure in obtaining good G-bands.

3. C-Banding

C-bands represent the regions of constitutive heterochromatin (Sumner, 1977) and predominantly contain transcriptionally inactive, highly repeated DNA sequences. Most C-banding techniques involve chromatin depurination (with acid), denaturation (with base) and preferential extraction of non-heterochromatic DNA in hot salt solutions (Comings, 1978). C-banding helps in revealing the distribution of constitutive heterochromatin and also to determine its role in karyotype evolution, speciation and differentiation of sex chromosomes. C-banding has been quite successful in fish chromosomes. The C-heterochromatin has been documented for about 125 spp. of fishes spread over different families with primary emphasis on documenting the existence and location of C-bands on fish chromosomes.

C-banding technique for C-heterochromatin in fish chromosomes was applied for the first time by Abe and Muramoto (1974) in two salmonid fishes (*Salvelinus leucomaenis* and *S. malma*) and Zenzes and Voiculescu (1975) in *Salmo trutta*. Subsequent to these reports, several workers successfully obtained C-bands in different fish species.

Lloyd and Thorgaard (1988) and Cau *et al.*, (1988) reported that treatment of metaphase chromosomes with specific restriction endonuclease enzymes followed by Giemsa staining produced C-bands on the chromosomes of rainbow trout and the muraenid, *Muraena helena* respectively. Some workers have used fluorochromes to resolve C-bands. AT-enhancing fluorochromes such as Quinacrine or DAPI have been used to resolve C-bands in salmonids, a Poeciliid and several European percids (Haaf and Schmid, 1984; Mayr *et al.*, 1987; Phillips and Hartley, 1988), whereas the GC-enhancing fluorochromes CMA have been used to resolve C-bands in North-American percid (Amemiya and Gold, 1986).

In India, Rishi and Mandhan (1990) obtained C-bands for the first time in *Labeo rohita*. Thereafter, several reports on C-banding in Indian fishes have been published (Rishi and Gill, 1992; Rishi and Thind, 1994 and Rishi *et al.*, 1994).

Most of the reports on C-banding in fishes reveal the distribution of C-heterochromatin to be of multisite type. In most of the fishes, C-heterochromatin is located on the centromeric and telomeric regions. However, intercalary C-bands and whole arm or whole chromosome C-bands have also been reported. Garcia *et al.*, (1987) have pointed out that constitutive heterochromatin may not be restricted to centromeric region in fishes. Rishi and Rishi (1992) reviewed C-heterochromatin in fishes and described a varied distribution of C-heterochromatin in fish chromosomes.

In *Mastacembelus pancalus*, *Amblyceps mangois* and *Ompok bimaculatus* centromeric and telomeric bands have been recorded, while *Channa gachua*, *Ptychobarbus conirostris*, *Schizothorax richardsoni* and *Trylophysa microps* showed whole long arm and whole chromosome C-bands in addition to centromeric and telomeric C-bands (Sharma and Sharma, 1998b).

General hypotheses have been put forth to explain the possible role of the C-heterochromatin in karyotypic evolution of fishes. Hinegradner and Rosen (1972) pointed out that loss of C-heterochromatin may be responsible for smaller genome size during the course of evolution. Kornfield *et al.*, (1979) suggested that elimination of C-heterochromatin accompanies the phyletic evolution of fishes. The presence of intercalary C-heterochromatin, however, suggests chromosomal rearrangements by

pericentric inversions (Zenze and Voiculescu, 1975). Phillips and Zajicek (1982) suggested that C-banding could be better device for intraspecific comparisons since C-heterochromatin is one of the most rapidly evolving part of the genome. In view of wide range of heterochromatin sites in fishes, it is proposed that most fish genomes are in a state of flux and are prone to loss or gain of DNA. The wide variation in C-heterochromatin has been considered responsible for the large scale chromosomal polymorphism exhibited by many fish groups (Rishi and Rishi, 1992). A continuous and random variation of DNA content in fish species, which otherwise exhibit similar karyotypes, may be entirely due to variability in heterochromatin fractions of their DNA which are also phenotypically inconsequential.

Some examples have come to light where sexes have differentiated in acryptic fashion and these can be visualised only by C-banding (Ojima and Ueda, 1982; Haaf and Schmid, 1984; Galetti Jr. and Foresti, 1986; Mayr *et al.*, 1987; Thode, 1987; Rishi and Gill, 1992). Ojima and Ueda (1982) could observe W and Z chromosomes in *Conger myriaster* in which they had earlier failed to identify these elements having small size and morphology. Haaf and Schmid (1984) reported an interesting case of homomorphic W and Z chromosomes in *Poecilia sphenops* va. *melanistica* with intense C-banding on the long arm of W-chromosome. Galetti Jr. and Foresti (1986) also found the W-chromosome having intense C-banding in species of the genus *Leporinus*. Mayr *et al.*, (1987) observed totally heterochromatized Y in *Luciperca luciperca*, *Gymnocephalus cernus*, *G. schraestee* and *Perca fluviatilis*. In 1987 they could also observe heterochromatic sex-chromosome in *Lepidogaster candollei* with Y being totally heterochromatic. In *Colisa fasciatus*, Rishi and Gill (1992) found the long arm of W to be fully heterochromatic in WZ sex chromosomes. Bertollo and Cavallaro (1992) found heteromorphic pair in *Triparthous guentheri*, where W was totally C-band positive and Z with heterochromatin only at telomeric and centromeric regions.

In fishes NORs have been found to be associated with C-band region such as in *Umbra limi* (Kligerman and Bloom, 1977), *Scorpaena notata* (Thode *et al.*, 1985) and *Colisa fasciatus* (Rishi and Gill, 1992). However, the significance of the association of NORs with C-positive regions is not clear yet.

4. NOR-Banding

NOR-bands (with silver) represent the chromosomal sites of 18s and 28s ribosomal RNA (rRNA) which presumably were actively transcribed at a preceding interphase (Howell, 1977, 1982). The silver-staining reaction is apparently specific for a NOR-associated, non-histone protein that selectively binds and reduces ionic silver. NOR-banding with GC-base pair binding fluorochrome chromomycin A3 (CMA) and mithramycin apparently stain DNA and differentiate NORs regardless of previous genetic activity or chromosomal stage (Amemiya and Gold, 1986; Schmid and Guttenbach, 1988). Both fluorochromes, however, can also selectively stain heterochromatin (Amemiya and Gold, 1986), suggesting that some caution is advisable before considering a CMA or mithramycin bright region on a chromosome as a NOR.

NOR-banding patterns are now known for well over 200 species of fishes (Gold *et al.*, 1990a). In most cases, emphasis has been laid only on documenting NOR bands on fish chromosomes. However, NOR-banded phenotypes have been used to address systematic, population or cytogenetic problems by workers like Amemiya and Gold (1988), Gold *et al.* (1988, 1990a), Amemiya and Gold (1990a, b), Li and Gold (1991), Jenkin and Gold (1992) on North American Cyprinids and studies by Phillips and colleagues on salmonid fishes (Phillips *et al.*, 1986, 1988, 1989).

The visualization of NORs on the chromosomes has now become an important parameter adding

to the structural details of the karyotypes. For silver staining, most researchers employ Howell and Black's (1980) one step method using a colloidal developer. The Ag-NOR staining technique of mammalian chromosomes (Goodpasture and Bloom, 1975; Hsu *et al.*, 1975) was first time applied in fishes in case of *Fundulus* chromosomes by Howell and Black (1979). Since then a lot of work has been done on localization of NORs in fishes. On the basis of available literature it has been observed that the number of NORs, the morphology of NOR-bearing chromosomes and the position of NORs on the chromosomes show marked diversity. Closely related species with very similar karyotypes may show different NOR sites. Therefore, the NORs can serve as an important aid for species differentiation and fish systematics.

Foresti *et al.*, (1981) studied NORs in 5 species of order Gymnotiformes viz., *Gymnotus carapo*, *Apteronotus*, *Sternopygus macrurus*, *Eigenmannia virescens* and *Eigenmannia* sp., and reported polymorphic nature of NORs in the group. In *Eigenmannia* sp. they reported intraspecific variability of the NOR-bearing pair and an increase in the length of that region, the larger one being about six times the size of the smaller. They suggested that an increase in the ribosomal gene in the NOR of certain species occurred during the evolution of this group.

Feldberg and Bertollo (1985) studied NORs in 10 neotropical species of family Cichlidae (Perciformes). In 8 species, the NORs were located on first pair of the complement, while in the remaining 2, these were on a relatively large, but not the first pair of the karyotype. The location of NORs showed variation from interstitial position in short arm /long arm to terminal on the long arm or short arm.

The ribosomal RNA gene expression in the genomes of evolutionary diploid (*Scardinius erythrophthalmus* *Leucaspis delineatus* *Tinca tinca*) and polyploid species (*Cyprinus carpio*, *Carassius carassius*, *C. auratus auratus* and *C. auratus gibelio*) of Cyprinidae were investigated by Mayr *et al.*, (1986) using silver nitrate and counter stain enhanced fluorescence technique. The diploid species exhibited only one pair of chromosomes with NORs, whereas in tetraploids (*Carassius auratus auratus* and *C. carassius*) 3 Ag-NORs were present and in hexaploid (*C. auratus gibelio*) 4 NORs were there. In *Cyprinus carpio* they observed the presence of only one pair of NORs. They suggested partial or complete functional inactivation of the third and the fourth NORs in the evolutionary tetraploid species of carp, *Cyprinus carpio* (Takai and Ojima, 1982), *Carassius auratus auratus*, *C. auratus* subsp., *C. auratus buergeri*, *C. auratus grandoculis* and *C. auratus cuvieri* (Takai and Ojima, 1982; Ojima and Yamono, 1980) consistently led to only 2 Ag-NORs associated with the 12th largest chromosome pair.

Takai and Ojima (1986) published a list of about 80 species showing the morphology of NOR bearing homologues, number of NORs, location and type of NORs. They categorised the NOR in fish into 7 categories. They observed that the NOR bearing chromosomes have various forms among the different species, but most of them belong to telocentric/ acrocentric or subtelocentric type (35.7%) with very vast range of size from largest (in many Pomacentrids) to smallest (in *Rhodeus ocellatus ocellatus*).

Most species of fish have only small NORs on a single pair of chromosomes. This usual condition has been considered as fundamental and original in fishes by Takai and Ojima (1986). Thode (1987) considered the fishes with one pair of NORs as most ancestral. The large NORs found in some Cyprinids and Gymnotids may be as a result of an increase in the DNA content by accidental translocation, duplication or other mechanisms (Takai and Ojima, 1986). Multiple NORs might have been induced due to partial translocation of NORs to other non-NOR bearing chromosomes. However, this assertion has been put to question by findings of multiple NORs in more and more species. Even a completely opposite explanation, though less plausible that single pair NORs might have been resulted by the aggregation of multiple site NORs, is also possible.

Some of the important reports about NOR studies are those of Kligerman and Bloom (1977), Kornfield *et al.*, (1979), Uwa and Ojima (1981), Uwa *et al.*, (1981, 1982), Takai *et al.*, (1987), Suzuki *et al.*, (1988), Lopez *et al.*, (1989), Ueda *et al.*, (1988), Oberdorff *et al.*, (1990), Sanchez *et al.*, (1990), Vitturi *et al.*, (1990), Sola *et al.*, (1990), Takai and Ojima (1991), Ren *et al.*, (1991), Rab *et al.*, (1991a, b), Almeida-Toledo *et al.*, (1992), Magtoon and Arai (1993), Padilla *et al.*, (1993), Zhang and Zeng (1993) and Tatewaki and Kitada (1994).

In Indian fishes, some work on the analysis of NORs has been done by Rishi *et al.*, (1991), Rishi and Manjusha (1991), Rishi and Thind (1992), Rishi and Gill (1992), Rishi and Girdhar (1992), Rishi *et al.*, (1993), John *et al.*, (1992), Rishi and Thind (1994) and Rishi *et al.*, (1994).

Gold and Amemiya (1986) worked out 14 species of Cyprinidae and observed 10 different NOR phenotypes. Based on their findings, they concluded that North American Cyprinids are far less conservative in terms of chromosomal evolution than previously believed. Amemiya and Gold (1988) found the study of NORs useful in both cytobotany and cytosystematics of North American Cyprinids. Gold *et al.*, (1988) studied NOR phenotypes of 8 species of North American Cyprinids genus *Notropis* (subgenus-*Cyprinella*). All of them had a single pair of chromosomal NORs. In 4 of the 5 *Cyprinella* species, the NORs were located terminally on the arm of medium sized metacentric chromosome pair, in the 5th *Cyprinella* sps, NORs were located on the short arms of the large submetacentric pair and in the remaining 3 species, the NORs were located on short arms of the medium sized submetacentric pair. Amemiya and Gold (1990a) worked out 7 species of North American Cyprinids and gave cytobotanical relationships among them.

The NOR studies may also throw light on the secondary diploidization, that succeeds the polyploid origin of some species. Phillips *et al.*, (1986) showed only one pair of NORs in each of the 6 species of Salmonid fishes (*Oncorhynchus*). Therefore, these species which are regarded as tetraploid in origin must have secondarily consolidated NORs only on one pair of chromosomes during the process of regressive diploidization. Two Cyprinid species, *Schizothoraichthys labiatus* ($2n = 98$) and *Ptychobarbus conirostris* ($2n = 84$), thought to be tetraploids in origin have been found to bear only one pair of NOR on a subtelocentric in each and the process of regressive diploidization seems to be cause of the presence of a single pair of NORs in these species (Sharma and Sharma, 1998b).

The occurrence of multiple NORs is not uncommon in fishes. This type of NOR distribution has been reported in some species of the family Cyprinidae (Takai and Ojima, 1984). Two pairs of NORs were reported in *Sacrocercilicthys variegatus variegatus*, *Tribolodon hakonensis*, *Moroco jouyi*, *Zacco platypus*, 3 pairs in *Ischikauia steenackeri* and 4 pairs in *Zacco temmincki*. Takai and Ojima (1986) again observed two pairs of NORs in *Pungtungia herzi* and *Hemibarbus barbus*. Gold *et al.*, (1990a) also observed multiple NORs in four species of North American Cyprinids. In one of the species, *Phenacobius mirabilis* they observed the presence of 3 pairs of NORs. Rishi and Gill (1992) reported three pairs of NORs in *Colisa fasciatus*. Again Rishi and Thind (1992) reported the presence of 2 pairs of NORs in case of *Puntius sophore*. Multiple NORs have also been reported in *Moenkhausia sanctifilomenae* (Foresti *et al.*, 1989). Rishi and Thind (1994) reported an interesting rare case of multiple NORs in *Notopterus notopterus*. They found that NORs were present on all the 42 elements of the complement. However, one pair possessed very prominent and large NORs than the others.

The heteromorphic NORs have also been reported in fishes (Gold, 1984; Amemiya and Gold, 1986; Philips *et al.*, 1986; Rishi and Manjusha, 1991; Rishi and Gill, 1992). The heteromorphism may be either functional (when one NOR shows more activity) or structural due to the presence of more rDNA cistrons present on one homologue and may be due to variable distribution or activity of ribosomal cistron (Takai and Ojima, 1986; Foresti *et al.*, 1989)

Various workers have tried to explain the varied location of NORs on the chromosomes of several

species. NOR location in interstitial region in many forms of Balistidae probably originated by tandem fusion between NOR-bearing chromosomes and other chromosomes (Kitayama and Ojima, 1984; Takai and Ojima, 1986). Rishi *et al.*, (1993) also reported the interstitial NOR pair in *Channa punctatus* (Ophiocephalidae). They suggested that the condition might have been produced through pericentric inversion or even through centric fusion of two acrocentric chromosomes, one of which had telomeric NOR. They considered this state of NORs in *C. punctatus*, a derived one.

Sharma and Sharma (1998b) recorded NORs in *Danio rerio*, *Ptychobarbus conirostris*, *Schizothoraichthys labiatus* (Cyprinidae), *Triplophysa microps* and *T. stoliczkae* (Cobitidae) from Jammu Kashmir state. In all, only single pair of NORs have been observed, which were homomorphic. In *Danio rerio* NORs were present on the telomeres of long arms of the largest subtelocentric pair, in *Triplophysa microps* and *T. stoliczkae*, these were present on the telomeres of the second pair of metacentric, whereas in *Ptychobarbus conirostris* and *Schizothoraichthys labiatus*, these were present on the telomeres of short arms of subtelocentric pair. The presence of NORs in second metacentric pairs in *Triplophysa microps* and *Triplophysa stoliczkae* suggests a close relationship of the two species (Sharma and Sharma, 1998b).

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12. Chromosomes in Birds

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Abstract: Birds are highly specialized morphologically, physiologically and biochemically with about 8600 known species. The work on chromosomes of this group started in the early twentieth century by using section cutting methods. With the development of hypotonic treatment and air drying techniques, many more species have been investigated. Avian karyotype is characterized by large number of chromosomes, presence of macro- and micro-chromosomes and female heterogamety. At present, the data are available for about 7% of the taxonomically known species. On the basis of available data cytogenetical relationships and mechanism of chromosome evolution have been derived by many workers. The bird karyotype has been found to be conservative and fusions, fissions or translocations seem to be the basis of chromosome evolution. Studies on NORs show that these are mainly located, on the microchromosomes. The sex-chromosomes are homomorphic in Ratites and heteromorphic in carinates. The wide variation of W-chromosome among carinates indicate various stages of W-chromosome differentiation from homomorphic state to the sex-chromosomes. Presently, genetic methods have been developed to sex the birds. Various molecular methods are now used to map avian genome including chromosome scraping, flow cytometry, zonal centrifugation, DNA markers etc. The analyses of *chd* genes present on Z and W sex-chromosomes of birds show that evolution in them is male driven, thus supporting a neutral model of molecular evolution. Chromosome painting techniques have revealed homologies of karyotypes of chick and emu indicating strong conservation of karyotypes.

Key words: Chromosomes, birds, evolution, sex-chromosomes.

1. Introduction

Birds are considered to be physiologically and morphologically highly specialized with about 8600 known species grouped in 26 orders with about 1600–2400 genera, being the second largest group of vertebrates.

They are present in almost every environment of the world. So far as the number, behaviour and morphology of its chromosomes are concerned, this class also occupies a unique and unrivalled position. The avian karyotype is characterized by high chromosome number, bimodality i.e., presence of macro-and micro-chromosomes and the female heterogamety.

The work on avian karyology was started as early in 1902 by Guyer. The bulk of earlier work (1902–1966) was based on microtome sectioned material. It was only in 1966 that bone marrow was tried by certain workers. The use of air drying technique opened new vistas in the field of avian cytogenetics. Although a remarkable progress has been made in the field of vertebrate cytogenetics in last few decades, it is still not complete. Our knowledge about avian chromosomes is only about 7% of the total taxonomically known species. It is mainly because of difficulty in analysis of large

number of chromosomes, particularly the microchromosomes. It is very hard to count them not only because of their small size, but also due to their agglutinating tendency. Moreover, earlier workers (Newcomer and Brant, 1954) did not even consider microchromosomes as the true chromosomes. However, the recent techniques have made the analysis of these chromosomes possible. The studies on DNA uptake during S-phase in metaphase nuclei by Bianchi and Molina (1967) and Comings and Mottacia (1970) proved the true chromosome nature of microchromosomes.

Earlier workers reported very low and varying number of chromosomes in a species without specifying the sex mechanism. By section cutting method they could never find constant number which could be assigned to a particular species, as is obvious from Makino's Atlas of Chromosome Numbers 1951, where the chromosome data of pigeons and doves alone show variation in chromosome number from 16 to 80. Therefore, work carried out in those years with section cutting techniques remains simply of historical importance as no confirmatory or satisfactory results were obtained and most of the species have been reinvestigated by the implication of feather pulp technique of Krishan (1962, 1964) and bone -marrow method of Ray-Chaudhuri *et al.* (1966). This followed investigation of chromosomes in a large number of other species of birds (Krishan, 1962; 1964; Stenius *et al.*, 1963; Ohno *et al.*, 1964; Krishan *et al.*, 1965; Krishan and Shoffner, 1966; Owen, 1965; Vegni-Talluri and Vegni, 1965; Hammar, 1966, 1970; Ray Chaudhuri *et al.*, 1966, 1969; Renzoni and Vegni-Talluri, 1966; Thorneycroft, 1966; Ray Chaudhuri, 1967, 1976; Takagi and Makino, 1966; Bloom, 1969; Castroviejo *et al.*, 1969; Itoh *et al.*, 1969; Jovanovic and Atkins, 1969; King, 1970; Piccinni and Stella, 1970; Srivastava and Misra, 1971; Hirschi *et al.*, 1972; Takagi *et al.*, 1972; Bulatova and Radzhabli, 1974; Takagi and Sasaki, 1974; De Boer, 1975, 1976; Kaul and Ansari, 1975; Sharma *et al.*, 1961, 1972, 1975, 1976, 1980; Theodorescu, 1975; De Lucca and De Aguiar, 1976; Misra and Srivastava, 1976; Mittal and Satija, 1978; Mittal and Sahuja, 1979 ; Mittal *et al.*, 1979; Mittal and Sharma, 1982, 1984, 1989, a, b, 1990a, b, 1993; Bhunya and Mohanty, 1985; Bhunya and Sultana, 1979; Bhunya, 1988; Sharma, 1989; Bhunya and Das, 1990; Das *et al.*, 1995; Roy and Kaul, 1990; Das and Jena, 1998; Das, 1996). Following is the overview of status of chromosomes in various species of birds.

2. Chromosome Number and Morphology

It appears that the chromosome number in birds vary from 52 to 126. These two extremes are represented by *Falco tinnunculus* (Renzoni and Vegni Talluri, 1966) of order Falconiformes and *Upupa epops* (Kanwaljit, 1975; Misra and Srivastva, 1975) from the order Coraciformes, respectively. The data on avian chromosomes reveal that maximum number of species have the diploid number of 80. The next peaks are seen at 78 and 82. There are certain gaps in diploid number of known species as the numbers 54, 56, 92, 96, 100–124 are not represented by any of the so far cytologically known species. Furthermore, the major bulk of species have 2n between 66–86 chromosomes, while lower and higher numbers are represented by one or two species each.

The chromosomes of birds can be distinguished into macro- and micro-elements *i.e.*, they show characteristic bimodality, as there are few large chromosomes with lengths more than 2 μm . On the other hand they have a high number of chromosomes with lengths less than 1 μm . In some species, it is possible to discriminate the larger chromosomes as macro- and the smaller as micro-chromosomes. The demarcation of macro- and micro-elements in many species is difficult to establish as the karyotypes form graded series. Even in those cases, morphology of the first 7–9 pairs of chromosomes is clearly distinguishable regarding the position of centromere, whereas the rest of the chromosomes are generally taken as telocentrics as their centromeric location cannot be depicted in dot-like chromosomes.

In the family Falconidae the number of microchromosomes is quite small. There are only 3–5 pairs of microchromosomes in the whole complement and remaining all are macros, except *Milvus migrans* that has 28–38 micros (Misra and Srivastava, 1976).

According to Rodionov (1996), in contrast to macrochromosomes, medium and small avian chromosomes lack the higher level of organization. They do not have spiral coiling. Microchromosomal euchromatin has GC rich regions. More than half of the mapped avian genes are located on microchromosomes as their crossing over frequencies are three fold higher than that of macrochromosomes.

The studies on systematics based on karyotypic similarities and diversifications in various families have been made by various workers from time to time (Yamashina, 1951; Hammar, 1966; Ray-Chaudhuri, 1973; Bhunya and Sultana, 1979; Bhunya, 1988; Mohanty and Bhunya, 1989 and others)

A glance at the karyotypes of various species of birds show that all are typical avian karyotypes with high somatic number and variation in chromosome length from the largest to the smallest. This may indicate that bird karyotype is very conservative. Theodorescu (1975) has put forth karyotype evolution in Pelecaniformes as a result of conservation of "shared chromosome pairs" and the convergent evolution of "non-shared pairs".

In some cases, centric fusion and fission, in others translocations or combinations of these processes have played a role in evolution. Sometimes, similarities in members of different families have been revealed leading to cases of parallelism in karyotypic evolution in completely independent groups (Dicruridae and Corvidae; Bhunya and Sultana, 1979)

Furthermore, the similarities between congeneric species and interfamilial similarities reveal the fact that it is not always the structural change of chromosomes, but point mutations have also played a major role in speciation of various taxonomical groups. According to Ray Chaudhuri (1973) and Ghosh and Prasad (1989) the three longest pairs of macrochromosomes are common to the karyotypes of many families and in others, they seem to be present only in slightly changed form. The relative conservatism of the bird karyotype in general leads to lack of conclusiveness of the cytological data with regard to relationships between higher avian taxa. Takagi and Sasaki (1974) showed that not only gross morphology but G- banding pattern of first 3 chromosome pairs in 9 species of birds are very similar. The existence of such cases of clear karyological similarities between only distantly related orders greatly hampers the drawing of unambiguous conclusions as to relationships between orders relying on cytological data.

Studies on Nucleolar Organizer Regions (NORs) in various species of birds (Mohanty and Bhunya, 1992; Bhunya and Mohanty, 1985 and others) reveal that NORs are localized on 8 to 10 microchromosomes of various species. In *Buteo albicaudatus* the NOR has been located on the larger satellite chromosome (the 7th pair). This is the only exception so far and has been attributed to some kind of translocation in this region.

The DNA replication studies on avian chromosomes by ^{3}H thymidine Galton and Bredbury (1966) or Brdu labelling have shown that C+ regions replicate early. The microchromosomes are also early replicating, while replication is late in larger chromosomes. In sparrow, the G + regions of all the macro-chromosomes start as well as finish replication almost simultaneously (Das and Jena, 1998).

Frequency, distribution and the density of microsatellites in bird chromosomes has been found to be less (Primmer *et al.*, 1997). It may be due to relatively less non-coding DNA in them as compared to mammals. The low microsatellite density in genome in general and microchromosomes in particular, imposes an obstacle for development of marker-rich genetic maps of birds and for localization of quantitative trait genes (Primmer *et al.*, 1997). The presence of microchromosomes has great evolutionary concern. Kaufman and Wallny (1996) has related the compact and simple nature of MHC genes to the

presence of microchromosomes and suggested that evolutionary origin of birds has been strongly influenced by the emergence of microchromosomes in this group.

3. Sex-Chromosomes

The avian karyotype reveals female heterogamety. This characteristic is shared with groups like Lepidoptera, Fishes, Amphibia and Reptilia. The sex chromosomes are designated ZW in female and ZZ in the male. The Z chromosome varies from 1st to 5th position in the complements of different species, while W is one of the microchromosomes, mostly the smallest. The identification of W chromosome is very difficult in majority of the species because of its small size.

There are certain exceptions to this rule. There are no heteromorphic sex chromosomes in Ostrich, Cassowary or Emu and no late labelling DNA replication has been observed (Takagi *et al.*, 1972). The studies on the ratite birds strongly indicated that female karyotype in them cannot be differentiated from the males alone on the basis of morphology. It is quite possible that an unpaired chromosome number 6 in Rhea may actually be representing an intermediate stage between homogametic sex pair in other ratites and grossly heteromorphic one in carinates. It has, however, been seen that in some species of carinates, the W chromosome is not necessarily quite small in comparison with the Z, whereas it is a minute element indistinguishable from micros in others. Such occasional occurrence of large W-chromosome in carinates and morphologically undifferentiated sex chromosomes in ratites seems to suggest that the sex-chromosomes of ancestral birds when diverged from the reptilian predecessor might have been homomorphic and conservative in some ratites, without further morphological differentiation. It appears that W-chromosome has reduced in size in most of the species of carinates although there is no reason to assume that carinate species invariably possess heteromorphic sex-elements. The wide variation in the size of W-chromosome among carinates may indicate various stages of W-chromosome differentiation from the homomorphic state of sex-chromosomes.

There is very little information available regarding the meiotic cycle of birds. Bammi *et al.* (1966) have shown that the sex chromosomes do not pair at all. Hence, they do not exchange genetic material. The Z and W univalents are also isopycnotic to the autosomal bivalents in the oocytes. However Ohno *et al.* (1960) have asserted the positive heteropycnotic Z in the fowl oogenesis. The lack of association between Z and W chromosomes in germ cells of heterogametic sex of these animals is in sharp contrast to the situation in mammals where there is end to end association of X and Y chromosomes. The XY bivalent is positively heteropycnotic at pachytene and has a characteristic morphology and location. Ohno *et al.* (1960) have postulated that heteropycnosis of XY bivalent in mammals is a manifestation by which crossing over is prevented between X and Y. It is possible that the Z and W in these bird species have differentiated too far from each other (assuming their origin from homomorphic chromosomes) to pair, hence making it unnecessary for natural selection to provide any other mechanism to restrict crossing over between these two chromosomes. This may be the reason for observed isopycnoticity of Z and W during oogenesis.

The Z - chromosomes in most of the species have been found to be between 4th and 6th pairs of autosomes in size. However, exceptions are presented by *Accipitar badius*, a falconiform, where it is considered to be the largest pair. In some cases it can be at 2nd/3rd position or even upto 11th pair. In the species *Hirundo smithii filifera*, Z is largest. W-chromosome has a wide range of morphology and size. It is generally the smallest macrochromosome or one of the micros. It can be a macrochromosome approximately, the size of Z. Extreme case is *Ardeola grayii* (Hammar and Herlin, 1975) where W chromosome is larger than that of Z. The W is of the size of Z-chromosome in *L. malacca* (Prasad and Patnaik 1977). Identification of W chromosome is sometime quite difficult

when its size is of one of the microchromosomes. That is why out of cytologically known avian species, W chromosome has been identified in lesser number.

In general none of the sex chromosomes in birds exhibit any special staining property with stains like Carbol Fuchsin, Giemsa, Orcein, Feulgen, Carmine, Ferric hematoxylin etc. They hardly show any differential staining properties at metaphase. Rothfels *et al.* (1963) found a heteropycnotic W-chromosome at metaphase and Stefos and Arrighi (1971) showed heterochromatic pattern of W in 8 species by heterochromatin staining method of Arrighi and Hsu (1971). Studies on DNA replication of bone marrow chromosomes in some species have shown W to be late replicating (Schmid, 1962). Takagi (1972) found other macrochromosomal segments also late replicating.

4. Sex Determination in Birds

According to Stevens, (1997) the sex determination in birds is by a genic balance mechanism in which ratio of autosomes to Z chromosome is a crucial factor. DNA sequences homologous to testis determining factor in humans have been detected in both male and female birds but it is not clear that they have a sex related function in birds. Methods have been developed to distinguish sexes in birds based on sex linked genes, the amount of DNA per cell and using DNA probes for sex-linked sequences. In sex reversed chicken, Abinawanto *et al.* (1998) identified some sperms with female specific chromosomes. Even the female germ cells have been found to differentiate into spermatozoa in male gonads (Tagami *et al.*, 1997).

5. Molecular Nature of Sex-Chromosomes in Birds

Recently, it has been attempted to differentiate sex-chromosomes on the basis of molecular techniques. The female sex can be differentiated by the presence of an unambiguous female specific band in the region EE 0.6 of W chromosome (Ogawa *et al.*, 1997). CHD-W gene has also been found to be helpful in sexing the birds (Ellegren, 1996; Griffiths *et al.*, 1996). According to Griffiths *et al.* (1998) two chromohelicase DNA binding (*chd*) genes are located on avian sex-chromosomes of all the birds with possible exception of ratites. The Z chromosome *chd-Z* gene is found in both the sexes whereas *chd-W* is present in females only. The sex specific DNA sequence has been developed by PCR (Itoh *et al.*, 1997). *Chd* gene codes for conserved protein with a putative role in chromatin architecture (Griffiths and Korn, 1997).

The Z-chromosome has been conserved in birds during evolution. This has been shown by sex-chromosome linkage of chicken and duck type I interferon genes (Nanda *et al.*, 1998). These genes are expressed in response to viral infection. In mammals, these are present on autosomes. Zimmer *et al.* (1997) developed chicken Z-chromosome painting probe by using microdissection and FISH techniques. The technique has provided appropriate probes which can be used in the analysis of chromosome rearrangements, in studies of cases of heteroploidy involving Z-chromosome and positional cloning of Z-linked genes and in studies on mechanism of sex-chromosome evolution in birds.

Griffiths and Orr (1999) proposed the identification of sex-specific markers by AFLP technique to sex organisms directly.

Fridolfsson *et al.* (1998) have suggested that evolution of avian sex chromosomes occurred from an ancestral pair of autosomes and thus, occurred independently in mammals and birds. Ellegren and Fridolfsson (1997) analyzed the molecular evolution of the gene *chd*, present in Z and W sex chromosomes of birds. The substitution rate at synonymous positions, as well as intron DNA was considerably higher on Z chromosome than on the female specific W-chromosome, with an estimated male-to-female bias in mutation rate of 3.9–6.5. Thus, evolution appears to be male driven in birds

supporting a neutral model of molecular evolution. Dickman (1997) and Lessells (1997) also reported that there are more mutations in the male birds.

The genetic analysis of *chd1* genes on avian Z and W sex chromosomes by Fridolfsson and Ellegren (2000) has also revealed that these two genes evolved independently, but are highly conserved at nucleotide as well as amino acid level thus not indicating female specific role of *chd1* W gene.

The chromosome painting of chicken chromosomes and the emu chromosomes showed remarkable homology between two species indicating strong conservation of karyotypes through evolution. Shetty *et al.* (1999) found that chicken Z chromosomes paint to hybridize to emu Z and most of the W, confirming that ratite sex chromosomes are largely homologous. The centromeric region of W which hybridizes weakly may represent location of sex-determining genes. The studies on recombination nodules (RNs) of Z and W chromosomes in *Rhea americana* shows that ZW pair of this species is in a primitive stage of chromosomal differentiation in which recombination is restricted only in the small short arm and in pericentric region. ZW pair is mostly euchromatic (Pigozzi and Solari, 1997, 1998). Ogawa *et al.*, (1998) reported that the homomorphic sex-chromosomes of ratites, show signs of genetic differentiation between Z and W chromosomes. They used two marker genes and found a deletion in female ostrich, the initiation of differentiation of W-chromosome.

Nishida-Umehara *et al.* (1999) identified sex chromosomes in *Casuarius casuarius* by a replication banding method. The characterization of sex chromosomes by FISH with different probes revealed that structural rearrangements such as deletions and inversions might have been the initial step of W-chromosome differentiation from ancestral homomorphic pair in this species.

Crooijmans *et al.* (1995) chromosome mapped the chromosomes of chick and marked and located linkage groups in different chromosomes based on microsatellite markers.

Chromosome mapping has also been done in some birds although various molecular methods are now used to map chicken genome including chromosome scraping, flow cytometry, zonal centrifugation construction of chromosome specific libraries, genetic analysis with polymorphic DNA markers and *in situ* hybridization. It has been seen that classic genetic maps, physical maps of chromosomes and new genetic maps constructed on the basis of polymorphic DNA markers do not coordinate with one another (Sazanov *et al.*, 1996). Application of cytogenetic methods to chromosome mapping in birds is limited because of some specific features characteristic of the organization of avian genome.

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13. Chromosomal Analysis in Human Population Monitoring

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Abstract: Frequencies of chromosomal aberrations in human peripheral lymphocytes of nuclear power plant workers, dry cleaners and controls are presented. Methodological aspects concerning analysis of chromosomal aberrations in human populations are discussed.

Key words: Chromosomal aberrations, lymphocytes, human population monitoring.

1. Introduction

Soon after the first cytogenetic analysis of the effects of acute overexposure to ionizing radiation (Bender and Gooch, 1962) cytogenetic methods were applied to look for chromosomal damage in workers occupationally exposed to genotoxic agents at low level exposures. Numerous human population monitoring studies were published in people exposed to ionizing radiation or chemical substances. For about 20 years human population monitoring with cytogenetic methods was done in our laboratory. Among others we investigated employees of waste disposal sites (Fender and Wolf 1998), long-haul flight personnel (Wolf *et al.*, 1999) and Ukrainian children living in areas contaminated by Chernobyl fallout (Wolf *et al.*, 1994). Here we present cytogenetic studies in radiation exposed nuclear power plant workers, in perchloroethylene exposed dry cleaners and in non-exposed persons (controls). In addition some methodological investigations regarding the number of cells that should be scored per person are presented.

2. Material and methods

2.1 Groups of persons investigated

2.1.1 Nuclear power plant workers

From 1984 to 1986, cytogenetic investigations of nuclear power plant (NPP) workers from an East German nuclear power plant were performed. At that time the plant operated for about 10 years. We investigated 186 men with known life-time doses and 18 nonexposed trainees as site controls. Information on health status and life-style were obtained by questionnaires. As additional control group, 49 male clerks from a Berlin administration were recruited.

In these groups 500 conventionally stained cells were scored per person for chromosomal aberrations.

2.1.2 Dry cleaners

On request of the industrial hygiene authority, 10 nonsmoking female dry cleaners with long-term

exposure to high concentrations of perchloroethylene and 9 nonsmoking female control persons were investigated. Perchloroethylene concentrations in the work environment were determined by personal air sampling and analyses were performed by gas chromatography. The trichlorethylene contamination of technical grade perchlorethylene was about 0.3 %. The employee's topical dose was calculated as the geometric mean of about ten average shift concentrations at the individual workplaces. As an estimate of life time exposure to perchlorethylene, a mean total concentration was calculated in each exposed woman regarding the time spent at different workplaces. In this study, 1500 conventionally stained cells were scored per person.

2.1.3 Controls

Blood samples from unexposed persons were continuously investigated to collect cytogenetic data of the normal population. Mostly people working in administrative jobs were asked to give blood for cytogenetic investigations on occasion of routine medical checks. We compiled two control groups, namely, 83 persons with 500 cells scored per person from conventionally stained slides (control group A), and 99 persons with 1000 first *in vitro* division metaphases (M1 cells) scored per person on differentially stained slides (control group B).

2.1.4 Methodological investigations

Intraindividual variability was established in four nonsmoking control persons by scoring up to 6000 cells from the same blood culture at consecutive samples of 1000 cells. To test the influence of sample size in 43 males, subsamples of the first 250 and 500 cells scored in each person were compared with the full sample of 1000 cells.

2.2 Cytogenetic Methods

Venous blood was taken with sterile heparinized monovets (Sarstedt) and the samples were coded. Cultures were set up in 50 ml Erlenmeyer flasks. Each culture contained whole blood, RPMI 1640 medium supplemented with 10% fetal calf serum, phytohemagglutinin and, in case of control group B, 8 µg/ml 5-bromodeoxyuridine was added (BrdU, Serva). Antibiotics were not used. The cultures were incubated for 48 hours including a 3 hours treatment with 0.33 µg/ml colchicine. Preparations were made using a routine protocol, including hypotonic treatment with 75 mM KCl for 15 min at 37°C and fixation in methanol - acetic acid (3:1). Slides from BrdU-containing cultures were stained differentially and exclusively M1 cells were analysed. Slides from lymphocyte cultures without BrdU were block stained with 3% Giemsa. All types of chromosomal aberrations were recorded according to Evans and O'Riordan (1975). Dicentric and centric ring chromosomes were pooled and were given as dicentric equivalents (dic). Polycentric chromosomes were converted to dic by subtracting one centromere from the polycentric and taking the remaining centromeres as the number of dic. Double minutes and acentric rings are included in the excess acentric fragments. Atypical monocentric chromosomes which were found in small numbers, were not given in the tables separately, but were included in the categories "cells with chromosomal aberrations" and "percentages of aberrant cells". Gaps were scored, but not included in the percentage of aberrant cells. Cells with more than two dic or more than four chromosome breaks were considered as multiaberrant cells. These cells were listed separately since it was often impossible to properly analyse all aberrations. Aberrations in multiaberrant cells were excluded from data showing the frequencies of single aberration types, but they were included in the cells with chromosomal aberrations and percentages of aberrant cells.

2.3 Statistics

Data were statistically analyzed with the nonparametric U-test of Wilcoxon, Mann and Whitney. This

test avoids making assumptions concerning the intercellular and interindividual distributions of aberrations in consideration of the heterogeneity between individuals within the groups and possible deviations from the Poisson distribution. Possible correlations between cytogenetic findings, exposure parameters and age were investigated using the rank correlation analysis of Spearman. The Pearson χ^2 test was applied to test the adequacy of Poisson and binomial distributions.

3. Results

Tables 1 to 7 summarize the population characteristics and cytogenetic data of the groups investigated.

3.1 NPP workers

The mean lifetime dose of 186 NPP workers was 58.5 ± 67.1 mGy (0–238 mGy). Compared to the control group of male persons, significantly more dic, cells with chromosomal aberrations and aberrant cells were found in NPP workers (Table 1). Stratification of the NPP workers by radiation exposure (Table 2) results in significantly higher yields of dic even in the lowest dose group (life time dose < 10 mGy). As compared to the lowest dose group, the difference in frequencies of dic between the dose groups is significantly higher only at lifetime doses > 100 mGy. The mean yield of excess acentric fragments and cells with chromosomal aberrations was highest at life time doses from 10 mGy to < 100 mGy. Chromatid aberrations are significantly more frequent in the lowest dose group, and stratification by smoking status (Table 3) showed that this is caused by high frequencies of chromatid aberrations in 14 smokers who stated to smoke more than 10 cigarettes per day. Multiple correlation analyses regarding the effect of age and parameters of exposure on chromosomal aberrations shows a significant positive correlation between life-time dose and number of dic ($r = 0.169$, $p < 0.05$), as well as of cells with chromosomal aberrations ($r = 0.151$, $p < 0.05$). The dose accumulated during the year before the cytogenetic investigation did not influence the frequencies of chromosomal aberrations.

The group of NPP workers includes 97 nonsmokers and 84 smokers, 26 of them stated to smoke more than 10 cigarettes per day. There is no significant difference in mean age (32.4 ± 8.7 years in nonsmokers versus 29.8 ± 7.5 years in smokers), mean exposure time (8.0 ± 4.0 versus 7.3 ± 3.8 years), mean life-time dose (62.8 ± 68.7 versus 47.1 ± 59 mGy) and mean dose of the year before the cytogenetic investigation (6.1 ± 8.5 versus 5.3 ± 7.4 mGy). Only in the subgroup of 26 persons smoking more than 10 cigarettes per day the number of cells with chromatid aberrations was significantly elevated as compared to nonsmokers or to the light smokers. In controls with known smoking habits (15 nonsmokers and 19 smokers) there is the same tendency of elevated number of cells with chromatid aberrations in smokers, but the difference is not significant.

3.2 Dry Cleaners

In one person from the exposed group unusual high numbers of chromosomal aberrations were observed most probably resulting from radiation therapy some years ago. This person was excluded from the study. Compared to female control persons the mean aberration yield in the remaining nine perchlorethylene exposed women is significantly elevated for dic, excess acentric fragments, cells with chromosome aberrations, and percentage of aberrant cells (Table 4). There was no correlation between frequency of most aberration types and age, seniority, topical dose and mean total concentration of perchlorethylene. Only excess acentric fragments were positively correlated with seniority ($r = 0.68$; $p < 0.05$).

3.3 Control Groups

The control group A (Table 5) comprises of 67 men and 16 women aged 20 to 56 years (mean 36.6

Table 1 Population characteristics and cytogenetic results of NPP workers and concomitant male control persons

Groups of persons analyzed	Mean ± SD (min – max)			Total number of aberrations			Mean ± SE per person		Mean ± SE % aberrant cells
	Age	Duration of employment Years	Life time dose mGy	Dose last year	Number of cells scored	Dic	Ace	Ma cells	
NPP workers n = 186	31.2 ± 8.2 (18–62)	7.7 ± 3.8 (1–18)	58.5 ± 67.1 (0–238)	5.8 ± 7.9 (0–30.7)	93.000	140	347	7	604 500
Site control n = 18	27.5 ± 7.1 (20–46)	—	—	—	9.000	0.8 ± 0.1 ^a 4	1.9 ± 1.5 25	0.04 ± 0.02 1	3.3 ± 0.2 67
External control n = 49	38.2 ± 7.0 (25–56)	—	—	—	24.500	0.2 ± 0.1 8	1.4 ± 0.4 59	0.06 ± 0.06 2	3.7 ± 0.8 135
Combined control n = 67	35.2 ± 8.5 (20–56)	—	—	—	33.500	0.2 ± 0.1 12	1.2 ± 0.2 84	0.04 ± 0.03 3	2.8 ± 0.5 212
						0.2 ± 0.1 ^a	1.3 ± 0.2	0.04 ± 0.03	3.2 ± 0.5 100
									1.5 ± 0.2 ^b 0.9 ± 0.1 ^c

Dic: dicentric and ring chromosomes; Ace: excess acentric fragments; Ma cells with multiple aberrations, sd: standard deviation; se: standard error;
^{a,b,c}: difference significant ($p \leq 0.05$)

Table 2 Population characteristics and cytogenetic results of NPP workers: Stratification according to lifetime doses
(Abbreviations see Table 1)

Stratification of groups	Age	Duration of employment (Years)	Life time dose (mGy)	Mean ± SD (min-mix)		Cells scored	Dic	Ace	Ma cells	Total number of aberrations Mean ± SE per person		Mean ± SE % of aberrant cells
< 10 mGy n = 75	31.1 ± 9.1 (18–62)	6.4 ± 4.2 (1–18)	2.5 ± 3.1 (0–9.6)	37.500	40	110	1			275	156	1.1 ± 0.1
10-< 100 mGy n = 56	30.7 ± 8.1 (19–51)	7.4 ± 3.4 (2–12)	42.5 ± 26.5 (10.1–99.3)	28.000	0.5 ± 0.1 ^a 36	1.5 ± 0.2 ^b 137	0.01 ± 0.01 4			3.7 ± 0.3 ^c 183	2.1 ± 0.2 ^d 177	1.3 ± 0.1
> 100 mGy n = 55	31.8 ± 9.1 (23–51)	9.8 ± 2.9 (6–18)	151.3 ± 37.2 (100.6–238.4)	27.500	0.6 ± 0.1 64	2.5 ± 0.3 ^b 101	0.07 ± 0.04 2			3.3 ± 0.4 146	3.2 ± 0.3 ^d 168	1.1 ± 0.1
					1.2 ± 0.2 ^a	1.8 ± 0.3	0.04 ± 0.04			2.7 ± 0.3 ^c	3.1 ± 0.4	

^{a,b,c,d}: difference significant ($p \leq 0.05$)

**Table 3 Population characteristics and cytogenetic results of NPP workers: Stratification according to smoking status
(Abbreviations see Table 1)**

Stratification of groups	Mean ± SD (min–max)			Cells scored	Total number of aberrations Mean ± SE per person			Mean ± SE %
	Age	Duration of employment (Years)	Life time dose (mGy)		Dic	Ace	Ma cells	
Nonsmokers n = 97	32.4 ± 8.7 (19–62)	8.0 ± 4.0 (1–18)	62.8 ± 68.7 (0–238.4)	48.500	73	179	6	296 ± 0.1 3.1 ± 0.3 ^a
All smokers n = 84	29.8 ± 7.5 (18–56)	7.3 ± 3.8 (1–18)	47.1 ± 59.0 (0–215.5)	42.000	64	160	1	260 ± 0.3 2.7 ± 0.3
Smokers, > 10 cig./d n = 26	30.4 ± 6.7 (22–45)	6.8 ± 3.5 (1–13)	47.0 ± 56.3 (0–146.3)	13.000	18	40	0	286 ± 0.1 3.4 ± 0.3
Smokers, < 10 cig./d n = 58	29.5 ± 7.9 (18–56)	7.6 ± 3.9 (1–18)	47.2 ± 60.6 (0–215.5)	29.000	46	120	1	118 ± 0.1 4.5 ± 0.5 ^{a,b}
No information n = 5	30.8 ± 3.7 (25–34)	9.4 ± 1.8 (7–11)	166.6 ± 77.6 (31.1–215.9)	2.500	3	9	0	169 ± 0.4 2.9 ± 0.4
							20	12 ± 0.2
							4.0 ± 2.5	2.4 ± 0.2

^{a,b}, difference significant ($p \leq 0.05$)

Table 4. Population characteristics and cytogenetic results of female dry cleaners and concomitant female controls
(Abbreviations see Table 1)

Group	Mean ± SD (min-mix)			Cell scored	Total number of aberrations Mean ± SE per person		Mean ± SE % aberrant cells	
	Age	Duration of employment	Topical dose ^a (Years)		Dic	Ace		
Dry cleaners n = 9	47.9 ± 5.5 (40–56)	16.4 ± 3.9 (8.6–20.5)	257 ± 62.8 (144–348)	4842 ± 2282 (1909–8277)	13.100 ^c 3.8 ± 0.7 ^d	34 6.7 ± 1.2 ^e	60 10.8 ± 1.1	97 11.2 ± 17 ^f
Controls n = 9	40.0 ± 16.5 (21–56)	—	—	13.500	3 0.3 ± 0.1 ^d	18 2.0 ± 0.8 ^c	66 7.3 ± 0.9	22 2.4 ± 10 ^f
Dry cleaners, radiation therapy	55	16.0	188	2154	1.500 18	15 7	7 33	0.7 ± 0.1 ^g 2.7

^a: geometric mean of about 10 shift average concentrations; ^b: estimate of life-time exposure; ^c: in 1 person only 1100 scorable cells were found
^{d,f,g}: difference significant ($p \leq 0.05$)

Table 5 Frequency of chromosomal aberrations in a control group (500 cells scored per person)

	Sex			Smoking status			Age		
	Male	Female	Nonsmokers						
				Smokers	No information	< 40 y	44	51	26
Persons analysed	83	67	16	20	19	44	51	26	6
Age [y]									
Mean ± sd	36.6 ± 8.6	35.2 ± 8.5	41.8 ± 7.3	32.1 ± 7.6	34.5 ± 8.4	38.9 ± 8.4	31.3 ± 5.2	46.0 ± 4.8	
(min–max)	(20–56)	(20–56)	(21–52)	(20–49)	(21–47)	(20–56)	(20–38)	(40–56)	
Cells scored	41,500	33,500	8,000	10,000	9,500	22,000	25,500	13,000	3,000
Aberrations									
Total number of aberrations mean ± SE per person									
Ctb	244	223	21	45	84	115	147	74	23
Cte	3.0 ± 0.4	3.3 ± 0.5 ^a	1.4 ± 0.5 ^a	2.3 ± 0.5	4.4 ± 1.0	2.7 ± 0.6	2.9 ± 0.5	2.9 ± 0.9	3.8 ± 1.9
Cte	5	3	2	2	2	1	3	1	1
Dic	0.06 ± 0.02	0.04 ± 0.03	0.1 ± 0.1	0.1 ± 0.07	0.1 ± 0.1	0.2 ± 0.02	0.06 ± 0.04	0.04 ± 0.04	0.2 ± 0.2
Dic	23	12	11	11	0	12	7	10	6
Ace	0.3 ± 0.1	0.2 ± 0.1 ^b	0.7 ± 0.3 ^d	0.6 ± 0.2 ^d	0 ^d	0.3 ± 0.1	0.14 ± 0.1	0.4 ± 0.2	1.0 ± 0.5
Ace	98	84	14	24	29	45	60	29	9
Ma cells	1.2 ± 0.2	1.3 ± 0.2	0.9 ± 0.3	1.2 ± 0.3	1.53 ± 0.4	1.0 ± 0.2	1.2 ± 0.2	1.1 ± 0.2	1.5 ± 0.6
Ma cells	3	3	0	0	1	2	3	0	0
CTA	0.04 ± 0.02	0.04 ± 0.03		0.05 ± 0.05	0.05 ± 0.03	0.06 ± 0.03			
CTA	233	212	21	45	79	109	143	66	24
CSA	2.8 ± 0.4	3.2 ± 0.5 ^c	14 ± 0.4 ^c	2.3 ± 0.5	4.2 ± 1.0	2.5 ± 0.5	2.8 ± 0.4	2.5 ± 0.8	4.0 ± 1.8
CSA	126	100	26	37	30	59	71	40	15
%	1.5 ± 0.2	1.5 ± 0.2	1.6 ± 0.5	1.9 ± 0.4	1.6 ± 0.4	1.3 ± 0.2	1.4 ± 0.2	1.5 ± 0.3	2.5 ± 1.0
%	0.9 ± 0.1	0.9 ± 0.1	0.6 ± 0.2	0.8 ± 0.2	1.2 ± 0.3	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.2	1.30 ± 0.4

^{a,b,c,d} difference significant ($p \leq 0.05$)

Ctb: chromatid breaks; Cte: chromatid exchanges; Dic: dicentric and ring chromosomes; Ace: excessacentric fragments; Ma cells: cells with multiple aberrations; CTA cells with chromatid aberrations; CSA: cells with chromosome aberrations; %: percentage of aberrant cells

Table 6 Frequency of chromosomal aberrations in a control group (1000 M1 cells scored per person) (Abbreviations see Table 5)

	Sex			Smoking status			Age		
	Male	Female	Nonsmokers	Exsmokers	Smokers	No information	< 40 y		No Information
							26	2	
Persons analysed	99	41	58	55	16	26	2	46	52
Age [y]	39.6 ± 9.8	39.7 ± 9.5	39.5 ± 10.1	39.0 ± 10.2	41.8 ± 9.7	39.7 ± 8.6	30.9 ± 5.1	47.1 ± 6.1	
Mean ± sd (min–max)	(17–58)	(23–58)	(17–58)	(17–58)	(21–58)	(24–58)	(17–39)	(40–58)	
cells scored	99.000	41.000	58.000	55.000	16.000	26.000	46.000	52.000	1.000
Aberrations	Total number of aberrations Mean ± SE per person								
Ctb	642	280	362	282	131	214	15	266	366
	6.5 ± 0.5	6.8 ± 0.8	6.2 ± 0.7	5.1 ± 0.5 ^{a,b}	8.2 ± 1.3 ^a	8.2 ± 1.3 ^b	(5; 10)	5.8 ± 0.7	7.0 ± 1.0
Cte	58	17	41	19	20	19	0	18	40
	0.6 ± 0.1	0.4 ± 0.1	0.7 ± 0.2	0.4 ± 0.1	1.3 ± 0.5	0.7 ± 0.3	0	0.4 ± 0.2 ^j	0.8 ± 0.1 ^j
Dic	119	44	75	54	18	45	2	44	73
	1.2 ± 0.1	1.0 ± 0.2	1.3 ± 0.2	1.0 ± 0.2 ^c	1.1 ± 0.3	1.7 ± 0.4 ^c	(0; 2)	1.0 ± 0.2	1.4 ± 0.2
Ace	207	90	117	94	56	48	9	83	123
	2.1 ± 0.3	2.2 ± 0.4	2.0 ± 0.3	1.7 ± 0.2	3.5 ± 0.9	1.9 ± 0.5	(8; 1)	1.8 ± 0.3	2.4 ± 0.3
Ma cells	5	0	5	4	0	1	0	1	4
	0.05 ± 0.03	0.09 ± 0.04	0.07 ± 0.04	0.07 ± 0.04	0.04 ± 0.04	0.04 ± 0.04	0	0.02 ± 0.02	0.08 ± 0.01
CTA	665	275	390	289	139	223	14	278	278
	6.7 ± 0.5	6.7 ± 0.8	6.7 ± 0.7	5.3 ± 0.5 ^{d,c}	8.7 ± 1.3 ^d	8.6 ± 1.3 ^d	(5; 9)	6.1 ± 0.7	7.3 ± 1.0
CSA	391	154	237	187	88	105	11	151	237
	4.0 ± 0.3	3.8 ± 0.6	4.1 ± 0.4	3.4 ± 0.4 ^{f,g}	5.5 ± 1.0 ^f	4.0 ± 0.7 ^g	(8; 3)	3.3 ± 0.4	4.6 ± 0.5
%	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	0.9 ± 0.1 ^{h,i}	1.4 ± 0.2 ^h	1.3 ± 0.2 ⁱ	1.3 ± 1.2	0.9 ± 0.1	1.2 ± 0.2

^{a,b,c,d,e,f,g,h,i,j}; difference significant ($p \leq 0.05$)

Table 7 Intraindividual variability of cytogenetic scores in consecutive samples of 1000 cells from the same lymphocyte culture (Abbreviations see Table 5)

Persons analyzed sex, age	Total number of cells scored	Total number of aberrations				Mean + SE (min-max) % of aberrant cells			
		Ctb	Cle	Dic	Ace	Ma cells	CTA	CSA	
A f, 53 y	4000	19	1	6	4	1	20	9	0.7 ± 0.4 (0-1.6)
		4.8 ± 2.3 (0-10)	0.3 ± 0.3 (0-1)	1.5 ± 1.0 (0-4)	1.0 ± 0.6 (0-2)	0.3 ± 0.3 (0-1)	5.0 ± 2.2 (0-10)	2.3 ± 1.4 (0-6)	
B f, 52 y	5000	34	6	7	34	2	37	45	1.6 ± 0.3 (1.1-2.5)
		6.8 ± 2.1 (2-14)	1.2 ± 0.7 (0-4)	1.4 ± 0.4 (0-2)	6.8 ± 2.4 (2-14)	0.4 ± 0.4 (0-2)	7.4 ± 1.9 (2-13)	9.0 ± 2.4 (0-17)	
C f, 57 y	6000	40	4	6	17	0	41	22	1.1 ± 0.1
		6.7 ± 0.6 (5-8)	0.7 ± 0.5 (0-3)	1.0 ± 0.5 (0-3)	2.8 ± 1.0 (0-7)	0	6.8 ± 0.7 (4-9)	3.7 ± 0.3 (0-9)	
D f, 54 y	6000	41	6	8	20	0	42	30	1.3 ± 0.4 (0.4-2.8)
		6.8 ± 2.9 (1-22)	1.0 ± 0.6 (0-4)	1.3 ± 0.5 (0-3)	3.3 ± 1.2 (0-8)	0	7.0 ± 3.2 (1-24)	5.0 ± 1.6 (0-10)	
Total	21000	132	17	27	76	3	140	106	1.2 ± 0.2 (0-2.8)
		6.3 ± 1.1 (0-22)	0.8 ± 0.3 (0-4)	1.3 ± 0.3 (0-4)	3.6 ± 0.8 (0-14)	0.1 ± 0.1 (0-2)	6.7 ± 1.1 (0-24)	5.0 ± 1.0 (0-17)	

± 8.6 years). Mean frequencies and standard errors of the means in samples of 500 cells scored per person were 0.3 ± 0.1 for dic, 1.2 ± 0.2 for excessacentric fragments, 2.8 ± 0.4 for cells with chromatid aberrations, and 1.5 ± 0.2 for cells with chromosome aberrations. The percentage of aberrant cells were 0.9 ± 0.1 . In men, chromatid aberrations were significantly more frequent than in women, whereas dic occur more frequently in women. Smoking status was known in a subgroup of 39 persons (20 nonsmokers and 19 smokers) and we observed a nonsignificant tendency of elevated numbers of chromatid aberrations and percentages of aberrant cells in the smokers. Dic were exclusively found in the group of nonsmokers. The frequencies of aberrations were not significantly different between groups of persons up to 40 years or older. There was no significant correlation between age and frequencies of aberrations. We observed 3 multiaberrant cells, all in the subgroup of men younger than 40 years.

Control group B (Table 6) consists of 99 persons, 41 men and 58 women, aged 17 to 58 years. In samples of 1000 M1 cells scored per person, we observed mean frequencies of 1.2 ± 0.1 for dic, 2.1 ± 0.3 for excessacentric fragments, 6.7 ± 0.8 for cells with chromatid aberrations and 4.0 ± 0.3 for cells with chromosomal aberrations. The percentage of aberrant cells was 1.1 ± 0.1 . Stratification according to sex revealed no significant differences between men and women.

Smoking status was known in all, but two persons. There were 55 nonsmokers, 16 exsmokers, and 26 smokers. Compared to nonsmokers significantly elevated frequencies of chromatid aberrations and dic were found in smokers. In exsmokers significantly more cells with chromatid aberrations occurred as compared to nonsmokers, cells with chromosome aberrations were more frequent in this group than in nonsmokers and smokers. Compared to nonsmokers the percentages of aberrant cells were significantly elevated in exsmokers as well as in smokers.

Although there was a tendency of increased mean values of all aberration types in the subgroup of persons older than 40 years, the differences were not significant. Correlation analyses revealed significant correlations between age and number of cells with chromosomal aberrations ($r = 0.215$; $0.05 > p > 0.02$) as well as percentage of aberrant cells ($r = 0.23$; $0.05 > p > 0.02$). In contrast to control group A, five multiaberrant cells were found in females, four of them in the subgroup older than 40 years.

In both control groups, the distributions of aberration frequencies fit well to the negative binomial distribution as described by Bowman and Kastenbaum (1992), whereas there were significant deviations from the Poisson distribution (Figs. 1 and 2).

3.4 Methodological investigations

Scoring of four to six consecutive samples of 1000 cells from the same lymphocyte culture in four female control persons revealed a considerable intraindividual variability (Table 7). Although the means fitted well with the data of the female subgroup of control group B, considerable differences occurred in single samples, such as 0 to 4 dic per sample in the same individual. In 43 elder men, the means and standard deviations of the percentages of aberrant cells in samples of 250 cells, 500 cells and 1000 cells were 1.98 ± 1.32 , 1.92 ± 1.13 , and 1.88 ± 0.71 respectively. There are only small differences of the means, but a marked increase of the standard deviations occurs if compared with the mean of the whole sample of 1000 cells. Figure 3 shows the observed distributions and the expected normal distributions for the mean percentage of aberrant cells.

4. Discussion

Cytogenetic investigations, especially chromosomal analyses, have been used for more than 30 years

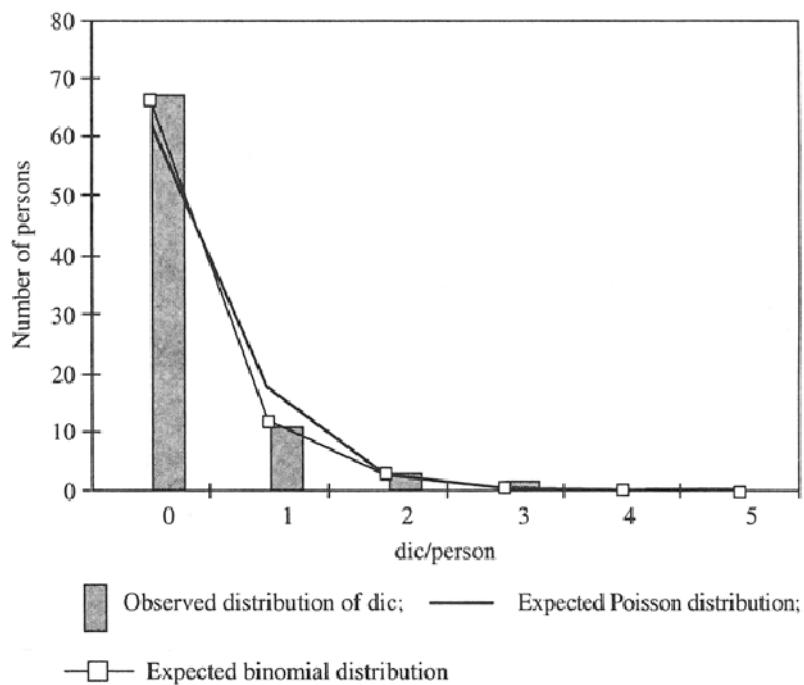


Fig. 1 Observed and expected frequencies of dic in control group A (500 cells scored per person)

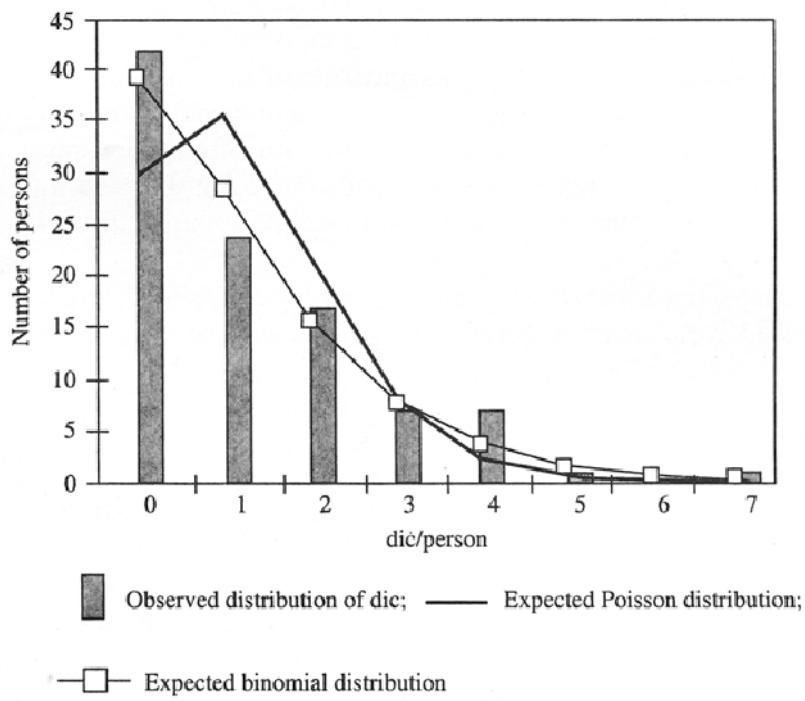


Fig. 2 Observed and expected frequencies of dic in control group B (1000 cells scored per person)

to monitor persons exposed to known or suspected genotoxic agents. Studies of occupationally radiation exposed personnel revealed an increase of mean frequencies of chromosomal aberrations, especially dic as well as acentric fragments (Evans *et al.*, 1979; Lloyd *et al.*, 1980; Balasem *et al.*, 1992; Barinquero *et al.*, 1993; Fender *et al.*, 1994). Results of our investigation of nuclear-power plant workers agree well with studies of others (Bauchinger *et al.*, 1980; Leonard *et al.*, 1984; Tawn

and Binks 1989; Chung *et al.*, 1996), where 500 cells were scored per person too. The mean frequencies of dic per person in NPP workers were reported to be 0.89 ± 0.1 (Bauchinger *et al.*, 1980), 0.84 ± 0.1 (Leonard *et al.*, 1984), 0.84 ± 0.1 (Chung *et al.*, 1994) and 0.8 ± 0.1 in this study. The respective control values are 0.2 ± 0.1 , 0.56 ± 0.2 , 0.24 ± 0.1 , and 0.2 ± 0.1 . Tawn and Binks (1989) found in 71 NPP workers with accumulated doses > 0.5 Sv a mean frequency of 1.6 dic per person. With the exception of the study of Leonard *et al.* (1984), where the mean yield of dic in controls was quite high, there are significantly more dic in exposed than in the control groups. Stratification in dose groups did not result in significant positive correlations between lifetime dose and frequencies of chromosomal aberrations in four studies (Bauchinger *et al.*, 1980; Leonard *et al.*, 1984; Tawn and Binks, 1989; Chung *et al.*, 1994), whereas we observed a significant elevation of mean yields of dic and mean number of cells with chromosomal aberrations in the lifetime dose group > 100 mGy as compared to the dose group < 10 mGy. Lack of evidence for a dose-dependent increase in aberration frequencies might be caused by small group size especially in the high dose groups (Bauchinger *et al.*, 1980; Leonard *et al.*, 1984) and truncation of the dose range by neglecting low doses (Tawn and Binks, 1989, Chung *et al.*, 1994). Evans *et al.* (1979) described a significant dose-dependent increase in mean aberration frequencies in a cytogenetic study of nuclear-dockyard workers. We did not observe an effect of heavy smoking on frequencies of dic and acentrics as described by Tawn and Binks, (1989), but chromatid aberrations were more frequent in the subgroup of heavy smokers than in nonsmokers and light smokers.

Ikeda *et al.*, (1980) investigated 10 workers exposed to perchlorethylene from three month up to 18 years. Six persons working in degreasing workshops were exposed to mean air concentrations of 92 ppm (≈ 628 mg/m³) perchlorethylene, the four others were exposed to air concentrations of about 10 to 40 ppm (≈ 70 to 270 mg/m³). The authors scored 50 metaphases of the second cell cycle *in vitro* (M2 cells) and did not find a difference in the percentage of aberrant cells between the exposed group and 11 control persons. In our opinion, this study suffers from serious drawbacks, for instance scoring of M2 cells and a sample size of only 50 cells per person in small groups, which compromises the result. Böttger and Elstermeier (1989) investigated 38 dry cleaners and 45 age matched control persons. The sample size was 200 cells per person. They did not find a significant increase in dry cleaners as compared to controls.

In our study we tried to compensate for the small group size by scoring 1500 cells per person and found a significant increase in aberration frequency in dry cleaners, which is mainly caused by elevated numbers of dic and excess acentric fragments. Frequencies of chromatid aberrations did not differ significantly between both groups. Although the mean frequencies of dic are unusual low in the control group, comparison with aberration frequencies of control group B confirm the increase of chromosomal alterations in perchlorethylene exposed dry cleaners.

The results of our investigations of nonexposed persons show the considerable interindividual variation occurring in the normal population. Moreover we confirmed the overdispersion of aberrations in aggregated cytogenetic data as described by Bowman and Kastenbaum (1992). A correlation with age as described by Ganguly (1993) and Tonomura *et al.* (1983) was only seen in control group B where 1000 cells were scored per person. Like Bender *et al.* (1988, 1989) we did not find differences in aberration frequencies between men and women. Regarding the influence of smoking on aberration frequencies, we confirmed the observations of Obe and Herha (1978); Obe *et al.* (1982), Littlefield and Joiner (1986) and Tawn and Binks (1989).

Comparing the two control groups A and B by normalizing the results of control group A to an aberration rate per 1000 cells, the mean frequencies of most aberration types are higher in control group B than in group A. Especially the mean value of dic in group B is nearly twice as high as in

group A, whereas the mean frequencies of excess acentric fragments are not different. Control data from literature extend over a broad range (Lloyd *et al.*, 1980; Bender *et al.*, 1988, 1989; Galloway *et al.*, 1986; Sinha *et al.*, 1986; Tawn, 1987; Ganguly, 1993). Interlaboratory comparisons showed that in chromosomal analyses, methodological differences may influence the result considerably (Zavala *et al.*, 1979; Lloyd *et al.*, 1988; Dobson *et al.*, 1991). In their extensive review Lloyd *et al.* (1980) discussed the variability of control data and concluded that unstable chromosome aberration rates are not systematically affected by the size of the sample of cells scored. They regarded the total numbers of cells scored in single studies and did not consider possible influences of inter- and intra-individual variability as well as of the number of cells scored per person. From our own experience, the variability increases with decreasing numbers of cells scored per person (Fig. 3) as well as with decreasing numbers of persons investigated. Therefore, at least 1000 cells should be scored per person and it seems reasonable to score equal cell numbers per person. Only if this is realized, it is possible to compute the inter-individual variability. It should be taken into consideration, that the "experimental unit" in human population monitoring is the individual and not the cell. Especially in studies of occupationally or environmentally exposed groups, where the increase of chromosomal damage will be small, it is essential to exclude avoidable sources of variability. Therefore, the recommendations concerning a proper study design for human population monitoring studies (Buckton and Evaus 1973; Natarajan and Obe, 1980; Bloom, 1981; Hook, 1982; IAEA, 1986; Carrano and Natarajan 1988) should be followed. Still there is no general agreement on standardization and quality control in chromosomal analysis of human populations.

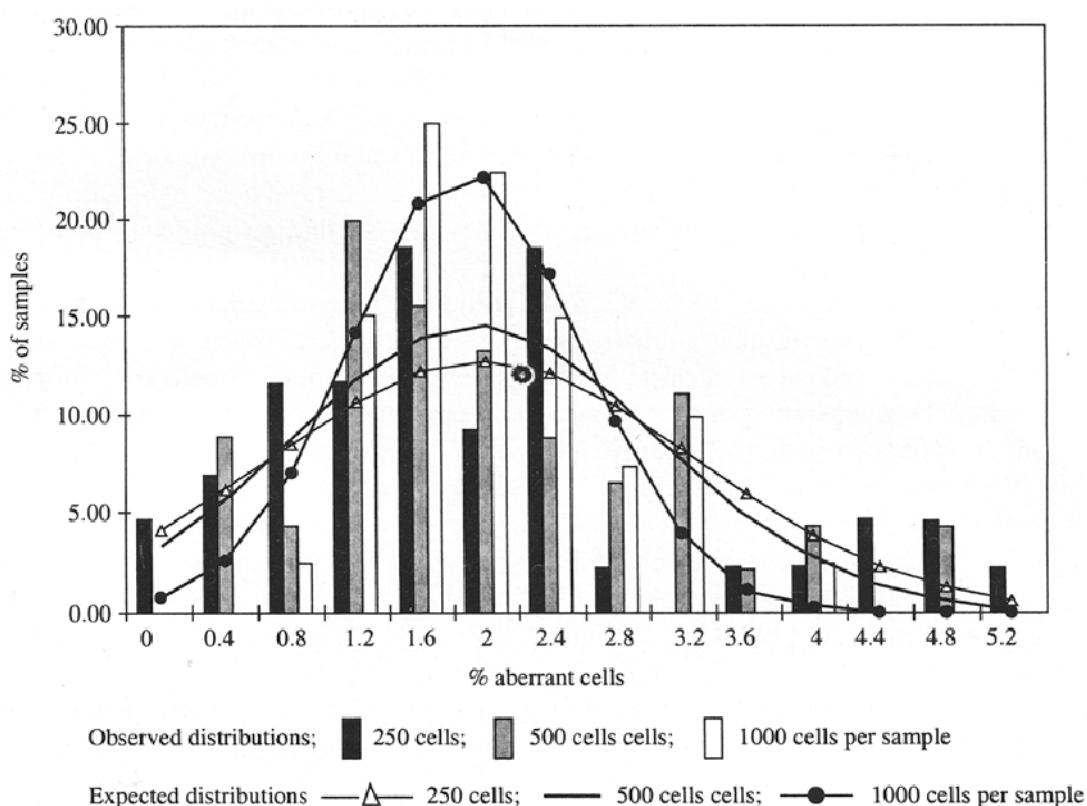


Fig. 3 Observed and expected frequency distributions of the percentages of aberrant cells in samples of 250, 500 and 1000 cells.

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14. Genotoxic Investigations on Occupationally Exposed Cattle Yard Workers

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Abstract: Genotoxicity of methane was investigated by assaying mitotic index (MI), chromosomal aberrations (CA), sister chromatid exchanges (SCE) and satellite associations (SA) in short term lymphocyte cultures of 60 male workers occupationally exposed to 56 ppm of methane and 60 matched controls compared with respect to age, sex, smoking and alcohol consumption. Various parameters showed significant increase ($p < 0.05$). MI (4.61-7.32), CA (0.95-3.81), SCE (3.98-7.22) and SA (8.78-18.12). Smoking and alcohol consumption were found to be confounding factors. Significant effect of period of exposure was also observed.

Key Words: Occupational exposure, methane, genotoxicity, chromosomal aberration, satellite associations.

1. Introduction

Genotoxic effect on workers exposed to X-rays (Yadav and Seth, 2000) and various gaseous chemicals and other toxic substances, in their occupational setting (Yadav and Seth, 1998a, b; Yadav *et al.*, 1999; Yadav and Thakur, 2000a, b) is noteworthy. This exposure results in damage to DNA, thus affecting its replicating ability. Its ability to carry information is also altered (Combes, 1992).

Atmospheric methane concentrations have more than doubled over the last two centuries (Thompson *et al.*, 1992), the major sources of methane in the atmosphere being fossil fuels, biomass burning, wetlands, rice cultivations, termites, oceans, etc. (Sajaak and Warneck, 1992).

Methane is produced discontinuously in the fore-stomach of ruminants during the fermentative process and about 95% of this gas produced is released into the atmosphere by the process of eructation (Murray *et al.*, 1976) Whereas CO₂ produced by respiration and eructation is consumed by plants, there is no such mechanism for methane absorption. Thus, the workers in the methane laden environment are at a great risk due to inhalation of this toxic hydrocarbon.

Mutagenic and lethal effects of halogenated methanes have been reported by Teressa and Pueyo (1993), Steward *et al.*, (1972) observed elevated level of blood carboxyhemoglobin level following exposure of human subjects to dichloromethane vapours. Four major trihalomethanes (CHCl₃, CHCl₂Br, CHClBr and CHBr₃) have been found to induce SCE in human lymphocytes *in vitro* and in mouse bone marrow cells *in vivo* by Morimoto and Koizumi (1983). Their studies report the genotoxic effect of derivatives of methane. However, no attempt has been made to assess the genotoxic effect of methane as such on human beings.

2. Materials and Methods

The present study was carried out on a total of 120 individuals, 60 of them were workers in the cattle

yard occupationally exposed to methane and 60 were matched controls with respect to sex, age, drinking and smoking habits (Table 1). The exposed group had an average exposure of 56 ppm of methane for 7 to 8 hours every day.

Table 1. Mitotic index (MI) in workers exposed to methane and control individuals

Duration of exposure (in years)	No. of samples	No. of cells screened <i>n</i> ₁	No. of metaphases <i>n</i> ₂	MI SD (<i>n</i> ₂ / <i>n</i> ₁) × 100
Control	60	296913	13706	4.6 11.08
Exposed	60	301907	22774	*7.3 21.04
0–5	21	102958	7806	7.5 80.97
6–10	21	107750	7748	7.1 91.21
11–15	7	35506	2960	8.3 30.05
16 above	11	55696	4260	7.6 40.66

**p* < 0.05.

An epidemiological survey was conducted and no severe external symptoms were observed in the exposed group. However, some of the workers reported suffocation and giddiness if they worked for more than 5–6 h at a stretch in the methane laden environment. All the 60 control individuals were healthy at the time of study.

Heparinized venous blood samples were obtained from each subject and samples transported to the laboratory in insulated ice boxes. Short term lymphocyte cultures were set up within 3 hours of sampling according to the method of Moorhead *et al.* (1960) with minor modifications. Briefly, 0.5 ml of whole blood was cultured in 5 ml of RPMI-1640 medium supplemented with 20% faetal calf serum and 0.1 ml phytohaemagglutinin (Sigma).

For mitotic index (MI) a minimum of 5000 cells per individual were counted for Giemsa-stained slides. Mitotic index was calculated by using the formula:

$$MI = \frac{\text{Number of dividing cells}}{\text{Total number of cells scored}} \times 100$$

The results were statistically analysed by applying the correlation test, coefficient of variance, chi square, and student's 't' test (Colton, 1980).

3. Observations

3.1 Mitotic Index

The mitotic indices (MI) in controls and workers exposed to methane over different periods of exposure are given in Table 1. The mean MI was 4.61 for controls and 7.32 for exposed personnel. The difference is significant at *p* < 0.05. It was the maximum (8.3) for the workers exposed to a period of 11–15 years. There was, however, no regular trend.

3.2 Chromosome aberrations and sister chromatid exchanges

The incidence of CA in the exposed group was found to be 2.21 as compared to 0.95 in the control group (Table 2). The frequency of SCE was 7.22 per cell in the exposed group and 3.98 per cell in the control group (Table 4). The difference in the frequencies of CA was significant at *p* < 0.01, whereas it was significant at *p* < 0.05 in the case of SCE.

Table 2. Total chromosome aberrations in exposed and control individuals

	Exposed			Control			't'
	n	Mean	SEM	n	Mean	SEM	
Total chromosome aberrations	60	3.81	0.54	60	2.22	0.44	p < 0.01
Gaps	60	1.60	0.64	60	1.27	0.59	„
Breaks	60	2.21	0.60	60	0.95	0.49	„

Table 3. Chromosome aberrations in relation to smoking and alcohol consumption

	Exposed			Control			't'
	n	Mean	SEM	n	Mean	SEM	
Alcoholics	4	2.00	0.50	13	1.15	0.57	p < 0.05
Smokers	17	2.11	0.47	16	1.13	0.64	„
Smokers-alcoholics	24	2.66	0.50	19	1.42	0.57	„
Non smokers-non Alcoholics	15	1.60	0.78	12	1.08	0.59	„

n = Number of individuals; SEM = Standard error of mean.

Table 4. Frequency of sister chromatid exchanges in workers to methane exposed and control individuals

	Exposed			Control			't'
	n	Mean	SEM	n	Mean	SEM	
Total SCEs	60	7.22	0.11	60	3.98	0.11	p < 0.
Alcoholics	4	7.04	0.32	13	3.41	0.14	„
Smokers	17	7.21	0.21	16	3.96	0.14	„
Smokers-Alcoholics	24	7.55	0.14	19	4.78	0.18	„
Non-Smokers-non Alcoholics	15	6.76	0.22	12	3.35	0.14	„

n = Number of individuals; SEM = Standard error of mean

3.3 Impact of alcohol and smoking

The incidence of CA and SCE among alcoholics (2.00 and 7.04 per cell), smokers (2.11 and 7.21 per cell), smoker alcoholics (2.66 and 7.55 per cell) and nonsmoker-non alcoholics (1.60 and 6.76 per cell) was quite high as compared to their respective controls (1.15 and 3.41), (1.31 and 3.96) (1.42 and 4.78) and (1.08 and 3.35) (Tables 3 and 4).

When these sub-groups of the exposed group were compared with the controls having similar habits it was observed that the frequency of both CA and SCE was much higher in the exposed group than the control group. The variations were significant at $p < 0.05$.

When the smokers (S), alcoholics (A), smoker-alcoholics (SA) and non smoker-non alcoholics (NSA) were compared to each other, it was observed that the incidence of CA and SCE was the highest among smoker-alcoholics than smokers and alcoholics followed by nonsmoker-non alcoholics (Tables 5 and 6).

Table 5. Comparison of chromosome aberrations in different sub-groups of personnel exposed to methane gas

	Mean Age (Years)	Mean exposure (Years)	Mean	SEM	SD	Coeff.of Variation	Range	Range Coeff.
S	31.52	9.00	2.11	0.50	1.23	56.86	0-4	1.00
A	33.50	7.75	2.00	0.50	1.15	50.00	1-3	0.50
SA	38.66	13.95	2.66	0.50	1.37	50.51	1-5	0.66
NSA	29.57	6.71	1.60	0.78	1.29	78.39	0-5	1.00

Coeff: Coefficient, S: Smokers, SA : Smokers-Alcoholics

SD : Standard deviation, A: Alcoholics, NSA: Non smoker non alcoholics

Table 6. Comparison of sister chromatid exchanges in sub-groups of personnel exposed to methane gas

	Mean Age (Years)	Mean exposure (Years)	Mean	SEM	SD	Coeff.of Variation	Range	Range Coeff.
S	31.52	9.00	7.21	0.21	0.89	12.40	5.8-8.40	0.10
A	33.50	7.75	7.04	0.32	0.64	9.17	6.4-7.80	0.09
SA	38.66	13.95	7.55	0.14	0.72	9.57	6.1-8.72	0.72
NSA	29.57	6.71	6.76	0.22	0.89	13.21	5.2-6.12	0.21

Coeff: Coefficient, S: Smokers, SA: Smoker- alcoholics

SD: Standard deviation, A: Alcoholics, NSA: Nonsmokers- nonalcoholics.

3.4 Impact of exposure period

When the CA were compared with the exposure period it was observed that their mean occurrence was quite high (3.28) in workers exposed to the cattle yard environment for 11–15 years as compared with exposure up to 5 years (1.57) (Table 7). However, frequency of CA increased up to the exposure of 15 years and then slightly decreased, whereas SCE frequency showed a positive correlation with exposure period.

3.5 Satellite associations

Table 8 shows satellite association patterns (SA) in exposed and control groups. There was nearly a

Table 7. Frequency of CA and SCE with duration of exposure

Duration of Exposure (Years)	N	CAs		SCEs	
		Mean	SEM	Mean	SEM
0-5	21	1.57	0.86	6.66	0.14
6-10	21	1.95	0.4C)	7.28	0.09
11-15	7	3.28	0.30	7.61	0.6
16 above	11	3.18	0.39	7.89	0.054%

N : Number of individuals.

two fold increase in the frequency of satellite associations in the exposed sample as compared with the controls. D G type of SA were the maximum (7.60), whereas 3D types were the lowest (0.63) in occurrence.

Table 8. Frequency of satellite association patterns in workers occupationally exposed to methane gas and control individuals

No. of indivi- duals	Total no. of cells	Types of satellite association between D and G group acrocentric chromosomes						Total no. of cells	Ass. per cell	SEM	
E	6000	D-D	D-G	G-G	2D-G	2GD	2G2D	3D	1088	18.13	0.92
60		(2.78)	(7.60)	(2.25)	(1.65)	(1.45)	(0.76)	(0.63)			
C	6000	114 (1.90)	236 (3.93)	72 (1.20)	28 (0.46)	28 (0.46)	23 (0.38)	26 (0.43)	527	8.78	0.31

Ass. per cell : Association per cell; E = Exposed, C = Controls.

4. Discussion

Methane has acquired importance recently as a green house gas responsible for global warming. Its presence in the cattle yard environment is easily felt by the peculiar smell, dung and rotting fodder being the main materials that generate this gas. Only those workers who have been working in the cattle yard at least for 7–8 h per day for at least two years were selected for the present study. Employees working in the same institute, but not connected with the yard, for a comparable period were taken as controls.

Significant variation in the frequency of CA between the exposed and control groups was observed. Gaps were not taken into consideration for obvious reasons. There was a positive correlation between the frequency of CA and the exposure period. Correlation was also noticed between the age and the incidence of CA in the present study. Picciano (1979), however, could not observe any relationship of induced aberrations with the age of the workers exposed to benzene. Low level of styrene exposure didn't show correlation either with exposure duration or with age (Makki-Pakkanen, 1987). However, Yadav and Kaushik (1995, 1997) working with SO₂ and NH₃ exposure, Sharma *et al.*, (1990) working with chemical exposure, Sobi and Bhardwaj (1991) working with stone dust and Rasmussen *et al.* (1988) working with exposure to trichloroethylene found that a highly positive correlation was apparent between CA and the increasing exposure duration. The latter authors also reported a positive correlation with age of the exposed workers as in the present study. In a way the increase in age indirectly refers to increase in the duration of exposure since the workers were regular employees who are recruited at a definite age. SCE analysis showed that there was significant variation in the incidence of SCE among the exposed and control groups. It was apparent that more the duration of exposure, more the number of SCE per cell. Like CA the SCE frequency was also positively correlated with the age of the sample. Similar results were obtained in SO₂ exposure by Yadav and Kaushik (1995), ethylene oxide exposure by Stolley *et al.* (1984) in workers using trichloroethylene for metal degreasing.

Smoking and alcoholism are confounding factors in chromosome aberrations and SCE. Lambert *et al.* (1978) found cigarette smokers in the control groups of their study to have significantly higher number of SCE than non smokers. However, they found no effect of smoking on the increased

frequency of SCE in laboratory workers. Sharma *et al.* (1990) and Sobti and Bhardwaj (1991) also reported nonsignificant effect of smoking and alcoholism on the frequency of CA and SCE. On the other hand Hansteen *et al.* (1984) Yadav and Seth (1998a,b) and Yadav *et al.* (1999) Yadav and Kaushik (1995, 1997) found the frequency of chromosomal breaks to be significantly increased in smokers compared with nonsmokers. Carbonell *et al.* (1990) observed that smokers, both the workers and the controls, had a high SCE frequency than nonsmokers and concluded that smoking is one of the most important factors influencing SCE induction in human lymphocytes *in vivo*. Batish (1983) also found that smoking and intake of alcohol had an increasing effect on the rate of chromosome aberrations and SCE in the personnel exposed to petrol exhaust, diesel exhaust and among bus drivers. A strong positive correlation between smoking and alcohol intake was observed during the present investigations. Smoker alcoholics showed the highest frequency of CA and SCE followed by smokers and alcoholics. The frequencies were quite low in exposed workers who neither smoked nor took alcohol but these were still higher than the control frequencies.

The formation of satellite association has often been attributed to the involvement of satellite chromosomes in nucleolar formation. The sticky nucleolar material would have a tendency to hold the associated chromosomes together through mitosis (Hsu, 1965). SA in exposed samples (8.18) shows a two fold increase, thereby increasing the risk of nondisjunction in exposed personnel.

The higher value of mitotic index in the exposed group as compared to the controls indicates higher division in the cells of exposed workers thereby making the workers cancer prone. The workers with duration of exposure between 11 and 15 years had the maximum MI (8.33).

It may be concluded from the above that cytogenetic end points are sufficiently sensitive to detect occupational level of exposure making it possible to evaluate the occupational environmental quality. Further, the genetic damage in the individuals exposed to methane may be detected using CA, SCE, SA and MI.

The present study also indicates that the cattle yard workers constitute a group which runs a risk of acquiring genetic injury. A regular cytogenetic monitoring of such workers is, therefore, needed.

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15. Telomeres, Telomerase and Cancer

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The enzyme telomerase is an object of much attention today. Telomerase activity has been found to be present in most malignant tumors (85%), but absent in most normal somatic tissues. Among normal tissues, activity is detected only in lymphocytes induced to proliferate, germline cells and stem cells. It also decreases with age. If telomerase could be used to detect a given stage of preinvasive neoplasia that could be treated with greater efficacy, it might have the potential to reduce cancer morbidity and mortality. Likewise, if telomerase could be used to identify invasive neoplasia earlier than is currently possible, it might have an impact on cancer mortality, assuming effective treatment is available. All these possibilities would be better understood, if the telomeres, their structure and functions are studied in detail.

Key words: Telomeres, telomerase, cancer, aging.

1. Introduction

Cellular immortality is the hallmark of cancer. Various factors have been associated with cellular immortality, along the continuum, which has created considerable interest in its potential for early detection of cancer (Rhyu, 1995; Greider, 1998). The goal of early detection is to identify carcinogenic lesions as early as possible in multistep pathogenesis (Mausner and Kramer, 1985; Srivastava and Kramer, 1995). The early detection of cancer is a dynamic process. It is driven by continually advancing insights into the biology of carcinogenesis and is limited by the availability of technologies. The nature of an ideal early detection biomarker, however, is unchanging. It should be expressed at a stage that allows interventions of greater efficacies during its pathogenesis.

The enzyme telomerase is an object of much attention today. Telomerase activity has been found to be present in most of the malignant tumors, but absent in most normal somatic tissues (Shay *et al.*, 1995). Benign tumors have telomerase activity in an intermediate percentage of cases. Among normal tissues, activity is detected only in lymphocytes induced to proliferate germline and stem cells (Rowley, 1998). At present “early in the multistep pathogenesis” is probably best defined by the histopathologic designation of preinvasive neoplasia. If telomerase could be used to detect a given stage of preinvasive neoplasia that could be treated with greater efficacy, it might have the potential to reduce cancer morbidity and mortality. Likewise, if telomerase could be used to identify invasive neoplasia earlier than is currently possible, it might have an impact on cancer mortality, assuming effective treatment is available. All these possibilities would be better understood, if firstly, the telomeres, their structure and functions and role of telomerase in tumor formation are studied in detail.

2. Telomeres—Their Structure and Function

Vertebrates have special structures at the ends of their chromosomes, known as telomeres. The

telomeres are organized as chromatin material, provide the chromosomes with genomic stability and protect them from degradation (Counter *et al.*, 1995). They

- help in correct pairing, initiation, movement of chromosomes at meiosis and serve as a buffer against loss of terminal sequences during DNA replication (Rubin, 1998),
- act as inert chromosomal caps preventing their rearrangements,
- have a special replicating mechanism.

Thus, a loss of telomeric function is associated with an increase in terminal fusion, dicentric chromosomes, mitotic instability and cell death.

Generally, telomeres consist of a tandem repeat of simple sequence DNA such as (TTAGGG)_n making up a total of 5–15 kilobase pairs. These DNA sequences are short (usually 5–8 nucleotides), precise and oriented 5' to 3' towards the end of the chromosome (Morin, 1989; Blessman and Mason, 1992; Counter, 1996). Furthermore, the length of the tandem repeats plays a role in the life-span and proliferation capacity of human cells. DNA replication does not allow completion of chromosome ends, so telomere length decreases with successive rounds of replication (Olovnikov, 1996). In culture and potentially *in vivo*, normal human somatic cells from a variety of tissues divide a finite number of times before they exit the cell cycle and arrest in a viable G₀ state called senescence (Goldstein, 1990). This system serves as a kind of mitotic clock and appears to be an important control mechanism for cellular proliferation capacity (Harley, 1991). Normal human somatic cells have a limited proliferative capacity, both in culture and *in vitro* and senescence. Transformation *in vitro* confers to cells an extended lifespan, but transformed cells eventually undergo a proliferative capacity accompanied by cell death, from which rare immortal clones emerge. Circumstantial evidence suggests that acquisition of extended proliferative capacity and even of immortality, can also occur *in vivo* during the development of tumors (Stamps *et al.*, 1992). In addition, since telomeres stabilize chromosome ends against recombination, their loss could explain the increased frequency of dicentric chromosomes observed in late passage senescent fibroblasts and provides a check point for regulated cell cycle exit.

While variation in subterminal telomere associated sequences has been described in many organisms, the structure of the terminal telomere repeat arrays at different chromosome ends within a cell is generally thought to be uniform. However, evidence from *in situ* hybridization experiments suggests that there may be some differences in telomere length between chromosomes in a single cell. Not only can the size and arrangements of variant and degenerate repeat regions differ between chromosomal termini, the sequence of the variant repeats can be chromosome specific. Sperm telomeres are longer than somatic telomeres and are maintained with age. It has also been shown that telomere arrays are much smaller in colonic mucosa and blood than in fetal tissue and sperm and that there is a reduction in average telomere length with age in blood and colon mucosa (Hastie *et al.*, 1990). Takagi *et al.* (1999) have shown that telomere length may represent the biologic behaviour of individual tumors and possibly the mode of development of colorectal carcinomas.

3. Telomerase

Cells must replicate their genes accurately whenever they divide so that each so-called daughter cell receives a complete set of genes without which the cell may malfunction and die. In 1972, James Watson noted that DNA polymerases, the enzymes that replicate DNA, could not copy linear chromosomes all the way to the tip. Hence, the replication machinery had to leave a single small region at the end (a piece of the telomere uncopied).

If cells had no way to compensate for this quirk, chromosomes would shorten with each round of cell division. Eventually, the erosion would eliminate the telomeres and certain critical genes in some generations of the cells. These cells would thus, perish, spelling the end of that cellular lineage. Telomeres do shorten during cell division, but they are also lengthened by the attachment of newly synthesized telomeric subunits. The researchers suspected that the source of these additional repeats was some undiscovered enzyme capable of a trick that standard DNA polymerases could not perform.

Telomerase, a ribonucleoprotein DNA polymerase, maintains telomere length with net telomere elongations (Greider and Blackburn, 1985; Yu *et al.*, 1990). The essential RNA component of telomerase contains a 9–30 nucleotide (nt) template region that dictates the synthesis of telomeric repeats on to DNA substrates, both *in vivo* and *in vitro*.

Telomerase activity was first identified and the genes encoding the RNA and protein components of telomerase were first cloned from unicellular ciliated Protozoa. In primary human and mouse cell strains, where telomerase activity is not detected, telomere length decreases with increasing number of cell divisions *in vitro* and with age *in vivo* (Harley and Villeponteau, 1995; Prowse and Greider, 1995). Primary human cells enter senescence after a characteristic number of divisions in culture (Hayflick and Moorhead, 1961). By contrast, telomere length is maintained in vertebrate germline cells and in immortalized cell lines in which telomerase is active (Counter *et al.*, 1992; Kim *et al.*, 1994). Thus, telomere length and telomerase activity appear to be markers of the replicative history and proliferative potential of cells; the intriguing possibility remains that telomere loss is a genetic bomb and hence casually involved in cell senescence and immortalization (Harley, 1991). Telomerase was able to build extensions to single strands of DNA from scratch, without benefit of an existing DNA template. This led to the proposal of a new solution to what has been called the end replication problem. This so-called end replication problem arises from the inability of standard mechanisms for replicating chromosomes to do a complete job. When DNA polymerases copy the two original, or “parent” DNA strands in a chromosome, they leave each new “daughter” strand shortened at one tip (at the end traditionally labelled 5'). If cells did not compensate for this flaw in the replication mechanism, chromosomes would inexorably shrink (Greider and Blackburn, 1996). Telomeric DNA is lost every time somatic cells divide, presumably because of the inability of DNA polymerase to fully replicate the ends of a linear DNA template and because of the lack of telomerase activity, the enzyme that elongates telomeric DNA *de novo*. Such shortening may act as a mitotic clock regulating the number of divisions a normal cell can undergo (Harley *et al.*, 1992; Baccheth and Counter, 1995). The link between shortening of telomeres and cellular aging and that telomeres are stably maintained in cancer cells has prompted intense investigations into the pathways connecting cellular aging and cancer (Shay, 1995).

Telomerase adds DNA to chromosomes before replication begins. The added DNA consists of one or more telomeric subunits, the short sequences of nucleotides that are repeated over and over in telomeres. It therefore, carries its own template for synthesizing telomeric DNA. It adds nucleotides that are “complementary” to those in the template *i.e.*, it aligns T nucleotides with As and G with Cs. The addition ensures that a daughter strand will be at least as long as its parent.

Telomerase activity has been demonstrated in culture cells from 18 different human tissues, 98 of 100 immortal populations, but not in 22 mortal populations. Similarly, Kim *et al.* (1994) demonstrated telomerase activity in 90 of 101 biopsies representing 12 human tumor types, but not in 50 normal somatic tissues. No telomerase activity is detectable in most normal somatic human tissues. In contrast, extensive telomerase activity is demonstrated in lung, colorectal, gastric, prostate, bladder, breast, cervical and vaginal cancers (Counter *et al.*, 1994; Hiyama *et al.*, 1995; Chandeneau *et al.*, 1995; Hiyama *et al.*, 1995; Pao *et al.*, 1997; Zheng *et al.*, 1997). Telomerase activity in normal

leukocytes is low as compared with that in immortalized cell lines and malignancies (Counter *et al.*, 1995). Shay *et al.* (1995) have found high telomerase activity in malignant tumors as compared to absent in normal somatic tissues. Among normal tissues, activity is detected only in lymphocytes induced to proliferate, germline cells and stress cells (Rowley, 1998).

Transfection of primary cells with SV40 T-antigen, EBV, E6/E7 of HPV, or mutant *p53* extends the life span of cells in culture (Counter *et al.*, 1994; Klingelhutz *et al.*, 1996). After a period of further growth, cultures enter a crisis and while most of the cells die, some become immortal and continue to grow. It has been demonstrated that HPV *E6* might directly induce low levels of telomerase in pre-crisis cell populations. After crisis, the telomerase activity increases suggesting the selection for telomerase positive cells in the population. Telomerase activity was also observed in exfoliated cells in urine from patients with bladder cancer (Kinoshita *et al.*, 1997) in colonic luminal washings (Yoshida *et al.*, 1997) and in lung cancer cells obtained from bronchial washings (Yahata *et al.*, 1998).

Bodnar *et al.* (1998) transfected telomerase negative normal human cell types with vectors encoding the human telomerase catalytic subunit. It was observed that they showed elongated telomeres, divided rigorously and showed reduced staining for β -galactosidase, a biomarker for senescence. It was concluded that introduction of telomerase into normal human cells could extend their life-span. Telomerase allowed the cells to divide for an additional 20 generations, past the time they normally would stop dividing.

4. Role of Telomerase in Malignancy

The telomere-telomerase hypothesis of aging has become a new model for some aspects of cancer progression (Kim *et al.*, 1994; Counter *et al.*, 1995; Hiyama *et al.*, 1995; Shay *et al.*, 1995; Piatyszek *et al.*, 1995; Wu *et al.*, 1999). This theory is based on the findings that:

1. progressive erosion of telomeres with age occurs in normal somatic cells.
2. most human tumors have shortened telomeres and express telomerase activity.
3. aging has been associated with an increased risk of cancer.

The presence of telomerase activity indicates that the cell has the ability to inactivate the telomeric "clock" that limits the proliferative capacity of normal somatic cells (Shay *et al.*, 1995). But the presence of telomerase activity in a cell implies very little about malignancy although it reflects its potentially immortal state. Cell immortality does not confer unregulated rates of cell proliferation, invasiveness or metastasis, but only gives the cells the proliferative capacity to accumulate the necessary mutations to become malignant. Cancer is diverse, thus, some tumors may need only a few mutations to become malignant and may not exhaust the normal limits of proliferation before they cause disease. These types of tumors could be expected to be both mortal and negative for telomerase activity. Escape from proliferative limits can occur at any time during cancer progression. So, the reactivation of telomerase would be expected to occur early in tumors, arising from cells near the limits of their proliferative capacity and late in tumors arising from a telomerase positive stem cells with long telomeres. In many cases of cancer, the inactivation of TSGs such as *p53* and *pRB* allows the cells to overcome M1 (Mitotic phase of cell-cycle) and have extended lifespan (Shay and Wright, 1996). This directly leads to the genomic instability that contributes to the accumulation of additional mutations.

5. Quiescence and Stem Cells

Although hematopoietic stem cells and their immediate progeny are telomerase competent, the telomerase of white blood cells decline with the donor's age (Vazin *et al.*, 1994) and in the very elderly are as short as in cultured cells at senescence (Mantell and Greider, 1994). The hematopoietic lineage appears to be both telomerase competent and mortal. This may represent a mechanism for supplying the extraordinarily high proliferative needs of regenerative tissues such as blood, the intestinal tract and the epidermis of the skin in which the normal rates of telomere shortening would prevent adequate replenishment. This may be particularly true for lymphocytes for which repeated clonal expansions might be required in response to repeated antigenic exposure. A regulated level of telomerase activity may be established to reduce the rate of telomerase shortening, but not prevent it. The organism may, thus, achieve the anticancer benefits of a limited cellular lifespan in these tissues without sacrificing the need for large number of cell divisions.

Evidence has shown that telomerase activity is repressed in quiescent immortal cells (Avilion *et al.*, 1996). This leads to the working hypothesis that there are at least two pathways for the repression of telomerase activity. In one, telomerase is repressed in somatic tissue late in development (Wright *et al.*, 1996). These telomerase-silent cells fail to express telomerase activity regardless of their proliferative state. In the second pathway, telomerase activity is reversibly regulated in telomerase-competent cells so that it is present during cell proliferation. Since equal telomerase activity is found in all stages of the cell cycle (Vazin *et al.*, 1994), it should be proportional to the fraction of telomerase competent cells in the proliferative pool rather than their rate of cell division. A recent study done by Morales *et al.* (1999) have shown the absence of cancer-associated changes in human fibroblasts immortalized with telomerase. They said that although ectopic expression of telomerase in human fibroblasts is sufficient for immortalization, it does not result in changes typically associated with malignant transformation. Another study by Jiang *et al.* (1999) has shown that telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype.

Telomerase activity is not found in sperms and oocytes, but it is present at very low levels in the most primitive stem cells, all of which might be expected to be telomerase positive. However, these cells are in a quiescent state and their proliferating progeny express higher levels of telomerase activity (Counter *et al.*, 1995; Hiyama *et al.*, 1995; Wright *et al.*, 1996). Wu *et al.* (1999) have correlated telomerase activity with p53 protein expression and genetic instability in lung cancer. Their study suggested telomerase to be a good tumor marker. Excess p53 protein expression was associated with telomerase activation. Thus, telomerase could be related to p53 status. The absence of telomerase activity, thus, can indicate either the presence of telomerase present/absent in cells or the quiescent state of telomerase competent cells. Variations in the levels of telomerase activity in tumor biopsies could reflect-

- heterogeneity in the number of telomerase—competent tumor cells versus telomerase negative normal cells,
- differences in the percentage of telomerase—competent tumor cells that are actually telomerase positive because they are dividing.

The prognostic value of the levels of telomerase activity may thus relate to-

- whether or not the barrier to unlimited cell division has been overcome and
- to the fraction of tumor cells that are in the proliferative compartment.

This may explain why many types of cancers display increased levels of telomerase activity as the cancer progresses. It also explains why the survival rate of cancer patients with high levels of

telomerase activity is significantly lower than that of cancer patients who have low or undetectable levels of telomerase activity (Hiyama *et al.*, 1995; Shay *et al.*, 1995; Zhang *et al.*, 1996).

6. Role of Telomerase in Diagnosis

Early detection of cancer has two components: early diagnosis and screening. Most work of telomerase at this time deals basically with how well the presence of telomerase in tissues or samples correlate with histopathology. Further studies are needed to determine the sensitivity and specificity of telomerase.

Little is known regarding the factors that regulate the expression and activity of the enzyme telomerase. (Feng *et al.*, 1995). The levels of the telomerase RNA component in telomerase-negative and telomerase-positive cells vary only slightly in many instances, which indicates that the detection of the level of the RNA component may not be an accurate predictor of the level of telomerase activity (Blasco *et al.*, 1996). A human gene *hTERT* that shares significant similarity with the telomerase catalytic sub-unit gene of yeast and ciliates has been identified. (Kilian *et al.*, 1997; Meyerson *et al.*, 1997; Nakamura *et al.*, 1997) Upregulation of *hTERT*-gene expression might be a rate-limiting mechanism regulating telomerase activation (Meyerson *et al.*, 1997). Therefore, telomerase protein and the activity of the telomerase holoenzyme may be important elements for detection and diagnosis of human cancers with the advent of the highly sensitive telomeric repeat amplification protocol (TRAP) (Kim *et al.*, 1994). Discovery of early stages of cancer through the detection of telomerase activity may increase the chance for patient survival. Telomerase activity is detected in almost 70% of stage 1 breast tumors and in > 95% of patients having stages II-IV tumors (Hiyama *et al.*, 1995). Park *et al.* (1999) have detected telomerase activity in ovarian tumors and differentiated between carcinomas, borderline tumors and fibromas. Their study concluded that telomerase activation can be used as a valuable diagnostic parameter that could help identify potentially progressive lesions.

While telomerase activity has been detected mostly in surgically removed tissues and tumors, detection of telomerase activity in fine-needle aspirated breast samples correlates well with detection of cancer by conventional cytology, indicating that telomerase activity may be detected in samples taken prior to surgery also. Furthermore, using a recent modification of the TRAP assay (Avilion *et al.*, 1996), cells obtained from bronchial brushings, urine or colonic effluent samples can be used to screen for telomerase activity and in some instances, telomerase activity can be detected where other cytological and molecular assays have failed to identify cancer cells. Therefore, it can be said that incorporation of a clinical assay to detect telomerase activity in tumor biopsies may aid in the management of cancer patients. Agents able to hobble telomerase might kill tumor cells without disrupting the functioning of many normal cells. In contrast, most existing anticancer therapies disturb normal cells as well as malignant ones and thus are often quite toxic. Further, because telomerase occurs in numerous cancers, such agents might work against a broad array of tumors.

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16. Chromosomal Radiosensitivity as an Indicator of Genetic Predisposition to Breast Cancer

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Abstract: An abnormally high frequency of chromatid breaks, representing unrepaired DNA double-strand breaks, in cells entering metaphase 30 to 90 min after irradiation, is a good indicator of the donor's predisposition to breast cancer. The unrepaired DNA double-strand breaks resulting from deficient repair of the radiation-induced DNA damage are evident in non cancerous cells like skin fibroblasts or blood lymphocytes from breast cancer patients carrying predisposing genes, with familial and some sporadic cancer partients and individuals at a risk to develop cancer. Such deficiency to repair DNA double-strand breaks resulting in persistent chromatid breaks, at least twofold higher than normal, after G₂ phase X-irradiation may therefore be used as a marker of cancer susceptibility in cancer families.

Key words: Chromatid breaks, DNA double-strand breaks, X-irradiation, DNA repair, Breast cancer predisposition.

1. Introduction

Breast cancer is the third most frequent cancer in the world and by far the most common malignancy of women (Parkin *et al.*, 1999). It is estimated that about 1.5 million women will be diagnosed with breast cancer in the 1990s (Cancer Facts and Figures. 1998). Furthermore one in eight women will develop breast cancer in her lifetime. As with many cancers, the etiology of breast cancer is multifactorial but a family history of breast cancer is one predisposing factor. Thus, a woman with a first-degree relative with breast cancer in premenopausal age is three times more at risk while in a woman with a first-degree relative with bilateral breast cancer in premenopausal age, the risk increases 10-fold (Bilimoria and Morrow, 1995). The risk factor of second and third degree relatives with breast cancer, although not fully understood, is apparent in family studies.

Although a number of genes are associated with genetic predisposition to breast cancer, only 5 to 10 percent of breast cancers are thought to be due to specific mutations that confer an extremely high risk of breast cancer development (Bilimoria and Morrow, 1995). While a number of breast cancers are familial, still the majority is sporadic. It has been proposed that high proportions of individuals with sporadic cases carry predisposing genes of low penetrance and that these predisposing genes may be involved in the processing of DNA damage (Scott *et al.*, 1994). In the present article we present evidence that G₂ phase of the cell cycle, is an indicator of predisposing genes in hereditary and familial as well as in a large number of sporadic breast cancers.

2. G₂ Chromosomal Radiosensitivity, DNA Double Strand-Breaks and DNA Repair

X-irradiation of cells in culture during G₂ phase of the cell cycle produces several DNA lesions including strand breaks and damage to the bases. DNA breaks can arise directly from irradiation or indirectly during repair processes (Hanawalt *et al.*, 1979). During this nucleotide excision repair process (NER) DNA is enzymatically incised to remove damaged sites. This incision step in NER introduces a DNA single-strand break which in the absence of a later step of repair synthesis or ligation would be converted into a double-strand break by a single-strand nuclease. Because each chromatid contains a single continuous molecule of DNA, unrepaired DNA double-strand breaks at the subsequent metaphase can be quantified as chromatid breaks. Chromatid breaks show either non-alignment and displacement of the broken segment (displaced breaks) or no displacement, but a discontinuity longer than the chromatid width (non-displaced breaks). The latter have been sometimes referred as chromatid gaps. The extent of chromatid damage in metaphase cells arrested by colcemid directly after irradiation (0 to 30 min) provide a measure of the sensitivity of cells to irradiation. Differences in the rate of decline of chromatid break frequencies during the subsequent hour (30 to 90 min after irradiation) reflect the capacity of cells to repair the radiation-induced DNA damage (Sanford *et al.*, 1989a; Sanford and Parshad 1989a). The persistence, increase or decrease in frequencies of chromatid breaks in cells arrested 30–90 min after irradiation, thus, provides a measure of the cell's capacity to repair radiation-induced DNA damage (Sanford and Parshad 1999; Sanford *et al.*, 1989a). This concept is supported by the fact that addition of a DNA repair inhibitor, 1-β-D arabinofuranosylcytosine (ara C), which inhibits the polymerase step in excision repair, can prevent the decline and result in increased chromatid breaks at intervals after X-irradiation (Sanford *et al.*, 1999). Cells from clinically normal donors typically showed a level of < 60 chromatid breaks per 100 cells entering metaphase 30 to 90 min after irradiation (58–60 R) at a standardized inoculum size and under the culture conditions used (Sanford *et al.*, 1989a). In the cancer prone individuals, cells under similar experimental conditions showed, an abnormally high, at least twofold higher number of breaks. We, thus, propose that in cultures of peripheral blood lymphocytes, an abnormally high frequency of chromatid breaks, representing unrepaired DNA double-strand breaks in cells, entering metaphase 30 to 90 min after irradiation, is a good indicator of the donor's predisposition to cancer. An abnormal frequency of persistent chromatid breaks, at least twofold higher than normal, after G₂ phase irradiation may, therefore, be used as a marker of breast cancer susceptibility in breast cancer families.

3. Breast Cancer Genes

3.1 AT

The first gene associated with genetic predisposition to breast cancer was related to ataxia-telangiectasia (AT) which is a rare, autosomal recessive, multisystem disorder manifesting neurologic, cutaneous and immunological abnormalities and an exceptionally high susceptibility to cancer particularly, but not exclusively, of the reticuloendothelial tissues. The AT gene was localized to chromosomal region 11q22–23 (Gatti *et al.*, 1988) and has been cloned (Savitsky *et al.*, 1995). Heterozygous carriers of mutated AT gene (ATM), which are estimated at 2.8% of the US population (Swift *et al.*, 1986) and are clinically indistinguishable, may have a twofold or threefold overall excess risk of cancer (Swift *et al.*, 1987). Breast cancer in women is the cancer associated with AT heterozygosity. It is estimated that approximately 8.8% of patients with breast cancer are carriers of the ATM gene (Swift *et al.*,

1987). At the cellular level AT is characterized by radioresistant DNA synthesis (de Wit *et al.*, 1981), hypersensitivity to ionizing radiation as determined by cell killing (Chen *et al.*, 1978) and defective cell cycle checkpoints (Painter and Young, 1980). It has been suggested that AT cells are deficient in the ability to recognize, repair or process such damage (Lehmann *et al.*, 1989) and that mutations in AT gene result in disequilibrium in rejoicing and endonuclease digestion of DNA termini are basis for misrepair (Cox *et al.*, 1986).

Skin fibroblasts or peripheral blood lymphocytes from AT heterozygotes differ from those of normal individuals in their cytogenetic responses to irradiation during G₂ (Parshad *et al.*, 1985a; Parshad *et al.*, 1985b; Shiloh *et al.*, 1986 and 1989; Sanford *et al.*, 1991; Mitchell and Scott, 1997; Tachirkov *et al.*, 1997). When collected at metaphase by colcemid from 30 to 90 min after irradiation, cells from AT heterozygous individuals show twofold to threefold higher frequency of chromatid breaks than that in cells from normal controls. In cells collected during the first 30 min, however, the frequencies of chromatid breaks were similar in cells from AT heterozygous and control individuals (Sanford *et al.*, 1991). In metaphase cells collected from 30 to 90 min postirradiation frequencies of chromatid breaks decreased rapidly in cells from normal individuals, but persisted at significantly higher levels in both AT patients and heterozygotes, presumably due to deficient or unbalanced DNA repair during G₂ (Sanford *et al.*, 1991).

3.2 p53

The association of p53 with breast cancer is apparent in Li-Fraumeni syndrome (LFS), which is a rare autosomal dominant syndrome, characterized by the occurrence of diverse mesenchymal and epithelial neoplasms at multiple sites (Li and Fraumini, 1969). In some instances, the neoplasms seem to be related to exposure to carcinogenic agents including ionizing radiation (Chang *et al.*, 1987). Breast cancer at a very young age is a very common neoplasm in women with this syndrome.

Unlike AT, skin fibroblasts from patients with LFS show an unusual radiation-resistant phenotype as evident in cell survival curves (Bech-Hansen *et al.*, 1981; Sproston *et al.*, 1996). DNA derived from skin fibroblasts of patients with this syndrome has mutations in p53 gene. In certain families a single mutation in p53 was present even in the germline (Srivastava *et al.*, 1990; Binch *et al.*, 1994; Sedlacek *et al.*, 1998; Davison *et al.*, 1998) while in one family the same mutation was found spanning two generations (Srivastava *et al.*, 1990). Mutations in the p53 gene seem to be the most common somatic changes found in breast tumors (Hollstein *et al.*, 1994). The p53 gene has been implicated in cell cycle control (Levine, 1997). The p53 tumor suppressor protein appears to be a central coordinator of cellular response to DNA damage and associated with genomic instability in tumors (Eyfjord *et al.*, 1995). Expression of wild-type p53 seems to be required for efficient global genomic nucleotide excision repair of UV-induced cyclobutane pyrimidine dimers and 6–4 photoproducts in LFS fibroblasts (Ford and Hanawalt, 1997). The p53 also modulates DNA repair via DNA binding of hMSH2, a mismatch repair gene (Scherer *et al.*, 1996) or interacting with human homologue of yeast Rad 51 (hRad 51) protein that is involved in recombination and DNA double-strand break repair (Buchhop *et al.*, 1997). It, therefore, seems that mutations in p53 gene modulate DNA repair by introducing lack of cell cycle checkpoints to monitor DNA damage or interact with hRad 51 protein involved in DNA double-strand break repair. Noncancerous skin fibroblasts from members of the families with LFS show significantly higher than normal frequencies of chromatid breaks after G₂ phase X-irradiation (Mitchell and Scott, 1997; Parshad *et al.*, 1993). In cells from six members of a partial LFS family all showed a high frequency of chromatid breaks compared to cells from an unrelated spouse and two control individuals. Five of these six members of this family had diverse cancers including five with breast cancer while the sixth member had polycythemia vera, a premalignant

neoplasm (Parshad *et al.*, 1993). The abnormally high frequency of chromatid breaks seen at 30–90 min after G₂ phase irradiation of cells from LFS patients, thus, represents unrepaired DNA double-strand breaks possibly arising from an interaction of p53 protein with Rad 51 protein.

3.3 BRCA1 and BRCA2

Studies of large families having many members with breast cancer led to the identification of two genes BRCA1 and BRCA2, on chromosome 17q21 and 13q12–13 respectively, that when mutated predispose the individual to breast cancer (Miki *et al.*, 1994; Wooster *et al.*, 1995). Heterozygous carriers of a germ-line mutation, in these families, have a high probability of developing breast cancer at a younger age than in the general population (Rowell *et al.*, 1994). Mutations in the BRCA1 gene are linked to half of all cases of familial breast cancers (Miki *et al.*, 1994; Futureal *et al.*, 1994) which constitute 2% of all breast cancers (Easton *et al.*, 1995). Carriers of BRCA2 mutations may have a smaller increase in the risk of early-onset of breast cancer (Krainer *et al.*, 1997).

There are more than 200 different mutations in the two BRCA genes, which hampers the ability of the putative tumor-suppressor gene to block the development of cancer (Healy, 1997). BRCA2 gene encodes a large protein found mutated in 45% of familial breast cancer (Wooster *et al.*, 1994). It has been shown that the carboxy-terminus of the BRCA2 protein interacts with the protein product of Rad 51, a DNA double-strand break repair gene (Mizuta *et al.*, 1997). Furthermore disruption of this interaction leads to radiation sensitivity in mouse blastocytes (Connor *et al.*, 1997) which suggest that BRCA2 protein helps to maintain genomic stability protecting cells against the disastrous effect of DNA double-strand breaks (Kinzler and Vogelstein 1997). Thus cells deleted for BRCA2 COOH terminus exhibit hypersensitivity to gamma radiation and premature senescence (Morimatzu *et al.*, 1998) and BRCA2 defective cancer cells are highly sensitive to agents that cause double-strand breaks in DNA (Abott *et al.*, 1998). Although the protein products of BRCA1 and BRCA2 genes have no apparent sequence similarity, circumstantial evidence relating mainly to their size and overlapping expression pattern, suggest their similar function. An insight into such similarity is provided in that like BRCA2, BRCA1 is associated with hRad 51 (Scully *et al.*, 1997), the human homologue of *E. coli* protein Rec A and of yeast ScRad 51 (Shinohara *et al.*, 1993). Rad 51 protein is involved in the repair of DNA double-strand breaks. It, therefore, seems that like p53 gene BRCA1 and BRCA2 proteins interact with Rad 51 protein, which is required in the repair of DNA double-strand breaks.

Although cells with mutated BRCA genes are hypersensitive to ionizing radiation, precise information on cytogenetic responses of such cells to G₂ irradiation is unavailable. Breast cancer patients carrying mutated BRCA1 or BRCA2 gene belong to high-risk families with multiple breast and or ovarian cancer in their relatives. In one study chromosomal response to irradiation was examined in cells of patients from such families with multiple breast and or ovarian cancer and, thus, presumably with mutated BRCA gene (Parshad *et al.*, 1996b). Exposure of PHA stimulated peripheral blood lymphocytes, to X-irradiation during G₂ from cancer patients of these families showed two to three fold higher than normal frequency of chromatid breaks. This abnormally high frequency of chromatid breaks was again seen in cells entering metaphase 30–90 min after irradiation.

4. Breast Cancer Families

Almost 20% of breast cancer patients have a family history of disease; only one-fourth of these appears to be inherited with an autosomal dominant mode of inheritance (Lynch *et al.*, 1988). Most of these cancers are without any inherited phenotype. Cells from familial breast cancer patients and proportionally high frequency of their first-degree relatives exhibit deficiencies in the repair of

damaged DNA Kovac and Almendral, 1987; Pero *et al.*, 1989; Parshad *et al.*, 1996b; Patel *et al.*, 1997; Rao *et al.*, 1998).

Exposure to ionizing radiation is associated with an increased risk of breast cancer (Miller *et al.*, 1989; Mattson *et al.*, 1993; Elkind, 1996). A statistically significant increase in the incidence of breast cancer following radiation treatment of various benign breast diseases was observed even among women older than 40 years at the time of first treatment (Miller *et al.*, 1989). It has been suggested that the target cells in a sizeable fraction of women who are susceptible to breast cancer harbor a radiation repair deficiency (Elkind, 1996). In four studies (Knight *et al.*, 1993; Helzlsouer *et al.*, 1996; Parshad *et al.*, 1996b; Patel *et al.*, 1997), peripheral blood lymphocytes from patients with a family history of breast cancer when X-irradiated during G₂ phase showed a significantly higher frequency of chromatid breaks than unrelated control cells. A family history of breast cancer has been defined as having one first degree relative with breast cancer or two or more second-degree relatives with breast or ovarian cancer and at least one with breast cancer (Parshad *et al.*, 1996b). A similar high frequency of X-ray-induced chromatid breaks was also observed in women with preinvasive lesions, including lobular or ductal cell carcinoma in situ, with or without a family history of cancer (Parshad *et al.*, 1996b).

71–79% of the healthy first-degree relatives of familial breast cancer patients showed higher than normal frequency of chromatid breaks comparable to that in the patients (Patel *et al.*, 1997; Helzlsouer *et al.*, 1996). Frequencies of chromatid breaks in cells from breast cancer patients, their first-degree relative and normal controls were comparable when harvested 30 min after X-irradiation (Patel *et al.*, 1997). Unlike cells from control individuals, where there was a precipitous decline in the frequency of chromatid breaks during 30–90 min after irradiation, cells from breast cancer patients or some of their first degree relatives maintained a much higher frequency of breaks indicative of suboptimal repair of the radiation-induced DNA damage (Patel *et al.*, 1997). In two studies (Patel *et al.*, 1997; Helzlsouer *et al.*, 1996) first-degree relatives of familial breast cancer patients were 5.2 or 7 times more likely to have this suboptimal DNA repair. On an average, first-degree relatives of these cancer patients were 2.3 to 2.7 times more risk of developing breast cancer compared to the control women (Patel *et al.*, 1997; Helzlsouer *et al.*, 1996). It, therefore, seems that abnormally high frequency of chromatid breaks after G₂ irradiation manifesting suboptimal DNA repair is a predisposing factor in familial breast cancer.

5. Sporadic Breast Cancer

The majority of breast cancers occur in the absence of a family history and only 8.8% of this show heterozygosity in mutations at the AT locus (Swift *et al.*, 1987). Of the 16 breast cancer patients with severe response to radiotherapy, none showed mutation typically found in obligate carriers of AT (Appleby *et al.*, 1997). Furthermore the epidemiological data supports that a substantial proportion of breast cancer patients may be predisposed via low penetrance genes that do not confer a strong family history (Teare *et al.*, 1994).

Lymphocytes from sporadic breast cancer patients when compared to those from normal controls show, on an average, higher sensitivity to chromosome damage by ionizing radiation. This high sensitivity was seen after exposure of cells to irradiation during G₀ (Scott *et al.*, 1998) or G₂ phases of the cell cycle (Scott *et al.*, 1994; Parshad *et al.*, 1996a). Whereas G₂ chromosomal radiosensitivity was evident as higher than normal frequency of chromatid breaks in metaphase cells arrested at metaphase 30 to 90 min after X-irradiation, G₀ radiosensitivity was manifest as micronuclei in cells irradiated by Cs gamma rays during G₀ period of the cell cycle *i.e.*, 6h before stimulation of lymphocytes

with PHA (Scott *et al.*, 1998). Twenty-four hours after stimulation, the cytosine kinase-blocking agent cytochalasin-B was added to identify first generation post mitotic cells as binucleated (Fabry and Coton, 1985). The frequency of micronuclei presumably representing acentric fragments is enumerated as an indicator of chromosomal radiosensitivity in binucleated cells harvested at 90h. A comparison of G₀ and G₂ assays show that while G₂ assay identified 45% (Scott *et al.*, 1994) and 54% (Parshad *et al.*, 1996) sporadic breast cancer with higher than normal level of chromosomal radiosensitivity, only 31% showed elevated radiosensitivity in G₀ assay (Scott *et al.*, 1998). There was no correlation between G₂ and G₀ sensitivity; most of the sensitive patients were either G₂ or G₀ sensitive and only 4% sensitive in both assays (Scott *et al.*, 1999). This lack of correlation between the G₂ and G₀ sensitivity is possibly due to differences in the type of DNA lesions introduced by these two sources of ionizing radiation, X-irradiation and Cs gamma radiation, or different mechanisms in DNA repair the deficiency of which could result in chromosomal radiosensitivity. Chromatid damage, seen at the first post treatment metaphase, after DNA damage in the pre S (G₀ or G₁) or post S (G₂) results from different DNA repair mechanisms as shown by exposure of cells to X-rays or fluorescent light. Fluorescent light like X-rays damages DNA through the generation of free hydroxyl radicals (Sanford *et al.*, 1986), but unlike X-rays fluorescent light does not induce a mitotic block or cell cycle delay (Parshad *et al.*, 1985a). Majority of the DNA damage, induced by X-rays or fluorescent light during G₂, leading to chromatid breaks at the subsequent metaphase is repaired by a sensitive nucleotide excision repair (Sanford and Parshad, 1999; Parshad *et al.*, 1996b, 1998). In the absence of nucleotide excision repair, DNA damage during G₁ can bypass by caffeine sensitive daughter strand repair mechanism during the subsequent S phase (Parshad *et al.*, 1996b, 1998).

6. Breast Cancer Development: An Interaction Between Chromosomal Radiosensitivity and Radiation Exposure

The interaction between increased chromosomal radiosensitivity resulting from deficient DNA repair and radiation exposure in the development of breast cancer becomes apparent from a cluster of breast cancer cases (four of the six sibs) occurring in one generation of a family. The characteristics of this family differed from those of families with breast cancer linked to a major gene locus, but certain members had a history of exposure to repeated chest fluoroscopic examination during adolescence and early adulthood (Helzlsouer *et al.*, 1995). DNA repair proficiency manifest as frequency of chromatid breaks after G₂ X-irradiation was measured within the sibship and among close relatives and examined in conjunction with the history of radiation exposure and breast cancer occurrence. The cancer patients had been exposed to repeated chest fluoroscopic examinations during early childhood and adolescence. The results strongly suggested that a deficiency manifest as abnormally high frequency of chromatid break after G₂ radiation may be a susceptibility factor in the pathogenesis of breast cancer. The pattern of breast-cancer occurrence and DNA repair proficiency in the family suggest that this susceptibility factor may interact with exposure to low-level radiation to increase the risk of developing breast cancer.

A similar role of deficient DNA repair manifest as chromosomal hypersensitivity to radiation was evident during malignant transformation of normal human mammary epithelial cells in culture (Sanford *et al.*, 1992). Normal epithelial cells established in culture from reduction mammoplasty tissue were proficient in the repair of DNA damage with chromatid break frequency equivalent to that in normal fibroblast cells after G₂ irradiation. Of two continuous cell lines, derived after benzo [a] pyrene treatment of these normal mammary cells, one (A1) maintained the efficient repair phenotype of the parent line through passage 50. A subline from A1 developed the repair-deficient phenotype characterized

Table 1 Responses of blood lymphocytes from breast Cancer patients with or without predisposing genes and from individuals at risk of breast cancer to ionizing radiation

Category of Individuals	Cellular Responses to Radiation	References
I. Individuals with predisposing genes AT gene, No Cancer	<ol style="list-style-type: none"> 1. Radioresistant DNA synthesis 2. Hypersensitivity to cell killing 3. Defective cell cycle checkpoints 4. Deficient repair of DNA damage 5. Disequilibrium in rejoining and endonuclease digestion of DNA repair 6. G₂ Chromosomal Radiosensitivity 	de Wit <i>et al.</i> (1981) Chen <i>et al.</i> (1978) Painter and Young (1980) Lehmann <i>et al.</i> (1989) Cox <i>et al.</i> (1986)
<i>p53</i> gene		Parshad <i>et al.</i> (1985b); Shiloh <i>et al.</i> (1986, 1989); Sanford <i>et al.</i> (1991); Mitchell and Scott (1997); Tachorkov <i>et al.</i> (1997)
Cancer Patients with Li-Fraumeni Syndrome	<ol style="list-style-type: none"> 1. Radioresistance to cell killing 2. Cell cycle checkpoints control 3. Genomic instability 4. Modulation of DNA repair via binding to hMSH2 5. Interact with hRad 51 protein involved in DNA double strand break repair 6. G₂ Chromosomal Radiosensitivity 	Ben-Henson <i>et al.</i> (1981); Sprotton <i>et al.</i> (1996) Levine, (1997) Erfjord <i>et al.</i> (1995) Scherer <i>et al.</i> (1996) Buchhop <i>et al.</i> (1997)
BRCA1 and BRCA2 genes	<ol style="list-style-type: none"> 1. BRCA1 and BRCA2 proteins interact with the protein product of Rad51, a DNA double strand break repair gene 2. Mutation in BRCA2 results radiation hypersensitivity in mouse blastocysts 3. Mutations lead to genomic instability 4. G₂ Chromosomal Radiosensitivity in individuals fitting into the Category with BRCA1 or BRCA2 	Parshad <i>et al.</i> (1993); Mitchell and Scott (1997) Mizutta <i>et al.</i> (1997) Connor <i>et al.</i> (1997) Kinzler and Vogelstein (1997) Parshad <i>et al.</i> (1996b)
II. Familial Breast Cancer Patients	1. G ₂ Chromosomal Radiosensitivity	Knight <i>et al.</i> (1993); Helzlsouer <i>et al.</i> (1996); Parshad <i>et al.</i> (1996b); Patel <i>et al.</i> (1997)
Familial Breast Cancer Patient's First degree Relatives without Cancer	1. 71–79% with G ₂ Chromosomal Radiosensitivity	Hozlesouer <i>et al.</i> (1996); Patel <i>et al.</i> (1997)
III. Sporadic Breast Cancer	<ol style="list-style-type: none"> 1. 45–54% with G₂ Chromosomal Radiosensitivity 2. 31% with G₀ Radiosensitivity 	Scott <i>et al.</i> (1994); Parshad <i>et al.</i> (1996b) Scott <i>et al.</i> (1998)

by a 3-to 5-fold higher frequency of chromatid breaks. This line was transformed to tumorigenic cells by HaMSV and SV₄₀ T antigen. The second continuous line (B5) and the derivative sublines became repair deficient early in culture and were transformed to tumorigenic cells by KiMSV. The following sequence of events appears to characterize the malignant neoplastic transformation of human mammary epithelial cells in culture: 1) 'immortalization' resulting from recessive gene mutation (Perira-Smith and Smith, 1983) and clonal selection from carcinogen treatment and/or prolonged culture; 2). acquisition of deficient DNA repair during the G₂ phase arising spontaneously during prolonged culture or induced by KiMSV containing the *ras* oncogene; (3) DNA damage leading to further chromosomal aberrations which can facilitate the accumulation of genetic changes required for malignant transformation. Acquisition of DNA repair deficiency manifest as chromosomal radiosensitivity phenotype appears to be an early step in the malignant transformation of human cells in culture.

Conclusions

Table 1 lists the responses of blood lymphocytes from different categories of breast cancer patients and individuals at risk of breast cancer to ionizing radiation. Cells from individuals with predisposing genes, which have been studied most extensively, show diverse response with only G₂ chromosomal hypersensitivity resulting from unrepaired DNA double-strand breaks as a common feature. Cancer patients from breast cancer families, 71–79% of their healthy first-degree relatives and 45–54% of sporadic breast cancer patients also share this feature of DNA repair deficiency. It, therefore, seems that deficient DNA repair manifest as an abnormally high frequency of chromatid breaks is a valuable indicator of genetic predisposition to breast cancer and to some extent predisposition to sporadic breast cancers.

The breakdown of a precise system for monitoring and repairing DNA damage sustained during G₂ phase, just prior to distribution of chromosomes to progeny cells in mitosis, would lead to genetic instability, thereby increasing the probability of nonlethal mutations (Sanford *et al.*, 1989b). Unrepaired DNA strand breaks lead to chromatid breaks with potential loss of acentric fragments during the subsequent mitosis. Strand breaks persisting from G₂ through mitosis into G₁, if not sealed, may rejoin at random to form interchanges, inversions, duplications, deletions and translocations with consequence gene arrangements associated with the genesis of cancer. Genetic instability, in turn, depends on cell cycling, because the repair deficiency is manifested only when DNA damage is sustained. Thus, the repair deficiency and rapid cell cycling resulting from activation of genes controlling cell proliferation such as oncogenes in the presence of DNA damage would produce new variants. Natural selection of these variants *in vivo* would favor those with increasing independence of growth controls. This sequence of events may constitute a stepwise mechanism for development of cancer in general. Deficient DNA repair during G₂ might, therefore, be the defect at the molecular level that results in the enhanced radiosensitivity and breast cancer proneness.

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17. Molecular Genetics of Gastrointestinal Tract Cancers

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Abstract: Cancer has been intimately related to a large number of environmental and genetic factors. All these ultimately result in change of cellular behaviour which proliferate uncontrollably thus, leading to malignancy. Age, sex, geographical variation patterns, social differences, varied dietary habits are various environmental factors while oncogenes/ antioncogenes, chromosomal aberrations are various genetic factors that play an important role. Human papillomavirus has been associated with benign squamous papillomas which may progress to invasive lesions in all types of malignancies of the gastrointestinal tract. Various tumor suppressor genes have been linked with malignancy leading to metastasis. Instability due to loss of chromosomal fragments and microsatellites allows sufficient amount of genetic damage that leads to imbalanced cellular proliferation. Thus, an insight of such factors would help in better understanding of the early activation events leading to malignancy and may be helpful in early identification of disease in high risk patients.

Key words: Gastrointestinal cancers, esophageal cancer, rectal cancer, oncogenes, antioncogenes.

The malignancies of the gastrointestinal tract include carcinoma of esophagus, stomach, intestine and colon. Their incidence varies throughout the world. A high incidence of esophageal cancer is seen within the Asian esophageal cancer belt, extending from the Southern shore of Caspian sea on the west to Northern China on the east, encompassing parts of Iran, Soviet Union (Earstwhile) Central Asia, Afghanistan, Siberia and Mongolia. Additionally high incidence pockets of the disease are present in Finland, Ireland, South-Eastern Africa and North-Western France (Crespi *et al.*, 1979; Yang, 1980). Colorectal carcinoma is the fourth most prevalent and second most frequent cause of death from cancer in the USA (Jessup *et al.*, 1997; Potter, 1997). In northern societies, colon cancer is one of the major causes of death. Every year in USA 110,000 new cases are diagnosed and 55,000 people die annually because of this disease. Mohandas and Jagannath (2000) estimated that in the year 2001, there will be approximately 1,45000 new cases of digestive tract cancers in India.

A variety of benign tumors occur in the esophagus - rarely exceeding 3 cm in diameter and appear mostly as solid, grey masses, but fibromas, lipomas, hemangiomas, neurofibromas, lymphangiomas and squamous papillomas may also arise. A vast majority is represented by malignant tumors. These are classified according to their cell types. Squamous cell carcinomas constitute between 80–85% of esophageal cancers, adenocarcinomas make up about 5–10% and the remainder comprise undifferentiated or rarer cancers.

Esophageal carcinoma has a poor prognosis with a 5 year survival rate in less than 20% of the patients. Many of the conventional prognostic indicators such as lymph node metastasis, depth of invasion and histological type have been inconsistent predictors of survival which may reflect the

relatively small number of patients included in most studies. There have been a number of premalignant lesions, the most important is the columnar epithelium -lined esophagus or Barrett's esophagus.

Esophageal cancer is believed to have a multifactorial origin, with some factors being highly significant. It has been categorized as a disease of affluence, as it occurs more often in upper socio-economic populations, living in urban areas (Gammon *et al.*, 1997). Consumption of food contaminated by certain fungi, chronic nutritional deficiencies of vitamins A, C and B complex group; trace metals like zinc and molybdenum, presence of esophagitis and esophageal stasis (Oelette *et al.*, 1986), of chronic alcohol and tobacco abuse and a racial or genetic predisposition (Munoz and Crespi, 1983; Enterline and Thompson, 1985, Mufti, 1998) are possible etiological factors in such cancers (Lagergren *et al.*, 2000). It has been shown that there is a 40-fold increase in the incidence of esophageal cancer in individuals with a history of alcohol intake of more than 80gm/day and cigarette smoking of more than 40 packs/year. Terry *et al.*, (2001) observed that heavy smoking over a long period was associated with a statistically significant three fold increased risk over those who had never taken to smoking. In areas where alcohol and tobacco are not excessively consumed, dietary and environmental factors play a significant role. Zhang *et al.* (1997) and Brown *et al.* (1998) have shown that high dietary vitamin C intake appears to protect against gastric carcinoma by scavenging free radicals that may be enhanced by *Helicobacter pylori*. According to Vecchia *et al.* (1997), a varied diet may protect against gastric cancer and that a more diversified and richer diet is a relevant underlying correlate of the decline in gastric cancer rates. The intake of raw fruits and vegetables provide protection against squamous cell esophageal carcinoma (Brown *et al.*, 1998).

A geographic variation pattern has also been observed. It is felt that migrant groups tend to assume the large-bowel cancer incidence rates of their adopted countries, thus, negating the role of genetic variations (Mayer, 1989). Hereditary predisposition, having a family history of the disease is another factor linked with this cancer. Dhillon *et al.*, (2001) have found no increased risk of esophageal cancer in individuals having a family history but they were prone to gastric adenocarcinoma. Case control studies have shown a positive association of gastric and colon cancer with fried foods, salted pickles, salted fish and negative association with fresh fruits, dairy products, lettuce and vitamin C (Cotran *et al.*, 1989).

Viruses

The role of viruses in the etiology of various cancers has been highlighted in various studies. Human Papilloma Virus has been associated with benign squamous papillomas which may progress to invasive lesions in almost all the malignancies.

Papillomaviruses are small double stranded DNA viruses that infect epithelial cells to induce a variety of lesions. These viruses display tremendous diversity as more than 70 different HPVs have been described (Durst *et al.*, 1995). HPVs 6, 11, 16, 18, 31, 33, 34, 40, 42, 72 and 73 are known to infect mucosal epithelium, whereas HPVs 1, 3, 5, 8, 36, 48 and 49 are cutaneous in nature. The mucosal epithelium associated HPVs can further be subdivided into low risk HPVs 6 and 11 and the high risk ones 16 and 18 (Zur Hausen, 1991a). Viral genome is organized into three major regions: two protein coding regions [early (E) and late (L) gene] and a non-coding upstream regulatory (URR) region (Park *et al.*, 1995). A series of E proteins are able to induce the cell to maintain an active DNA replication machinery that can be subverted for use during viral replication (Howley, 1996). Viral infection normally occurs through a disturbed epithelial barrier, as only basal replicating keratinocytes are infected by HPVs. A receptor $\alpha 6\beta 4$ integrin on keratinocytes is a strong candidate for viral attachment (Evander *et al.*, 1997). The clinical manifestations of HPV positive lesions and associated molecular changes are given in Table 1 (Barbosa *et al.*, 1991).

Table 1 Clinical manifestations of HPV positive lesions and associated molecular changes (Barbosa *et al.*, 1991)

HPV Infection/Early Viral Replication
(a) Viral entry
(b) Viral replication
(c) Viral release
Productive HPV Infection
(a) Viral replication
(b) E6 and E7 oncoproteins expressed
(c) Premalignant changes observed
(d) Abnormal p53/pRb functions.
Non Productive HPV Infection/High Risk Type
(a) Viral integration
(b) Dysregulated expression of E6 and E7
(c) Loss of viral regulatory proteins E1 and E2
(d) p53/pRb non-functional
(e) Other acquired mutations (Chromosome 3q).

The viral genome replicates as an episome in the differentiated cells and leads to malignant progression and non-permissive infection (Cullen *et al.*, 1991). This occurs more frequently in carcinomas associated with HPV 16 and 18 (72% and 100% respectively). Viral integration typically occurs within the viral E1 or E2 genomes. Disruption of E1 or E2 genes during viral integration allows for dysregulated expression of the E6/E7 viral proteins which have a direct relation in the process of cellular transformation (Zur Hausen, 1991a). Thomas and Laimins (1998) have shown that HPV oncoproteins E6 and E7, independently can abrogate the mitotic spindle check point, although the paths which they both follow are different.

The most important biologic function of high-risk E6 protein that has been identified to date involves inactivation of the p53 tumor-suppressor protein. It is known that E6 promotes cell growth by abrogating p53 related effects on cellular proliferation as well as effects related to apoptosis in cells that have undergone genomic abnormalities because of HPV infection. Thus, E6 has been implicated as the major HPV-associated viral protein responsible for the elevated mutation rate in high risk HPV infections (Zur Hausen, 1996). The high-risk E7 proteins are also able to form complexes leading to the degradation of cellular proteins like Rb (Jones and Milnger, 1997). It has been implicated that the E6-E7 gene expression is not sufficient for the development of malignant growth. Additional factors like exposure to radiations, chemicals, etc. along with HPV 16 and 18 infection are required for tumorigenesis (Kim *et al.*, 1993; Durst *et al.*, 1995). Len Ritter (1997) also reported that synthetic pesticides contribute significantly to overall cancer mortality. The greater biological activities observed in high risk HPV types may, therefore, be attributed to differences in expression of their E6 and/or E7 genes, in intrinsic potency of E6 and/or E7 protein products or a combination of these factors (Barbosa *et al.*, 1991).

The status of HPV in esophageal cancers is not well defined. There are contradictory reports varying from their complete absence (Loke *et al.*, 1990; Benamouzig *et al.*, 1995) to a relatively high occurrence (Chang *et al.*, 1990; Benamouzig *et al.*, 1992; Togawa *et al.*, 1994; Dillner *et al.*, 1995; Fidalgo *et al.*, 1995; Shindoh *et al.*, 1995). In a study conducted on French patients suffering from esophageal carcinoma, PCR was done for different HPV types 6, 11, 16, 18, 31 and 33 directed to E6 gene. Benamouzig *et al.* (1995) could not detect HPV DNA in tumors or their adjacent tissues.

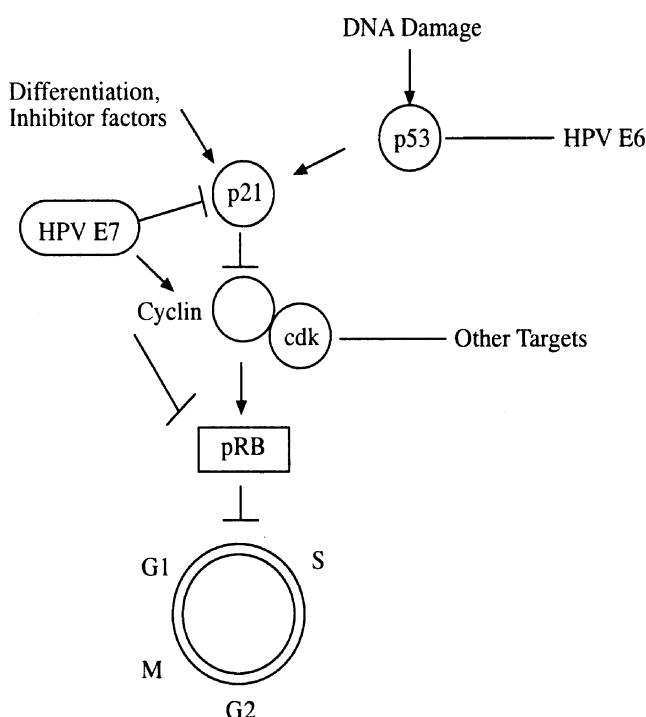


Fig. 1. HPV oncoproteins and their effect on cell-cycle machinery (Adapted from Alani and Miinger 1998, *J. Clin. Oncol.* 16: 330–337, 1998).

Similarly, Loke *et al.* (1990) had not detected HPV 6, 11, 16 and 18 in esophageal squamous cell carcinoma cases.

Different methods have been used for the detection of HPVs. These include *in situ* hybridization (Heino *et al.*, 1989; Nuovo and Riochart, 1989; Rakoczy *et al.*, 1989; Jourdan *et al.*, 1995), dot/slot blot (Leary *et al.*, 1983; Inoue *et al.*, 1989; Duggan *et al.*, 1990), Southern blotting, PCR, immunohistochemistry and electron microscopy (Choo *et al.*, 1987; Couturier *et al.*, 1991; Cullen *et al.*, 1991; Zur Hausen, 1991a; Yokoyama *et al.*, 1995). The latter two methods are of limited use as they are not very sensitive or specific. Most of the commercially available kits for *in situ* hybridization detect 20–25 copies of HPV-DNA/cell. Dot blot technique can detect 1–10 copies/cell, while Southern blotting about one copy/cell. Polymerase chain reaction can detect 1 copy/10 cells (Allan and Planner, 1995).

Using *in situ* hybridization techniques, HPV prevalence in esophageal cancerous lesions ranging from 33–43.1% has been reported (Chang *et al.*, 1990; Benamouzig *et al.*, 1992). Using dot blot, 41.6% occurrence of HPVs in esophageal squamous cell carcinoma was observed in western countries, while Chang *et al.* (1989) found their incidence to be 76.4% using Southern blotting in oral epidermoid carcinomas in Taiwan.

Using PCR for HPV amplification, primers for various regions such as L1, E6, E7, E2, LCR, URR have been used (Duggan *et al.*, 1995). Besides type specific probes, degenerate probes recognizing many HPV types simultaneously are also available. Fidalgo *et al.* (1995) have utilized type specific primers for HPVs 16 and 18 E6 regions in esophageal cancer. Shindoh *et al.* (1995) have designed a sense primer corresponding to non-coding region of HPV 16, which can amplify HPV 16, 18 and 33 (Chaves *et al.*, 1993; Togawa *et al.*, 1994; Benamouzig *et al.*, 1995; Miller *et al.*, 1997).

Oncogenes/Antioncogenes

p53 gene located on chromosome 17 serves as a cell cycle check point protein by transactivation of genes which encode proteins with growth suppressing activities. In addition to its role in controlling

the G1-S checkpoint, studies have suggested a role for *p53* in a G2 related checkpoint (Cross *et al.*, 1995; Powell *et al.*, 1995). A study by Poele and Joel (1999) has shown that cell cycle arrest was coincident with increased *p53* expression in each phase of the cell cycle and not at a particular point of cell cycle. *p53* also acts as a transcriptional factor for various genes including *waf/cip/Gadd 45* (Harris, 1993). *Mdm-2* also binds to wild type *p53* protein which results in inactivation of transactivational activity of *p53* (Momand *et al.*, 1992). Alterations in *p53* like point mutations or interaction with other factors leads to a stop on the cell cycle check, thus leading to an abnormal growth of cells. HPV oncoproteins and their effect on cell cycle machinery is shown in Fig. 1 (Alani and Miinger, 1998). Point mutations in *p53* TSG and *ras* protooncogenes are the most common specific gene changes known in human tumors (Bos, 1989). Hollstein *et al.* (1991) studied esophageal squamous cell carcinoma samples from high risk patients (alcoholic and tobacco smoking) where mutations were detected in *p53* gene, but none were detected in the *H*-, *K*-, or *N-ras* genes. Casson *et al.* (1991) have located mutations at codons 273 (CGT → CAT), 176 (TGC → TTC), 152 (CCG → CTG), 155 (ACC → GCC) and 175 (CGC → CAC) in esophageal cancer. A series of 20 point mutations in *p53* gene from various tumors including colon were seen by Nigro *et al.* (1989). Alterations in *p53* gene and/or its protein product frequently occur in preinvasive lesions of the esophagus. *p53* mutations have been found in Barrett's epithelium and oral squamous cell carcinoma (SSC) (Shindoh *et al.*, 1995). In a study on nine cell lines derived from human esophageal cancer, Barnas *et al.* (1997) reported impaired *p53* activity in all cell lines, including those containing wild-type *p53* sequences. However, Ogden *et al.* (1997) have shown that overexpression of *p53* in normal oral mucosa of oral cancer patients does not necessarily predict malignant disease in the future. Although molecular basis for *p53* overexpression has not been determined for all human malignancies, a mutational basis is true in case of colon, breast and lung carcinomas (Bartek *et al.*, 1990; Hinds *et al.*, 1990; Iggo *et al.*, 1990; Rodrigues *et al.*, 1990; Davidoff *et al.*, 1992). Evaluation of *p53* overexpression by multiparametric flow cytometry has indicated that it is frequently involved in carcinogenesis in Barrett's esophagus (Sasano *et al.*, 1992). Studies implicating *p53* in pathogenesis of esophageal cancer have also been carried out by Poljak *et al.* (1996). According to these authors, prognostic factors, such as *p53*, S phase and DNA index may be useful in stratifying patients for adjuvant therapies in future clinical trials of esophageal cancer.

Ribeiro *et al.* (1998) have performed *p53* analysis on 42 patients with esophageal carcinoma and 25/42 were found to be immunopositive. It appears that histopathologic and genetic analysis is a better determinant of *p53* mutational damage and can be used as a prognostic marker for esophageal carcinoma. Serum assay of anti-*p53* antibodies is a rapid and readily facilitated test for predicting tumor advancement, depth of invasion and liver metastasis. (Machara *et al.*, 1999). Igarashi *et al.*, (1999) have shown the predictive value of *p53* protein analysis as useful tool for the accurate diagnosis of early gastric carcinoma. *p53* mutations have been found to be events linked to processes of metastasis and local invasion in patients with colorectal carcinoma. (Giannatromanolaki *et al.*, 1999). In our laboratory, levels of *p53* protein were observed in sera of 15 patients and 3 controls using the monoclonal antibody PAb 1801, and these were found to be increased in as many as 67% patients. Of these, 47% were moderate and 20% were high expressors. The average *p53* expression of healthy controls was 3.27 ng/ml, while that of cancer patients was 4.13 ng/ml (Parashar and Sobti, 1998).

Apoptosis is induced in human keratinocytes containing mutated *p53* alleles (Magal *et al.*, 1998). It has been found that E6 and E7 oncoproteins of HPV lead to the inhibition of *p53* function which actually resulted in apoptosis. Koh *et al.* (1998) have shown that both HPV infection and/or *p53* mutations are implicated in oral squamous cell carcinomas, but are not mutually exclusive events. It is possible that decrease in apoptosis is more closely related to *p53* mutation rather than HPV

infection. In oral squamous cell carcinoma patients, HPV was detected in 52%, whereas 38% of patients had *p53* mutations.

Studies from our laboratory indicate no fixed correlation between *p53* expression and HPV prevalence (Sobti and Parashar, 1998). Although lesser *p53* expression was observed in patients positive for both HPV 16 and 18, the *p53* levels in HPV negative patients as well as those positive for HPV 16 only, were similar.

In a study on 70 Chinese patients with esophageal squamous cell carcinoma, *p53* overexpression and *p53* mutations were detected in 73% and 44% of the patients respectively. (Lam *et al.*, 1997). These were primarily point mutations common in codons 248, 273 and 285. There was no relationship with the age, sex, smoking/drinking habits of patients and tumor site, size or stage. Mutations in *p53* also appear to play a role in predicting the survival of patients with stage III esophageal squamous cell carcinomas. Gleeson *et al.* (1998) studied the association between *17p* allelic loss, *p53* gene mutation, *p53* protein expression and DNA aneuploidy in adenocarcinomas arising in the esophagus and gastric cardia. Allelic loss was detected in 79% of esophageal and 83% of gastric adenocarcinomas.

A follow-up study of up to 3–4 years on the expression of *p53* and *Rb1* proteins was done on 250 primary operable colorectal carcinomas. It showed that overall cancer-specific mortality was 34.8% with 87 cancer deaths and 35 deaths as a result of other causes (Poller *et al.*, 1997). This suggests that *p53* and *Rb1* proteins do not appear to be useful in determining the prognosis of operable colorectal cancer.

Genetic instability related to defective DNA mismatch repair genes may be involved in the pathogenesis of carcinoma in hereditary non-polyposis colorectal cancer. To detect somatic mutations in *K-ras* and *p53* genes, SSCP followed by DNA sequencing was done. *K-ras* mutations were detected in 17% and *p53* in 13% of the 23 patients under study (Losi *et al.*, 1997). This suggested that other genes are also involved in colon cancers. A relationship between *p53*-gene mutation, type and location of the tumor found that mutations in its conserved regions accumulate in distal but not in proximal tumours (Jernvall *et al.* 1997). This difference may be related to the more aggressive behaviour and to different etiological factors associated with distal tumors.

Shiao *et al.* (1998) examined *p53* mutation spectra which provided clues to molecular mechanisms involved in different histologic types of gastric cancer. 105 patients were studied for mutations in exons 5–8 by PCR-SSCP. They found a predominance of G:C → A:T transitions at CpG sites in all the tumor types showing that DNA methylations may be the major determinant for *p53* mutations and may also be equally important in gastric carcinogenesis regardless of histology. Genetic abnormalities and microsatellite instability was also observed by Iniesta *et al.* (1998) in 63 colorectal tumors. They found *p53* mutations in exon 7 in the later stages of tumor progression. Thus, the authors suggested that *p53* as TSG can be used as a prognostic indicator to determine metastatic potential of colorectal adenocarcinomas.

It has been suggested that cyclin-dependent kinase inhibitors including *p16* and *p15* are tumor suppressor genes. It has also been seen that deletions or mutations of *p16* and *p15* genes are uncommon in primary gastric carcinomas. It has been suggested that defective mRNA transcription, sometimes by aberrant DNA methylation might be one of the pathways of inactivation of the *p16* gene that leads to the development of gastric carcinoma (Lee *et al.*, 1997).

Gastrointestinal Tract Cancer in Relation to Tobacco and Alcohol Related Metabolic Enzymes: Role of Detoxifying Genes

Esophageal squamous cell carcinoma has been reported to be epidemiologically associated with tobacco and alcohol consumption. Aromatic hydrocarbons, including benzo [a] pyrene in tobacco

smoke first require metabolic activation by phase I enzymes, cytochrome P450s (Cyp450s), and then are subjected to detoxification by phase II enzymes, the glutathione-S-transferases.

Cyplal is well conserved among the xenobiotic metabolic enzymes. Formation of hydroxylated products from benzo [a] pyrene including aryl hydrocarbon hydroxylase (AHH) is linked with *Cyplal* gene (Guengerich *et al.*, 1993). Co-segregation of *Cyplal* high inducibility phenotype and polymorphism of *msp1* restriction site in the *Cyp* gene is related to carcinogenesis and induction of gastrointestinal tract cancer (Peterson *et al.*, 1991).

Another genetic polymorphism in the *Cyp1a1* gene at exon 7 results in the replacement of Ile by Val in the haem binding region (Kawagiri *et al.*, 1993). This indicates that genetic susceptibility plays an important role in the occurrence of cancer. The association of combined genotypes of Val/Val and homozygous mutant *msp1* results in more risk of esophageal carcinoma than the other two genotypes (Nimura *et al.*, 1997).

Cyp2el The ethanol inducible Cyp2eI metabolizes several known carcinogens including N-Nitrosoamines, styrene, butadiene and urethane. Genetic polymorphisms, identified by RFLPs using the restriction enzymes Dra1, Rsa1 and Taq1 of the *Cyp2eI* gene has been shown to be associated with esophageal cancer (Raunid *et al.*, 1995). In a French study of 260 control and 511 alcoholic patients, the frequencies of mutated *Cyp2* alleles were found to be 2.5 for Rsa1 and 7.9% for Dra1. Only significant difference was found in Dra1 polymorphism, whose frequency was enhanced in alcohol related esophageal cancer as compared to controls (Lucas *et al.*, 1996).

Glutathione-S-Transferase: GSTs are multifunctional enzymes that catalyze the reaction between glutathione (GSH) and electrophiles and hydrophobic compounds. These are known to be inactivated by *Gstm1* and *Gstt1* enzymes (Ketterer *et al.*, 1992). The *Gstm1* gene locus contains three alleles *GstmIA* and *GstmIB*, which differ by a single amino acid (Raunid *et al.*, 1995). About 50% of the Caucasian populations are known to inherit two deficient alleles that are homozygous for the null allele (Daly *et al.*, 1993). The GST null genotype frequencies show marked ethnic variations (Bell *et al.*, 1995). In esophageal cancers, the null genotype of *Gstm1* is reported to be a risk factor for susceptibility to esophageal cancer (Nimura *et al.*, 1997), but other studies have found no association between null *Gstm1* genotype and esophageal cancer (Morita *et al.*, 1997). However the combined polymorphisms *Cyp1AI*, *Msp1* and Ile/Val and null *Gstm1* are risk factors for gastrointestinal tract cancers (Nakajima *et al.*, 1996).

Gastrointestinal Tract Cancer related Chromosomal Instability

Our understanding of the pathogenesis of cancer has undergone a revolution. Tumors develop by the accumulation of damage to genes that regulate cell growth. One of the most important concepts that has facilitated our understanding of carcinogenesis is that of genetic or genomic instability which allows sufficient amount of genetic damage to accumulate thus, permitting neoplastic phenotype to emerge and evolve. Two mechanisms involved are loss of chromosomal fragments and microsatellite instability (Boland *et al.*, 1998).

Cancer is thought to appear as a result of multiple genetic alterations (Comings, 1973; Knudson, 1985; Weinberg, 1989). Loss of function mutations, which inactivate tumor suppressor genes, are also believed to play important roles in various types of tumors (Klein, 1987; Green, 1988; Ponder, 1988). This inactivation of (TSG) resulting from chromosomal deletion or point mutations allows a cell to escape from normal growth into tumorigenesis with uncontrolled cell growth. Loss of heterozygosity (LOH) on specific chromosomes or chromosomal regions has been reported in colon cancers (Fearon *et al.*, 1987).

Squamous cell carcinoma of the esophagus (SCCE) is an extremely aggressive form of human neoplasia. Complex structural and numerical changes have been reported in cells from esophageal tumors derived from cell lines (Wuu *et al.*, 1986; Whang *et al.*, 1990). In esophageal tumours frequent structural changes occur in chromosomes 1, 2, 3, 7 and 12 with most consistent abnormality being deletion of 12 p (Xiao *et al.*, 1991). In gastric and lower esophageal adenocarcinomas, several non-random structural abnormalities have been identified, notably a highly specific region of rearrangement, 11p 13–15, which may be of diagnostic value.

In Barrett's adenocarcinoma, aneuploid cells and karyotypic abnormalities are associated with its development (Reid *et al.*, 1987; Rabinovitch *et al.*, 1988; Garewal *et al.*, 1989; Blount *et al.*, 1990; Rodriguez *et al.*, 1990). In one series, 95% of Barrett's adenocarcinomas had aneuploid or tetraploid cell populations (Blount *et al.*, 1991). Another study reported commonly deleted region on the long arm of chromosome 5 in esophageal carcinoma (Oagasawara *et al.*, 1996). They hypothesized that this frequent 5q deletion may play an important role in majority of esophageal carcinomas. This deletion was thought to target the *irf 1* gene or other genes at 5q 31.1. *Apc* gene locus deletion observed was attributed to a larger deletion on 5q and is not important by itself in pathogenesis of esophageal cancer.

A homogeneously staining region (*hsr*) *i.e.*, cytogenetic sight of gene amplification in proximal 11q has been described in several cell lines from esophageal carcinomas. This band (11q. 13) corresponds to location of an oncogene *Prad1*. A complex karyotype with a homogeneously staining region (*hsr*) in 11q 13 in short term cultured SCC of the esophagus has also been reported (Jin *et al.*, 1995).

Deletion in 3q has been found to be the most common cytogenetic abnormality in esophageal and gastric adenocarcinomas (Rao *et al.*, 1995). In addition, rearrangements affecting 1p13, 6a15-q 23, 7p 22, 7q 22 and 11p 11.2-p 14 were also seen.

In tumors, including those of esophageal origin, allelic deletions of 17p have also been observed (Blount *et al.*, 1991; Wagata *et al.*, 1991; Rosenblum-Vos *et al.*, 1993). Allelic deletions of 17p and alterations of *p53* including elevated *p53* protein levels frequently occur in Barrett's adenocarcinomas. This suggests their role in carcinogenesis in Barret's esophagus (Blount *et al.*, 1991). There is substantial evidence that the target of 17p allelic loss is the *p53* gene. It has been demonstrated that Barrett's esophagus is frequently characterized by clonal proliferation of karyotypically abnormal cells (Garewal *et al.*, 1989).

In case of colorectal carcinoma, allelic deletions have been reported to occur at various sites of the genome (Vogelstein *et al.*, 1989; Leister *et al.*, 1990; Miyaki *et al.*, 1990). In a few studies, the relationship of aneuploidy to molecular genetic alterations in colorectal carcinoma has been investigated (Remvikos *et al.*, 1990; Wagata *et al.*, 1991). Association of aneuploidy with allelic deletion on the short arm of chromosomes 17 and 18 has been found (Delattre *et al.*, 1989). In another study on colorectal carcinoma, high fractional allelic loss, deletion of 17p and 18q were associated with distant metastasis (Offerhaus *et al.*, 1992). The mapping carried out on an inherited colon cancer syndrome, familial adenomatous *Polyposis coli* (APC) to the same region of 5q also implies that this part of the genome contains a gene or genes involved in an early and perhaps rate-limiting step in the mutational pathway to colon cancer.

A study conducted by Bonassi *et al.* (1995) found a lack of correlation between CA frequency and mortality from patients of digestive tract cancers, even though subset of five colon cancer cases showed clearly increasing trend in standardization mortality rates by levels of CA.

Sister chromatid exchanges (SCEs), involving the exchange of homologous or nearly homologous segments between sister chromatids have also been observed by various workers (Taylor, 1958; Latt, 1973; Chaganti *et al.*, 1974; Perry and Wolff, 1974; Wolff and Perry, 1974). The phenomenon of

SCEs has been widely used as an indicator of chromosome instability (Perry & Evans, 1975; Carrano *et al.*, 1978).

A study conducted in our laboratory has shown that the mean frequency of SCEs in esophageal cancer patients was elevated as compared to controls, the values in the patients being 6.47 ± 2.67 , as against 3.86 ± 2.34 in the controls (Sobti and Parashar, 1998). Similar observations were of Mufti *et al.* (1998) on the patients with oral carcinoma. Murthy *et al.* (1997) found that the SCE values deviated significantly from that of controls. According to Kurvink *et al.* (1978) patients with viral diseases revealed significantly elevated SCE scores. Bazopoulou-Kyrkanidos *et al.* (1986) found no significant SCE rate increase in cancer patients as compared to normal controls. Higher frequencies of SCE were observed in individuals who smoked more than 10 bidis or cigarettes/day compared with people who smoked less than 10 bidis or cigarettes/day. Interchromosomal distribution of SCE revealed an increased frequency of SCE in almost all the chromosomes in cancer patients when compared to controls. Comparison of cell cycles revealed a block at M1 in the PBLs of the patients as compared to the controls.

Conversely, a study by Adhvaryu *et al.* (1988) on esophageal cancer patients showed a significantly higher SCE per cell value in the patients compared with controls. It can, therefore, be concluded that the SCEs can be taken as prognostic marker of DNA damage, including onset of malignancy. The study of SCEs of an individual may indicate the potential risk of cancer to the individual. SCEs were studied in PHA-stimulated PBL from 81 patients with squamous cell carcinoma of the oral cavity.

In the study conducted in our laboratory the mean average generation time (AGT) of the patients was $43.07\% \pm 5.61$ as compared to 35.53 ± 3.01 in controls; the difference being statistically significant. Not much difference was observed between the CAs in patients with cancer and control subjects, with the mean frequencies being $1.70 \pm .50$ and 1 ± 0.71 respectively (Parashar, 1998).

The kinetics of cellular turnover in the lymphocyte cultures of the cancer patients may be another parameter responsible for genetic instability. Studies have shown an increase in proliferation index rates of cancer patients as compared to controls. Cell cycle analysis has revealed significantly longer cell cycle in patients with leukemic AML (Abe *et al.*, 1980), CML (Becher *et al.*, 1988) and Sezary syndrome (Limon *et al.*, 1995).

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18. Molecular Pathogenesis of Lung Cancer

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Abstract: Lung cancer is the largest cancer killer of men and women. In addition to the progress made from anti smoking campaigns, new tools to diagnose and treat patients are emerging which have made rapid advances in the knowledge of molecular pathogenesis of lung cancer. These tools have started to provide an insight into how the tumor cell, by altering oncogenes and tumor suppressor genes, achieves growth advantage, uncontrolled proliferation and metastatic behaviour. Now knowledge is being gathered in terms of the molecular definition of individual susceptibility to tobacco smoke carcinogens. This review summarizes current knowledge of the molecular pathogenesis of lung cancer.

Key words: Lung cancer, genes, pathogenesis

1. Introduction

Lung cancer is the most frequent cause of cancer deaths in both men and women in the United States (Esteller *et al.*, 1999) and tobacco smoking is accepted as the major cause. It is classified into two major groups: small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC) (Minna *et al.*, 1997). Squamous cell carcinoma, adenocarcinoma and large cell carcinoma are the major histological types of NSCLC (Lengauer *et al.*, 1998).

As with other epithelial malignancies, lung cancers are believed to arise after a series of progressive histopathological changes (preneoplastic lesions) in the bronchial epithelium (Levin *et al.*, 1995). These morphological steps include hyperplasia, metaplasia, dysplasia and carcinoma *in situ* (CIS). Many mutations, especially those involving recessive oncogenes have been described in invasive lung cancers (Minna *et al.*, 1997).

2. Molecular Alterations in Lung Cancer Cells

Tumor Suppressor Genes

(a) p53: *p53* maintains genomic integrity in the face of DNA damage from ultraviolet irradiation and carcinogens. DNA damage results in rapid increase in *p53* which acts as a transcription factor regulating downstream genes including *p21*, *mdm2*, *gadd45* and *bax*, thereby helping to regulate the G1/S cell cycle transition, G2/M DNA damage checkpoint and apoptosis (Takashi *et al.*, 1989). Dysfunction of *p53* allows the inappropriate survival of genetically damaged cells leading to tumor progression (Bennet *et al.*, 1999).

There are other genes homologous to *p53*, including *p51* at chromosome locus 3q28 and *p73* at 1p36, whose products can induce growth suppression and apoptosis. Mutations in *p51* appear to be infrequent in lung cancer (Yoshikawa *et al.*, 1999). Similarly *p73* mutations are absent or infrequent in lung cancers despite allele loss at 1p36 region (Mai *et al.*, 1998). *p21* is a *p53*-responsive gene that inhibits cyclin/cyclin-dependent kinase (CDK) complexes at the G1 phase. Although not somatically mutated in lung cancer, *p21* was overexpressed in 65 to 75% of NSCLCs, especially in well-differentiated tumors (Marchetti *et al.*, 1996). One NSCLC study reported that *p21* expression is linked to a favourable outcome (Caputi *et al.*, 1998), whereas another suggested that concordant expression of *p21* and transforming growth factor β 1 (TGF- β) predicts better survival than discordant expression (Bennet *et al.*, 1998).

(b) *p16*-cyclin D1-CDK4-Rb pathway: The *p16*-cyclin D1-CDK4-Rb pathway is central to controlling the G1-S transition of the cell cycle and its components are functionally altered or mutated in many cancers. The major growth suppressing function of *Rb* is to block G1-S progression and inactivation of both *Rb* alleles at chromosome 13q14 is common in lung cancer (Harbour *et al.*, 1998). Functional *Rb* loss includes deletion, non-sense mutations or splicing abnormalities, frequently leading to truncated *Rb* protein (Geradts *et al.*, 1999). Mutations in *Rb* have been detected in about 90% of SCLCs and 15 to 30% NSCLCs (Kvatalie *et al.*, 1996, Dosaka *et al.*, 1997). Two *Rb* related genes *p107* and *pRb2/p130* have been implicated in lung cancer. The decreased expression of the proteins of these genes is associated with more aggressive histological behaviour (Hibi *et al.*, 1992).

(c) Cyclin D1 and CDK4: Cyclin D1 inhibits the activity of *Rb* by stimulating its phosphorylation by Cdk4. Thus, cyclin D1 overexpression is an alternative mechanism to *Rb* mutation for disrupting the *p160* cyclin D1-CDK4 pathway (Caputi *et al.*, 1999). Cyclin D1 was overexpressed in 25 to 47% of NSCLCs and has been associated with poor prognosis (Betticher *et al.*, 1996, Mishina *et al.*, 1999). Co-inactivation of *Rb* and *p16* in any one tumor is rare, but cyclin D1 overexpression can co-exist with these abnormalities in the same tumor (Taga *et al.*, 1997).

(d) *p19^{ARF}*: The *p16* locus also encodes a second alternative reading frame protein *p19^{ARF}* that overlaps with *p16*. The amino acid sequence of the two is different. However, they both appear to be important in growth regulation. Immunohistochemical analysis suggests that *p19^{ARF}* protein expression was more frequently lost in tumors with neuroendocrine features (Gazzevi *et al.*, 1998).

3. Apoptosis

Tumor cells often escape the physiological response (programmed cell death or apoptosis) to cellular and DNA damage (Jiang *et al.*, 1999). Expression of the *bcl-2* anti-apoptotic proto-oncogene is higher in squamous cell carcinoma than in adenocarcinoma (Tezella *et al.*, 1993). It also correlates with neuroendocrine differentiation and is higher in SCLCs than in NSCLCs (Apolinaro *et al.*, 1997, Higashiyam *et al.*, 1997). Similarly, there is also a prolific expression of *fas L* in lung cancer cells (Neihans *et al.*, 1997). Moreover, *fas* expression has been shown to be reduced in adenocarcinomas which may account for resistance to *fas*-mediated apoptosis. Thus, it appears that lung can make *fas*, but do not show a response towards it as it lacks *fas* receptor, but the *fas* they produce inactivates T-cells, providing a mechanism of escape from a patient's immune response (Fong *et al.*, 1999).

4. Methylation

Abnormalities of DNA methylation occur in human neoplasia and promoter region. Hypermethylation

in 5 CpG islands may transcriptionally silence and inactivate TSGs like *Rb*, *p16* and *vhl* (Merlo *et al.*, 1995). In NSCLCs, *p16* hypermethylation contributes to its downregulation and occurs at an early stage in the development of lung cancer (Otterson *et al.*, 1995, Belinsky *et al.*, 1998).

A series of genes have been found to undergo promoter methylation, especially in NSCLCs, death associated protein kinase (DAP), glutathione-S-transferase P1 (GSTP1) and MGMT (Esteller *et al.*, 1999). Other sites of hypermethylation found in lung cancer include loci 3p, 4q34, 10q26 and 17p13 (Makos *et al.*, 1992; Kohno *et al.*, 1998).

Methylation also plays a role in mediating genomic imprinting, thus loss of genomic imprinting in genes like *IGF2* and *H19* at chromosome loci 11p15 occurs in lung cancers (Suzuki *et al.*, 1994; Kondo *et al.*, 1998).

5. Angiogenesis and Metastases

Tumors cannot exceed 1- to 2-mm³ volume without the development new blood vessels. They require angiogenic factors early in their pathogenesis (Angelelti *et al.*, 1996). Tumor angiogenesis is complex and is controlled by adverse family of inducers and inhibitors which regulate endothelial cell proliferation and migration (Hananan *et al.*, 1996, Chandrachud *et al.*, 1997). Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are the important angiogenic inducers (Mattern *et al.*, 1996). VEGF expression in squamous cell carcinoma is related with tumor cell proliferation (Fontanini *et al.*, 1997). VEGF expression was higher in lung cancer showing nodal metastasis and was associated with poor prognosis in NSCLC (Fontainini *et al.*, 1997). Fibroblast growth factor (FGF) is expressed in 70% of NSCLC tumors (Volm *et al.*, 1997). Expression of angiogenic-derived endothelial cell growth factor is correlated with tumor angiogenesis and worse prognosis in N-O stage of NSCLCs (Koukourakis *et al.*, 1997).

6. Telomerase Activity

Telomeres are specialized structures located at the end of chromosomes having TTAGGG tandem repeats (Counter *et al.*, 1992). These are maintained by an enzyme telomerase. Germ cells, some stem cells and most cancer cells have telomerase activity (Kim *et al.*, 1994). Nearly all SCLCs and 85% NSCLCs have high levels of telomerase (Hiyama *et al.*, 1995; Abanell *et al.*, 1997). The high expression of telomerase activity is associated with increased cell proliferation rates and advanced stage in NSCLCs (Albanell *et al.*, 1997). Telomerase activity or dysregulated RNA expression are frequent in carcinoma *in situ* lesions, implicating involvement in lung cancer development (Yashima *et al.*, 1997).

7. Growth Stimulation and Oncogenes

As a consequence of the expression of growth factors, regulatory peptides and their receptors by either the cancerous or adjacent normal cells, autocrine and paracrine growth stimulatory loops exist in lung cancers. Several, but not all components of these stimulatory pathways are proto-oncogene products.

Gastrin-releasing peptide (GRP)/bombesin (BN) autocrine loop: GRP is a 27 amino acid mammalian homologue of the amphibian peptide BN. GRP/BN functions include a role in lung development and repair (Sparzem *et al.*, 1997). Immunohistochemical studies have shown that approximately 20 to 60% of SCLC cancers express GRP/BN, whereas non-SCLCs (NSCLC) less frequently do so (Richardson

et al., 1993). The human GRP/BN receptor subtypes belong to the G-protein-coupled receptor superfamily and include GRP-, neuromedin B-, and BN subtype-3 receptors; all of these can be expressed in SCLCs, NSCLCs and in some biopsy specimens of bronchial epithelium of smokers (Fathi *et al.* 1996; Siegfried *et al.*, 1997). However, lung cancers so far have not been found to have mutations in either GRP/BN or the GRP/BN receptors, thus the mechanisms for “reactivation” of these embryonic regulatory loops are still not known. The GRP/BN autocrine is an important growth stimulatory loop in lung cancer, particularly SCLCs. The *in vitro* formation of soft agar clones and the *in vivo* growth of nude mouse xenografts of SCLC cell lines are inhibited by a neutralizing monoclonal antibody directed against GRP/BN, as well as by antagonists of BN (Cuttitta *et al.*, 1985, Halmos *et al.*, 1997). A clinical trial of the anti-BN monoclonal antibody has shown some antitumor activity in previously treated patients with SCLC (Kelley *et al.*, 1997). This system appears to play an early role in pathogenesis, since there is an increased likelihood of expression of the GRP receptor messenger RNA in the respiratory epithelium of some individuals with a history of prolonged tobacco exposure. The expression of the GRP receptor mRNA is accompanied by responsiveness of these respiratory epithelial cells *in vitro* to the mitogenic effects of BN-like peptides (Siegfried *et al.*, 1997). These effects also appear to persist after cessation of smoking.

8. Oncogenes Implicated in Lung Cancer

(a) ras: The *ras* protooncogene encodes plasma membrane proteins and is activated by point mutations, resulting in continued signaling for cell divisions (Bos *et al.*, 1989). K-*ras* mutations affect approximately 30% of adenocarcinomas and 20% of all NSCLCs, but rarely do so in SCLCs (Richardson *et al.*, 1993). K-*ras* mutations correlate with smoking (Slebos *et al.*, 1991) and most of them are G-T transversions caused by polycyclic aromatic hydrocarbons and nitrosamines in tobacco smoke (Greenblatt *et al.*, 1994). The presence of K-*ras* mutations result in poor prognosis in NSCLCs (Rosell *et al.*, 1993, Graziano *et al.*, 1999). To be active in cell, *ras* has to have lipid modification (Farnesylation) regulated by farnesyltransferase. Several farnesyltransferase inhibitors from Bristol Myers, Squibbs, Jansen and Merck are currently undergoing clinical trials against lung cancer.

(b) Myc: *Myc* family comprises *myc*; *myc N* and *myc L* (Nau *et al.*, 1985). *Myc* is the most frequently activated in SCLCs and NSCLCs, whereas the others usually only affect SCLCs. Activation occurs by gene amplification (~2-115 copies per cell) or by transcriptional dysregulation, both of which lead to protein overexpression. Richardson and Johnson (1993) concluded from 17 studies that 18 to 31% of SCLCs had amplification of one *myc* family member. Conversely, only 8 to 20% of NSCLCs were affected. *Myc* amplification appears to occur more frequently in patients undergoing chemotherapy and the “variant” SCLCs subtype (Johnson *et al.*, 1996) and may correlate with adverse survival. Lastly, *in vitro* growth inhibition of an SCLC cell line by all-trans-retinoic acid was associated with increased neuroendocrine differentiation, increased *myc L*, and decreased *myc* expression (Ou *et al.*, 1996).

(c) Erb b Family: NSCLCs but not SCLCs demonstrate abnormalities of receptors erb b2 and erb b1. On ligand binding, erb b receptors homodimerize or heterodimerize, thereby, induce intrinsic kinase activities that influence signal transduction cascades including MAP kinase pathway (Weiner *et al.*, 1990). *Erb b2* is highly expressed in approximately 30% of NSCLCs especially adenocarcinomas (Rachwal *et al.*, 1995). High *erb b2* levels are associated with multiple drug resistance (Tsai *et al.*, 1996) and increased metastatic potential in NSCLCs (Yu *et al.*, 1994). Erb b Blockers (CP3558774, ZD1839) are entering clinical trials.

9. Cytogenetic Analysis

Cytogenetic and allelotyping have revealed many hemizygous and some homozygous deletions at multiple chromosomal regions in lung cancers (Ohata *et al.*, 1993). The chromosomal regions showing hemizygous deletions include 1p, 1q, 2q, 3p, 4p, 4q, 5q, 6p, 8p, 8q, 11p, 11q, 14q, 17q, 18q, and 22q (Sato *et al.*, 1994; Shiseki *et al.*, 1994; Otsuka *et al.*, 1996; O'Briant *et al.*, 1997; Cleno *et al.*, 1998). Although several of these chromosomal arms contain known tumor suppressor genes (TSGs) like *apc* at 5q21, *wtl* at 11p13 and *nf2* at 22q12, these are not known to be mutated in lung cancer (Virmani *et al.*, 1998; Wistuba *et al.*, 1999).

Hemizygous loss of one chromosome allele occurs in lung cancer (Kok *et al.*, 1997) implicating the presence of multiple TSGs in regions of 3p *i.e.*, 3p25–26, 3p21.3–22 and 3p14.2, which encode dinucleoside hydrolase, found to be deleted in lung cancer (Sozzi *et al.*, 1996; Fong *et al.*, 1997). *Fhit* loss is more pronounced in smokers than non-smokers (Sozzi *et al.*, 1997).

Similarly, 11q23–24 chromosome locus is a region containing frequent allelic loss with two regions showing distinct loss, one contains the gene encoding the β -iso form of the A subunit of the human protein phosphate 2A (*ppp2rib*). Mutations in these regions in lung cancer suggest that *ppp2rib* gene acts as a tumor suppressor gene (Wang *et al.*, 1998).

Lung cancer cells (40–80%) frequently express abnormal mRNA transcripts of *fhit*, but nearly always also express wild-type *fhit* transcripts (Sozzi *et al.*, 1996; Fong *et al.*, 1997). However, unlike classic TSG inactivation, *fhit* point mutations are rare (Sozzi *et al.*, 1996; Fong *et al.*, 1997) and abnormal transcripts can be found in normal lung tissues (Tokuchi *et al.*, 1999). *Fhit* is expressed in normal lung, but in primary lung tumors, it is absent (Sozzi *et al.*, 1997). Moreover, *fhit* allelic loss is more common in smokers than non-smokers (Sozzi *et al.*, 1997) and may be associated with a poorer survival in NSCLC (Burke *et al.*, 1998). Furthermore, reintroduction of exogenous wild-type *fhit* suppressed tumorigenicity of lung cancer cell lines in nude mice (Siprashvili *et al.*, 1997; Fong *et al.*, 1999), but not in human cancer cell lines (Otterson *et al.*, 1998). The 3p21.3 region has been extensively examined for putative TSGs (Kok *et al.*, 1997), particularly at a 600-kb region homozygously deleted in three SCLC cell lines (Kok *et al.*, 1994; Wei *et al.*, 1996), while another 800-kb deletion region exists at 3p21.

Protein tyrosine phosphatases may play a role in tumor suppression because of their ability to antagonize the growth-promoting protein kinases. The phosphatase encoded by the *pten* gene at chromosome 10q23, is mutated in a few primary lung cancers and several lung cancer cell lines and involves homozygous deletions of the gene. Another candidate at 10q region 25.3–26.1, is *dbmti*, which is frequently down-regulated and occasionally homozygously deleted in lung cancer.

10. Detoxifying Genes

(a) Cyp genes: Polycyclic aromatic hydrocarbons (PAHs) like benzo (a) pyrene are believed to be oncogenic compounds in tobacco smoke and cause lung carcinomas (US Surgeon General, 1982). PAHs occur as inactive procarcinogens in tobacco smoke. The detoxification of these procarcinogens is carried out in two phases. In phase I, detoxification is mediated by *cyp 1a1* enzyme aryl hydrocarbon hydroxylase (AHH) of cytochrome *p450* enzyme family, PAHs (like benzo (a) pyrene) are metabolized to their ultimate carcinogenic intermediate, trans 7, 8-diol, 9–10 epoxide moiety (Conney *et al.*, 1989). These after being metabolized, form active PAH-DNA adducts which modify normal DNA structure and function (Pelkonen *et al.*, 1980) and have been associated with carcinogenic potency in several animals and *in vitro* systems (Brookes *et al.*, 1964; Goshmann *et al.*, 1967; Shen *et al.*, 1980; Nakajama *et al.*, 1995).

These active electrophile epoxides undergo detoxification reactions (Phase II) in which they are enzymatically conjugated with intracellular glutathione to form inactive, water soluble metabolites and are readily excreted. This detoxification reaction is catalyzed by glutathione-S-transferase (Stewart *et al.*, 1993). The GST enzymes are the most abundant non-protein thiols in mammalian cells. Maintenance of homeostatic GST is done by *de novo* synthesis and salvage pathways (Kenneth *et al.*, 1994). There are four distinct GST enzymes; α (basic) which include GST2, GST A μ (near neutral) GST TI, GST MI π (acidic) GST3, GSTP (Morgernstern *et al.*, 1988, Mannervik *et al.*, 1992) and the θ . These GSTs are classified according to their isoelectric points (Meyer *et al.*, 1991).

The *cyp 1a1* enzyme is encoded by *cyp 1a1 gene*. The human *cyp 1a1* cDNA has been cloned, sequenced and localized to chromosome 15 near *msp1* locus. Several *cyp 1a1* RFLP patterns have also been described (Jaiswal *et al.*, 1985; Bale *et al.*, 1987; Nerbert *et al.*, 1987; Spur *et al.*, 1987; Haugen *et al.*, 1991). In lung, *cyp 1a1* expression is seen in Clara cells, type II pneumocytes and pulmonary macrophages (Coombs *et al.*, 1986). *Cyp 1a1* enzyme aryl hydrocarbon hydroxylase is a risk factor in the etiology of lung cancer. A positive correlation has been seen between *cyp 1a1* mRNA and *ahh* activity in about 73% of NSCLC and 22% of SCLC cases, both in the presence and absence of benzopyrene (Jaiswal *et al.*, 1985).

If a high expression of *cyp 1a1* mRNA is detected in the lung tissues, it is postulated that these individuals could be at a higher risk for developing pulmonary carcinomas and this proves as an important marker for screening and identification of people having a risk for lung cancer (Kouri *et al.*, 1983; Kouri *et al.*, 1984, 1986; Mclemore *et al.*, 1987; Nerbert *et al.*, 1987). The expression of *cyp 1a1* mRNA was assessed in lung tissues using polymerase chain reaction. This suggests the possibility of using peripheral cells to indicate an individual's phenotypic characteristics (Omiecinski *et al.*, 1990). Induction of *cyp 1a1* expression in humans may be subjected to genetic polymorphism such that most responsive members may be at the highest risk for developing lung cancer (Jaiswal *et al.*, 1985; Nerbert *et al.*, 1989). A high susceptibility to lung cancer was associated with *msp1* polymorphism in the 3' flanking region of *cyp 1a1* gene (Nakachi *et al.*, 1993). Another genetic polymorphism is seen in the coding region of *cyp 1a1* gene which results in the replacement of isoleucine by valine at residue 462 in the haem binding region. The ile/val polymorphism resulted in two different p450 *cyp 1a1* proteins. The val-val type *cyp 1a1* showed higher AHH activity than ile/val type (Kawajiri *et al.*, 1991). The *msp1* polymorphism resulted in 3 genotypes. The genotype with a low mean cigarette consumption showed an increase in susceptibility to develop lung cancer (Nakachi *et al.*, 1993). Similarly people with val/val polymorphism of *cyp 1a1* gene had increased risk to develop lung carcinoma at low cigarette dose. This indicates that genetic susceptibility plays an important role in the occurrence of lung cancer at a low cigarette dose. This is true for a Japanese population but such association has not been seen for Norwegian and Finnish populations (Huvonen *et al.*, 1992). This discrepancy may be due to polymorphism as that *msp1* or ile/val polymorphism can vary among races.

Certain other *cyp* family genes have also been correlated with cigarette smoking (Randa *et al.*, 1997). Nitrosamines present in cigarette smoke induce *cyp2e1* gene polymorphism which plays an important role in the etiology of lung cancer (Raunio *et al.*, 1995). According to Czerwinski *et al.*, (1994) the expression of genes like *cyp 2b7*, *cyp or*, *cyp1a1* has not been seen in relation to cigarette smoking.

We have analyzed the *cyp2e1* gene polymorphism in lung cancer patients and controls using PCR-RFLP technique and the data are being analyzed for publication (Fig. 1).

(b) GST genes: A genetic polymorphism for the glutathione-S-transferase μ enzyme has also been described in humans (Strange *et al.*, 1984). *Gstt1* gene is polymorphic and is deleted in 30% Europeans (Pemble *et al.*, 1994) and 20% North Americans (Nelson *et al.*, 1995). This deficiency is correlated with the inability to conjugate glutathione with small molecular weight toxins (Randa *et al.*, 1997).

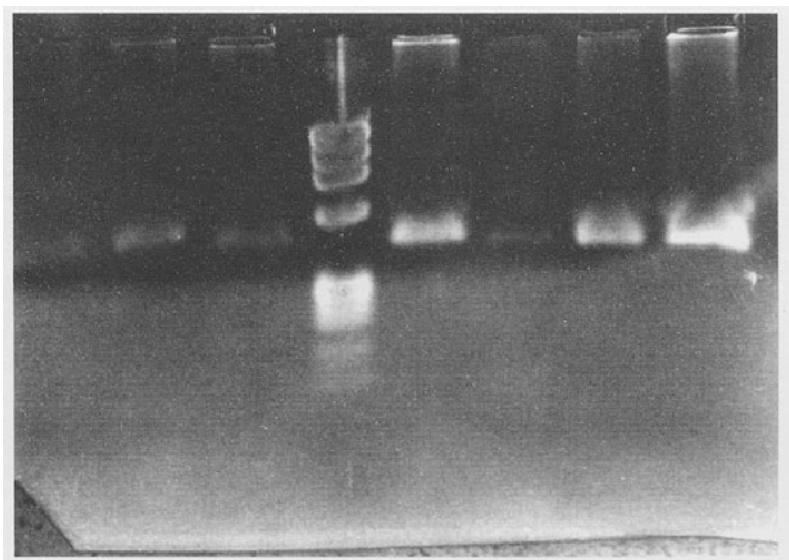


Fig. 1 PCR-RFLP analysis of CYP2E1 polymorphism. Lane 1: Wild type CYP2E1-410 base pair, Lane 2: PCR product cut with enzyme Pst 1-no cut wild type, Lane 3: Wild type CYP2E1-40 base pair, Lane 4: Marker - PHI 174 cut with Hae III, Lane 5: PCR product cut with enzyme Pst 1-No cut wild type, Lane 6: Wild type CYP2E1-410 base pair, Lane 7: PCR product cut with enzyme Pst 1-No cut wild type and Lane 8: Wild type CYP2E1-410 base pair.

The *gstm1* is also a polymorphic form of *gst μ* class isoenzymes, but lacks in about 38–70% of different ethnic populations. A null *gstm1* genotype is also seen to be associated with risk for squamous cell lung carcinomas (SCLCs). Individuals heterozygous for *gstm1* gene have reduced capacity to detoxify metabolites (Seidegard *et al.*, 1990; Nakajama *et al.*, 1995). They are more susceptible to mutagen induced sister chromatid exchanges (Norpra *et al.*, 1995) and are at increased risk for asbestosis (Smith *et al.*, 1994) and smoking induced bladder cancer (Bell *et al.*, 1996).

GST μ isoenzyme activity has been assessed by trans-stilbeneoxide (tso) in leukocytes. Low or no *gst μ* activity in mononuclear leukocytes of smokers is said to be 3 times higher as compared to those smokers who had a high *gst-tso* activity and are susceptible to develop lung carcinomas (Seidegrad *et al.*, 1990; Seidegrad *et al.*, 1996). The *gst* enzyme activity is differentially expressed across human tissues (Strange *et al.*, 1984, 1985, Awasthi *et al.*, 1987).

We have also studied *gst* gene polymorphism in lung cancer patients and controls by using multiplex PCR methods to analyse both *gst M1* and *gst T1* genes. The data are being subjected to computation.

11. Conclusions

Some examples of new molecular tools that may change clinical practice in the near future have been given. The potential of molecular epidemiology is aimed at identifying the individual genetic susceptibility factors to lung cancer and individuals at the highest risk for the development of lung cancer. Somatic mutational events occur in airways exposed to tobacco smoke carcinogens and may lead to future refinement of risk prediction in genetically susceptible individuals. Apart from this, detection of lesions remain a high priority for lung cancer patients; and highly sensitive molecular tools can now detect changes in them. Large prospective studies needed to reach at clinically valid conclusions will need to come from large prospective studies.

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19. Bladder Cancer: Genetic and Epidemiological Factors Involved in its Genesis

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Abstract: The present paper reviews the genetic and epidemiological factors in the risk of bladder cancer in humans. Tobacco smoking accounts for about 50% of bladder cancer cases in Western Countries. Occupational exposures are second in importance to bladder cancer development. Exposure to aromatic amines in dye stuff manufacturers, in rubber and textile industry, exposure to paints and leather dust, inks, some metals and diesel exhausts in association with dietary factors, specifically, coffee drinking, alcohol consumption, vegetable, fruits, meat and vitamins, parasites and drugs have been identified as risk factors for bladder cancer. The molecular studies have revealed mutations in a number of genes in the tumor cells which might be responsible for the genesis of this cancer.

Key Words: Genes, Epidemiological Factors, Bladder Cancer

1. Introduction

Bladder cancer is the fourth most common malignancies in the western male population (Qureshi *et al.*, 1999), tenth in women (Protheroe *et al.*, 1999) and represents 3% of cancer deaths. Bladder cancer is diagnosed in over 50,000 people yearly in the United States alone (Scher *et al.*, 1997). In Europe, the annual incidence is 100,000 with a higher frequency in industrialized areas (Qureshi *et al.*, 1999) and with approximately 12500 new cases in the U.K. (Protheroe *et al.*, 1999).

In Japan, the bladder cancer is the eighth malignancy in men and the sixteenth in women and, the peak incidence is in the age group of 70–80 years (The Research Group for population-based Cancer Registration in Japan, 1990).

Bladder cancer is the second commonest urological cancers in India and its incidence seems to be increasing now (Tongaonkar *et al.*, 1995).

In several developing nations in Africa and in the Middle East, most predominantly in Egypt, bladder cancer is one of the most common types of malignancies both in men and women (El-Bolkainy *et al.*, 1981). In Egypt, the most common histological type of bladder cancer is squamous cell carcinoma (SSC), which comprises 60% of cases: transitional cell tumors (TCC) are frequent, comprising only 30% of cases; the remaining cases are adenocarcinoma (ADC) or undifferentiated carcinomas (UDC) (Aly and Khaled, 1999).

The disease is of two types: superficial and invasive. Superficial bladder cancers (pTis, pTa and PT1) do not invade the muscle layer. They are usually low-grade (Grade 1 or Grade 2) tumors. These two types of bladder cancers exhibit significantly different clinical behaviour. These usually occur and develop in multiple and low-grade forms with their specific papillary shape and frequently occur at the original site or at other new sites in the urinary bladder after transurethral resection. Most

superficial bladder cancers have a good prognosis, but in 10 to 20% of cases, cancer cells become more malignant showing an increase in the grade and/or infiltration into the muscle layer. On the other hand, invasive bladder cancers are commonly nodular with high-grade malignancy as they are very aggressive, because they develop and progress rapidly and metastasize at an early stage (Fujimoto *et al.*, 1992). Of new tumors, 70% are superficial (pTa/pT1), 25% are muscle-invasive (T2-T4) and 5% are carcinoma *in situ* (*cis*) types. Patients with muscle invasive disease account for the majority of deaths due to bladder cancer (Qureshi *et al.*, 1999).

Most of the bladder neoplasms are transitional cell carcinomas (TCC). Only 10–20% of bladder carcinomas present with tumour invasion are limited to the lamina propria (pT1) (Richter *et al.*, 1999). Transitional cell bladder carcinoma include a heterogenous group of tumors that occupy a spectrum extending from papillary grade 1 lesion, which have been considered virtually benign, to poorly differentiated cancers, these often lead to a fatal outcome (Smith, 1999). Even within the category of tumors with depth of infiltration superficial to the detrusor muscle of the bladder, there is a marked variation in tumor behaviour. Thus, the term "superficial" can be misleading (Smith, 1999).

Transitional cell carcinoma (TCC) of the bladder is an important public health problem (Fleshner *et al.*, 1999). The major clinical problem among patients with superficial TCC is disease recurrence with three years recurrence free survival ranging from 35 to 70% (Fleshner *et al.*, 1996). This high rate of recurrence supports the concept of bladder cancer as a field disease. Patients with superficial TCC are also at risk for disease progression and other urothelial tract cancers.

The bladder is also a well-documented site of extrapulmonary small cell carcinoma (SCCB) but is an uncommon tumor with approximately 5-years survival for 8% patients and disease remains confined to the pelvis (Lohrisch *et al.*, 1999). SCCB accounts for 0.5–1.0% of the incidence of bladder malignancy (Blomjous *et al.*, 1989; Holmang *et al.*, 1995). It is characterized by early metastatic spread and a poor prognosis (Abbas *et al.*, 1995). The majority of long term disease free survivors had limited disease at presentation (no extrapelvic spread) and had been treated with single or combined-modality therapy.

Many epidemiological studies have shown that bladder cancer is associated with factors such as exposure to occupational exogenous carcinogens and/or related to personal habits such as cigarette smoking and coffee drinking.

2. Genetic Factors

(a) *p53* gene

(b) Several studies have indicated that inactivation of multiple tumor suppressor genes is required for tumor development. Tumor suppressor gene *p53* is located on chromosome 17 p13.1. Highly significant associations have been reported between *Tp53* protein accumulation detected by immunohistochemical methods and the mutations by polymerase chain reaction-single-strand conformational polymorphism–(PCR-SSCP) in bladder cancer (Esrig *et al.*, 1993; Cardon-Cardo *et al.*, 1994; Vet *et al.*, 1995; Abdel-Fattah *et al.*, 1998). Diffused immuno-staining for *Tp53* and *Tp53* gene mutations are detected in about 50% of muscle-invasive bladder tumors (Cardon-Cardo *et al.*, 1994; Spruck *et al.*, 1994). However, it is clear that significant accumulation of the *Tp53* protein can arise in the absence of detectable mutations in exons 4–9 of the *Tp53* gene (Vet *et al.*, 1995; Abdel-Fatah *et al.*, 1998).

(b) *Fas*

Loss of Heterozygosity (LOH) studies have suggested that loss of one or more tumor suppressor

genes at chromosome 10q 24.1.-24.3 may be involved in the development of bladder TCC (Cappellen *et al.*, 1997). One of the candidate genes in this region is *Fas*, which is located on chromosome 10q 241. The *Fas*-*Fas* ligand (*FasL*) system has been recognized as a major pathway for the induction of apoptosis in cells and tissues (Nagata, 1997). *Fas* is a member of the death receptor subfamily of the tumor necrosis factor superfamily. There is mounting evidence that disruption on the *Fas* system frequently occurs in non-lymphoid malignancies as well (Natoli *et al.*, 1995; Mizutani *et al.*, 1997). To date, however, somatic mutations of the *Fas* gene, one of the possible mechanisms that mediates the disruption of *Fas* system, have not yet been reported in non-lymphoid malignancies, including TCC of urinary bladder. Lee *et al.*, (1999), however, analyzed somatic mutations and loss of heterozygosity of *Fas* gene in transitional cell carcinomas of urinary bladder. They found that 28% of cases have *Fas* mutations.

(c) *C-erb-B-2*

The expression of *c-erb B-2* protein, which is a transmembrane growth factor receptor has been related to increased cell proliferation and metastatic potential in TCC as well as in other neoplasia (Lofts and Gullick, 1992). The *her/neu* 2-gene, which encodes *c-erb-B-2* protein, has an outstanding sequence homology to epidermal growth factor receptor (EGFR), although they are not identical (Schecter *et al.*, 1985; Yamamoto *et al.*, 1986). The epidermal growth factor proto-oncogene is located on chromosome 7p13 (Rosenkranz *et al.*, 1989). EGFR was overexpressed in 35% of bladder cancer cases (Lipponen and Eskelinan, 1994).

(d) *C-Ha-ras-1*

The *c-Ha-ras-1* gene, homologous to the oncogene present in the Harvey murine sarcoma virus, is located on the short arm of chromosome 11p15 (Ellis *et al.*, 1981). A *c-Ha-ras-1* gene has been found to be activated transforming gene in different human cancers, including bladder carcinomas (Der *et al.*, 1982; Parada *et al.*, 1982; Santos *et al.*, 1982). *C-Ha-ras-1* gene polymorphism in a bladder cancer affected population and in some cases, a loss of genetic material in the vicinity of this locus has also been detected. But as no specific genotype can be implicated in the predisposition to bladder carcinoma, *C-Ha-ras-1* genotyping appears to be of limited value in the clinical management of these patients (Bittard *et al.*, 1996).

(e) *Mdm 2*

The murine double minute-2 (*mdm-2*) gene is located on chromosome 12q 13.14 and encodes a nuclear protein capable of forming complexes with both wild type and mutant p53 protein (Oliner *et al.*, 1992). The *mdm2* protein inhibits p53-mediated tumor suppressive function (Momand *et al.*, 1992). The *mdm2* amplification and/or overexpression was frequently observed particularly in sarcomas and glial tumors indicating that mdm 2 protein might affect the carcinogenesis and tumor progression in human malignancy (Oliner *et al.*, 1992; Reifenberger *et al.*, 1993). Shiina *et al.* (1999) found clinical significance of simultaneous evaluation of *mdm 2* and *p53* immunostaining as it proved to be superior over that of cell proliferation and/or apoptotic markers when elucidating the biological characteristic of bladder cancer.

(f) *E-Cadherin* gene

Cadherins are calcium-dependent transmembrane glycoproteins mediating cell-cell adhesion by binding to cadherin molecules of the same type of neighbouring cell (Takeichi, 1990). *E-cadherin* has been reported in malignant bladder tumors with abnormal expression correlating with increasing grade and

invasiveness' (Bringuier *et al.*, 1993; Otto *et al.*, 1994; Lippinen and Eskelin, 1995; Ross *et al.*, 1995; Syrigos *et al.*, 1995) and is associated with reduced survival (Bringuier *et al.*, 1993; Syrigos *et al.*, 1995). Similarly in patients with pTa or pT1 bladder tumors, abnormal expression of *E-cadherin* gene has been associated with more rapid progression and reduced survival (Otto *et al.*, 1994; Lippinen and Eskelin, 1995).

3. Epidemiological Factors

(a) Tobacco Smoking

Tobacco smoking causes millions of cancer deaths annually. Fifteen per cent (1.1 million new cases per year) of all cancer cases are attributed to cigarette smoking, 25% in men and 4% in women (Parkin *et al.*, 1994). Tobacco smoke is a complex mixture of thousands of chemicals including many known animal carcinogens. Cigarette smoking is most prevalent especially in industrialized countries (Johansson and Cohen, 1997). It is accepted as a major cause of cancers including bladder cancer (Parkin *et al.*, 1994). Smoking accounts for about 50% of bladder cancer cases in Western countries (Priastu *et al.*, 1996). The results of Davanzo *et al.* (1995) suggested that more than 2500 of the 5400 deaths due to bladder cancer in Italy in 1990 could have been prevented by the elimination of cigarette smoking. Smoking is thought to contribute to 50 and 30% or more human bladder cancers in men and women, respectively. The risk of transitional cell carcinoma (TCC) associated with exposure to sidestream smoke is not well defined. The risk of bladder cancer increases linearly (two-to-three-fold) for persons who smoke at least 10 cigarettes per day and then increases subsequently in people who smoke 40 to 60 cigarettes per day (IARC; 1987, Hartage *et al.*, 1987). North American and European data estimate that the risk for bladder cancer is 43% in men and 36% in women for having ever smoked cigarette (McCredie *et al.*, 1999). It is agreed that there is a strong relationship between tobacco and bladder cancer in both sexes (Whynder and Goldsmith, 1997; Morrison and Buring, 1980; Morrison *et al.*, 1980; McCredie *et al.*, 1983; Mommsen *et al.*, 1983a; Schiffers *et al.*, 1987; Anton-Culver *et al.*, 1992) and there is a strong dose-response relationship (Akdas *et al.*, 1990).

Epidemiological evidence implicates tobacco at both early and late stages of bladder carcinogenesis. While ex-smokers retain a risk about 50% higher than never smokers, there is an approximately 20% reduction of risk, two to four years after cessation of smoking (Hartage *et al.*, 1987).

Continued smoking following diagnosis of bladder cancer is a significant clinical problem. Russo *et al.* (1996) reported that approximately 50% of patients who were smoking at the time of their TCC diagnosis, continued to smoke despite the diagnosis of tobacco-associated neoplasm, although data in literature of other smoking-associated tumors have shown that smoking cessation is beneficial in reducing second primaries (Silverman *et al.*, 1983; Richardson *et al.*, 1993). No published studies have shown that smoking cessation, prior to or following diagnosis alters the behaviour of superficial bladder carcinoma (Fleshner *et al.*, 1999). Momas *et al.* (1994) suggested that black tobacco may be more harmful than blend tobacco and may have an early non-reversible role in bladder carcinogenesis.

The mechanism for the relationship between smoking and bladder cancer risk has not yet been fully elucidated, but most likely is due to the presence in cigarette smoke of low levels of some carcinogenic arylamines known to induce bladder cancer in occupational settings (Taylor *et al.*, 1998). These arylamines undergo metabolic transformation and the activated form can form adducts with DNA, a probable essential step in the process of carcinogenesis (Ross *et al.*, 1996).

Aromatic amines contaminate the ambient of environmental tobacco smoke. There is an increasing evidence that the excess of bladder cancer in smokers is attributable to aromatic amines rather than to other contaminants of tobacco smoke such as polycyclic aromatic hydrocarbons (PAH). A modulating

role in the risk of bladder cancer associated with exposure to aromatic amines is played by metabolic polymorphisms, such as the N-acetyl transferase, raising important social and ethical issues (Vineis and Priastu, 1997).

Certain aromatic amines carcinogenic for the human urinary bladder, such as 4-aminobiphenyl, undergo hepatic metabolic activation to N-hydroxylamines which, are transported to the bladder (Shipper and Tannenbaum, 1994). During the transport process, these reactive species come in contact with hemoglobin and react with this blood protein. The principle hemoglobin adduct formed is a cysteine sulfonamide and qualitative methods have been developed for the analysis of sulfonamide adducts at the levels present in ordinary human blood specimens. N-acetylation is an alterative metabolic fate to N-hydroxylation. The amount of hemoglobin adduct is decreased to the extent that this pathway is increased relative to N-hydroxylation. So the hemoglobin adduct is sensitive to dose, cytochrome p-450-mediated activation and N-acetyltransferase-mediated detoxification. In addition, it has been shown that DNA adduct concentration of 4-aminobiphenyl present in human bladder epithelial cells is significantly associated with hemoglobin adduct levels. Thus, the hemoglobin adduct of 4-aminobiphenyl and perhaps several other aromatic amines, is a good dosimeter for the target tissue dose of the ultimate carcinogenic metabolite of these amines. The results of some studies indicate that the hemoglobin adduct of 4-aminobiphenyl is closely associated with three major risk factors for bladder cancer: cigarette smoking, type of tobacco smoked and acetylator phenotype. They also support a major etiologic role of aromatic amines in human bladder cancers (Shipper and Tannenbaum, 1994).

Molecular studies have shown that chromosome 9 alterations and tissue *p53* mutations are the most frequent events in bladder cancer. Zhang *et al.* (1997) suggested a link between smoking and chromosome 9 alterations in the etiology of bladder cancer. This indicated that potential tumor suppressor genes on chromosome 9 may be involved in smoking related bladder carcinogenesis. Genetic alterations in chromosome 17p and *p53* mutations also appear to occur more frequently in high grade and invasive bladder tumors. In fact cigarette smoking may possibly be a determination factor of mutations of the *p53* gene in bladder cancer (Uchida *et al.*, 1995).

The data from lung and bladder cancers suggest an increasing proportion of patients with *p53* mutations in non-smokers, former smoker and current smokers in that order in both cancer groups. Taken together, more than half (55 and 65% for lung and bladder cancers respectively) of the patients who continued smoking (CS), less than 40% of those who had stopped smoking (before 1–5 years) of clinical diagnosis (ES) and less than 30% (25% and 29%) of those who were non-smokers (NS) had *p53* mutations (Husgafel and Kannio, 1996). Zhang *et al.* (1994) observed a significant association between the number of cigarettes smoked per day and *p53* overexpression ($p = 0.02$). These data also support the hypothesis that certain carcinogens derived from cigarette smoking and occupations may include T *p53* mutation, which in turn are involved in early steps of bladder carcinogenesis. Pacchioni *et al.* (1997) could find the expression of the *bcl-2* gene in only 2 out of 13 *p53* positive smokers.

(b) Occupational Factors

Various industrial workers are exposed to a number of aromatic amines and they become more susceptible to urothelial cancers. The responsible amines include Benzidine, 2-Naphthylamine, 1-Naphthylamine and 4-aminobiphenyl. Several other chemically related compounds have also been associated with urothelial cancers and the carcinogenicity of these compounds is supported by experimental studies (Wallace, 1988).

Many epidemiological studies carried out during the last two decades have identified certain occupations with an excess of bladder cancer. If it is assumed that there is a causal relationship

between the work and the development of bladder cancer, then 8–20% of bladder cancers can be attributed to occupational exposure (Cole, 1973; Cartwright, 1982; Vineis and Simonato, 1986). In two plants in the USA that manufactured 4-aminobiphenyl, 16.1 and 18.5% of the workforce developed bladder cancer (Melick *et al.*, 1971) and 26.2% of a work force of 366 male employees developed bladder cancer after exposure to 1-Naphthylamine, 2-Naphthylamine and Benzidine in a coal tar dye factory (Goldwater *et al.*, 1965).

(b-1) The latent period: The studies of Case and Pearson (1954) showed that the mean latent period for urothelial tumors in the chemical industry was 18 years from first exposure to 1-Naphthylamine, 2-Naphthylamine and Benzidine. The range was noted to extend to 45 years and with further time, the mean latent period was increased as more cases of urothelial cancer had occurred. The mean latent period for tumors in the rubber and chemical industry was 25 years in 1982. Vineis *et al.* (1982) observed that the consequences of this long latent period are far ranging and is one of the major difficulties with occupational urothelial cancer today. After such a long interval of time, it becomes increasingly difficult to establish what exposure did occur to the patients as the factory might have been closed down, the records have been lost or destroyed and memories fade and become unreliable. In addition, most patients will be beyond retirement age and may not be motivated to go through complicated and protected proceeding (Wallace, 1988).

(b-2) High Risk Occupations: Epidemiological studies in the last two decades have shown that there exist excess risk of bladder cancer in the occupations listed in Table 1.

Table 1. Occupations where epidemiological studies showed an increased relative risk for bladder cancer in the last two decades

Chemical manufacture
Leather work
Rubber industry
Printing industry
Machinists
Truck drivers and diesel exhaust exposure
Painters
Furnace men
Aluminium refining
Textile industry

(b-2.1) Chemical manufacture (Dye-stuff workers): The occupational bladder cancer was first reported in chemical industry in the late 1940s by Case and Pearson (1954). The risks have still not been eliminated from this industry particularly in those involved in the manufacture of benzidine based dyes. In England, the relative risk for dye manufacturers was 3.5 (Cartwright, 1982) and 3 in Italy (Vineis *et al.*, 1982). A case control study has suggested that the risk in the chemical industry in England had peaked and that the calendar years during which exposure had occurred were the most important (Boyko *et al.*, 1985).

The analysis of industrial activities in which cases and controls were engaged suggests that very high risk of bladder cancer is present in the province of Torino for dyestuff workers. This confirmed previous indications of trade unions and of a follow-up study (Rubino *et al.*, 1982). Benzidine can be used to synthesize 254 dyes or pigments in addition to its use in the detection of blood. It is used as

a hardener for plastic and rubber, in security printing and in organic and inorganic chemistry (IARC, 1982). The dyes can be absorbed through the skin and excreted in the urine. They can be broken down to the parent benzidine by the action of gut bacteria (Rinde and Troll, 1975).

(b-2.2) Leather workers: The leather industry has been suggested to be a high-risk activity, particularly in finishing processes by Cole *et al.* (1972) in USA, Decoufle (1979), Howe *et al.* (1980) in Canada, Cartwright (1982) in England and Vineis and Magnani (1985) in Italy.

(b-2.3) Rubber industry: According to studies in England (Baxter and Werner, 1980) and USA (Monson and Fine, 1978; Checkoway *et al.*, 1981) high risks have persisted in the rubber industry, particularly in the jobs of warehouse shipping, milling, calendering and assembling. The risk is concentrated in the production of rubber goods other than tires among workers in the age group 40–59 (Vineis and Magnani, 1985).

(b-2.4) Printing industry: The printing industry comprises three main sections: news print, general publishing and security printing. Induline and nigrosine dyes were used which might have contained 4-aminobiphenyl as impurity. These were withdrawn in 1976, but it is not clear who might have been exposed, where and when and what the level of exposure might have been (Case, 1983).

Benzidine has been used in security printing but the workforce exposed is small and the use of other compounds is difficult to assess for obvious reasons. Some workers have reported a positive risk among printers (Cartwright, 1982). A small study in the USA (Najem *et al.*, 1982) found correlation between the printing industry and bladder cancer by relative risk of 2.7 (0.8–9.6), whereas in Detroit Silverman *et al.* (1983a) reported an estimate of 3.0 (0.6–14.8). There is no evidence, however, that printers in different parts of the world share similar exposures. In addition, when incident cases were only considered, no evidence risk was indicated by Veneis and Magnani (1985).

(b-2.5) Machinists: The job title of tuner and more generally the category of machinists, has been associated with bladder cancer. Machine tunning involves the use of oils as lubricants which contain certain additives. Some of these are aromatic amines such as PAN and BPN which may have contained 2-naphthylamine as impurity (Vineis *et al.*, 1982). Nitrates have also been added to stop corrosion and these may be combining with the amines in the heat of the process to form nitrosamines. Relative risks were 4.0 in the USA (Dunham *et al.*, 1968), 4.8 in England (Anthony and Thomas, 1970), 2.7 in Canada (Howe *et al.*, 1980) 5.0 in Finland (Cartwright, 1982) and 1.5 in the UK (Cartwright, 1982). In addition, Silverman *et al.* (1983) found an increased risk both for machinists in the car industry and for a related category of tool and dye makers. Veneis and Magnani (1985) reported a relative risk of 3.1 for machine tuner, mainly for those employed before 1940 with more than 10 years exposure.

(b-2.6) Truck drivers: Silverman *et al.* (1983a) and before them, Decoufle *et al.* (1977) and Milham (1983) had found increased relative risk of bladder cancer to truck drivers. Four studies have shown a small risk for road transport workers, especially drivers of diesel engine vehicles with relative risks of 1.33 to 2.2 (Hoar and Hoover, 1985; Smith *et al.*, 1985; Baxter and McDowell, 1986; Silverman *et al.*, 1986). A case-control study by Wynder *et al.* (1985), however, has not shown an increase in bladder cancer in occupations with exposure to diesel exhausts in six American cities. Motor exhaust emissions, especially from diesel engines contain polycyclic aromatic hydrocarbons which are mutagenic and genotoxic (Rosenkranz and Mermelstein, 1983, Sternberg *et al.*, 1983). The public health importance

of these findings is enormous in considering the ubiquitous nature of diesel exhaust exposure, but this in itself makes conclusive studies very difficult. Silverman *et al.* (1986) estimated that 4% of bladder cancer in the USA could be attributed to employment as a truck driver.

(b-2.7) Painters: For painters, the evidence in the literature points basically to a lack of association. Cole *et al.* (1972) have reported cases/controls and age adjusted relative risk (RR) at 1.4, Anthony and Thomas (1970) an RR lower than 1.0; Howe *et al.* (1980) an estimation of 1.0 for commercial painting and 1.8 for spray painting and Silverman *et al.* (1983a) estimated 1.0 for painters in general and of 0.5 for painters in the car industry. Vineis and Magnani (1985) suggested an increased risk for car painters which, however, is not statistically significant.

(b-2.8) Furnacemen: Vineis and Magnani (1985) suggested an association between bladder cancer and brick production and other activities related to the transformation of non-metallic minerals. This is in consonance with the finding of the Roswell Park Memorial Institute on furnacemen, founders and pourers (RR = 2.4, based on 7 cases). Both this category and truck drivers are heavily exposed to polycyclic aromatic hydrocarbons. It has been suggested that such exposures could be shared with other occupations also showing an increased risk of bladder cancer. These include gas workers (Doll *et al.*, 1972), roofers (Hammond *et al.*, 1976) and aluminum workers (Theriault *et al.*, 1984). A small outbreak of bladder cancer had occurred in the production of aluminium in Canada (Gibbs, 1981; Theriault *et al.*, 1981). This risk is in the primary production of aluminium by electrolysis, where carcinogens may be in the fumes and there is a clear relationship with the years of exposure.

(b-2.9) Textile industry: The textile industry has been suggested by Anthony and Thomas (1970) to entail a high risk of bladder cancer. Cartwright (1982) has reported a relative risk of 1.3 for dye users in the wool industry. For tailors, Howe *et al.* (1980) observed relative risks of 1.5 in males and of 2.0 in females.

(c) Coffee Drinking

Coffee is a complex substance whose chemical composition in raw or processed form is not fully known (Sivetz and Desrosier, 1979). The green (unroasted) coffee bean contains caffeine (1–2 per cent), coffee oil (10–15 per cent), sugars (8 per cent), proteins (11 per cent), ash (5 per cent) and chlorogenic and caffeic acid (6 per cent) (The Merck Index, 1976). The remaining constituents are other carbohydrates (starches, celluloses *etc.*) At the high temperatures attained during the roasting process, some chemical changes do occur within the bean.

It is possible that coffee contains some polycyclic aromatic hydrocarbons generated during roasting. Kurassune and Hueper (1958) demonstrated the presence of benzo (a) pyrene, a known carcinogen in coffee soot. Subsequently, Hueper and Payne (1960) showed that coffee soot produces bladder neoplasia in guinea pigs. Small amounts of benzo (a) pyrene have been found in roasted coffee (IARC, 1973). Additionally, coffee soot was at one time used to extract caffeine from coffee (Kuratsune and Hueper, 1958). Caffeine is the only individual constituent of coffee that has received any attention as a possible carcinogenic agent.

In 1971, Cole first reported an excessive risk of bladder tumors among coffee drinkers (one or more cups per day). The effect was stronger in females than in males. Since then, many studies have shown a positive relationship between coffee drinking and bladder cancer (particularly in males) both independently or after adjustments for age and smoking (Wynder and Goldsmith, 1977; Bonham,

1979; Howe *et al.*, 1980; Marret *et al.*, 1983; Weinberg *et al.*, 1983). The regular, instant decaffeinated coffee slightly increased the risk of bladder cancer (Howe *et al.*, 1980).

In 1976, an estimated 80 per cent of the U.S. adult population was of current coffee drinkers (Bonham, 1979). Since there were additional persons who were former or "non-current" drinkers, an even higher proportion might be thought to have been exposed to coffee during their lifetime. In addition, over 20 per cent of the current consumers reported usually drinking five or more cups per day (Bonham, 1979). Thus, it was important to continue to investigate the possibility of a causal relationship between coffee consumption and bladder cancer. Table 2 summarizes major features of some of these studies which have examined this association and achieved high rates of response (Cole, 1971; Schmauz and Cole, 1974; Howe *et al.*, 1980; Morrison *et al.*, 1982; Marrett *et al.*, 1983).

Table 2 Selected features of four population-based studies examining the association between coffee drinking and bladder cancer (Marrett *et al.*, 1983)

Study	Location	Year of diagnosis	Cases	Controls	Control selection	%controls <1 cup/day (Male/Females)
Cole (1971)	Boston and Brockton, MA	1967–1968	468	498	Random sample of residents lists approximately age and sex-matched	12–6 22–9
Schmauz and Cole (1978)	(SMSAs)					
Howe <i>et al.</i> (1980)	3 Provinces of Canada	1974–76	632	632	Neighborhood controls one to one age and sex matched	13.8 13.2
Morrison <i>et al.</i> (1982)	Boston, MA	1976–1977	587	528	Random sample of residents list approximately age and sex matched	5.9 12.2
Marrett <i>et al.</i> (1983)	Connecticut	1978–1979	412	881	Aged 21–64 random digit dialing; aged 65–84; random sample of HCFA files; approximately age and sex matched replaced	9.5 9.9

Piper *et al.* (1986) and Donato *et al.* (1977) suggested that increased levels of coffee consumption may be associated with an increased risk of bladder cancer, while Stenvold and Jacobsen (1994) reported on the contrary that there was no association between coffee consumption and overall cancer and with particular significance to that of pancreas and bladder. Similarly Bruemmer *et al.* (1977) and Probert *et al.* (1998) found no significant association with coffee and tea consumption to bladder cancer. Akdas *et al.* (1990) found a significant difference between the case and control groups on Turkish coffee drinking ($p < 0.01$). This was more pronounced in people who drank more than 2 cups/day for more than 20 years ($p < 0.01$). This indicated that Turkish coffee might play a direct or indirect role in the etiology of bladder cancer.

(d) Alcohol Consumption

Several studies have indicated that there exists relationship between consumption of alcohol and

bladder cancer, but reports on inverse association are also available. Murta *et al.* (1996) found a synergistic relationship with alcohol consumption, independent of tobacco smoking for upper aerodigestive tract and bladder cancer. The risk of bladder cancer for alcohol and tobacco users was statistically significant ($p < 0.001$). The risk increased with the quantity of alcohol consumed and people who had taken more than 175 cm³ of a strong drink (containing equal or more than 40% distilled alcohol) per day ($p < 0.001$). Frequency of alcohol drinking is another independent variable that influences the risk. People who drank every day were at the highest risk ($p < 0.001$). The duration of alcohol consumption was positively correlated with the increasing risk of bladder cancer (Akdas *et al.*, 1990). Subjects with an alcohol-drinking history of 11–20 years were at risk ($p < 0.01$) and those who drank longer than 20 years, were at higher risk ($p < 0.001$). The case control ratio of non-drinkers to ever drinkers was 1.67 (Schiffers *et al.*, 1987). Other results suggested that regular consumption of alcohol can be independently associated with an increased bladder cancer risk (Donato *et al.*, 1977; Mommsen *et al.*, 1983a). Probert *et al.* (1998) found significant association of occupational exposure, cigarette smoking and beer consumption (but not wine and spirits). Also important is the increased risk when alcohol consumption was adjusted to smoking. Since many people smoke cigarettes with alcohol, this may be a confounding factor for it can act as a promoter in the development of bladder cancer (Schiffers *et al.*, 1987). Most of the studies have not found association between the incidence of bladder cancer and intake of alcohol (Bruemmer *et al.*, 1997; Chatenoud *et al.*, 1998).

(e) Nutrition

It has been documented that high intake of vegetables and fruits reduces the risk of cancer of mouth, pharynx, larynx, oesophagus, lung, stomach, pancreas, breast and bladder (Biomhoff, 1988). High cruciferous vegetable consumption may reduce bladder cancer risk, but other vegetables and fruits may not confer appreciable benefits against this cancer (Michaud *et al.*, 1999a; La Vecchia and Negri, 1996). A diet rich in fresh fruit and vegetables and hence, possibly in carotenoids reduces bladder risk (La Vecchia and Nagri, 1996). Pohlabeln *et al.* (1999) found the protective effects and risk reductions of approximately 50% by the intake of raw carrots, salad and fruits.

Case-control studies have reported an increased association between bladder cancer and vitamin C (Patterson *et al.*, 1997) and the total fluid intake was inversely associated with the risk of bladder cancer (Michaud *et al.*, 1999b).

Bladder cancer risk is weakly associated with the duration of exposure of population to municipal water (Freedman *et al.*, 1997). According to Bruemmer *et al.* (1997), there is no association between the incidence of bladder cancer and intake of water or liquids from tap. This study suggested that the intake of water and specific beverage in overall is not associated with this risk of bladder cancer. There is, thus, limited evidence of an association between total fluid intake and bladder cancer among women. Calcium and sodium may be related to bladder cancer risk.

On the other hand, frequent consumption of meat increases the risk of colorectal cancer and possibly also of breast, stomach, pancreas and urinary bladder (Stog *et al.*, 1995). There has been a long debate on the carcinogenicity of artificial sweeteners. The use of artificial sweeteners either in beverages or as table top sweeteners was not associated with an increased risk of bladder cancer. This finding is in contrast to another study which indicated excess exposure restricted to low-risk in women (defined as non-smoking, non-occupationally exposed women) (Hoover and Stasser, 1980). While How *et al.* (1980) reported an increased risk for males, Mommsen *et al.* (1983) observed its risk for females. Other studies have failed to show such relationship (Wynder and Goldsmith, 1977; Kessler and Clark, 1978; Moller-Jensen *et al.*, 1983). Similarly Jensen and Kamby (1982) showed that exposure to saccharine did not increase the risk of human bladder cancer. Although Akdas *et al.*

(1990) found that artificial sweetners may be a causative factor for bladder cancer, the figures were small and this may be just by chance.

(f) Other Factors

Specific chemicals have also been considered as risk for causing bladder cancer, as occupational exposures to less well defined specific agents. Treatment with cytostatic drugs, especially cyclophosphamide is associated with increased risk of bladder cancer, as is treatment with radiotherapy for uterine cancer (Johansson *et al.*, 1997). Piper *et al.* (1986) found that bladder cancer in young women is associated with heavy use of phenacetin-containing drugs. The association between urinary tract cancers and phenacetin is biologically plausible since the kidney has the capacity to generate a potent nephrotoxin from a benign precursor which is a metabolite of phenacetin (Carpenter and Mudge, 1981). The fact that this metabolic reaction takes place in the kidney may explain why tumors are mainly found there rather than in the bladder. Twenty six per cent of women who regularly consumed phenacetin had bladder cancer in New South Wales (McCredie *et al.* 1983) (no data are available for men).

In developing countries, especially in the middle east and parts of Africa, infections and members of the genus *Schistosoma* are responsible for a high incidence of bladder cancer, 75% of which are squamous cell carcinomas (Johansson *et al.*, 1997). Bladder cancer in Egypt has been associated with chronic infection with the parasite *Schistosoma haematobium* (Bilharziasis), which is an endemic disease in that country (Aly and Khaled, 1999).

4. Conclusions

Bladder cancer is the fourth most common malignancies in the western male population and tenth in women and represents 3% of cancer deaths.

It has been found that personal habits, such as cigarette smoking, occupational factors (exogenous carcinogens), such as chemical substances, coffee drinking, alcohol consumption, nutritional factors, treatment with cytostatic drugs, especially cyclophosphamide and chronic infection with the parasite *Schistosoma haematobium* may play a direct or indirect role in the etiology of bladder cancer. These factors may be directly or indirectly responsible for causing mutations in various onco/anti-oncogens. It therefore, appears that continued studies may lead to finding out factors that may reduce the frequency of bladder cancer.

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20. Molecular Genetics of Prostate Cancer

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Abstract: The formation of histological prostate cancer appears to be very frequent, occurring in one third of the men older than age 45. Geographically, this incidence is roughly the same world wide. The number of clinical cancers, however, differs widely among various populations, suggesting important environmental factors in triggering the progression. On the other hand, studies of familial aggregation of prostate cancer have suggested the existence of an autosomal dominant allele that predisposes men to develop prostate cancer independent of environmental exposure.

The prostatic carcinogenesis and progression are regulated by both the activation of genes stimulating cellular growth and tumor progression (oncogenes and metastasis genes) and the inactivation of genes inhibiting these processes (tumor and metastasis—suppressor genes). Identification of these molecules and characterization of the mechanisms for their contribution to tumor formation and progression are the important areas of research because they can generate information for the improvement of diagnosis and therapy of prostate cancer.

Key words: Genes, Prostate Cancer

1. Introduction

Prostate is a complex organ with distinguishable anatomical and functional areas and composed of cells of different embryonic origin (Mc Neal *et al.*, 1988; Mc Neal, 1992). Prostate cancer is most common in Western men and is second only to lung cancer as a cause of deaths due to cancer (Van der Gulden *et al.*, 1994; Parker *et al.*, 1996).

Prostate cancer is characterized by an unpredictable clinical behaviour. Some tumors remain indolent for many years, whereas others progress rapidly to incurable metastatic disease (Schalken, 1997). While some patients die within 1–2 years after diagnosis, others presenting with ‘Latent’ tumors have no symptoms of prostate cancer during their life time. These clinical observations indicate the highly variable biological potential of prostate cancer. In 1998, 184,500 new cases and 41,800 deaths from prostate cancer in U.S. were estimated (American Cancer Society, 1998), comprising 29% of diagnosed cancer in men. With the westernization of dietary habits in Japan, the prostate cancer associated mortality has also increased in recent years (Tominaga and Kuroishi, 1997).

The overall life time probability of developing clinically evident prostate cancer is much lower than its prevalence at autopsy by the age of 80 years (16% vs 80%). With the increasing incidence of prostate cancer in the aging population, there is an urgent need for the identification of molecular markers that can serve as prognostic indicators (Cheng *et al.*, 1996).

Despite its high incidence and mortality rate, the exact molecular mechanisms underlying the tumorigenesis and progression of prostate cancer are still not clear (Latil *et al.*, 1997). More than 95% of prostate cancers are adenocarcinomas (Paulson, 1987; Stamey and Kabalin, 1989). The ability of prostate cancer to invade and to metastasize is high (Muss *et al.*, 1949; Eagan *et al.*, 1976; Chlebowksi *et al.*, 1978; Rubben and Altwein, 1987 ; Voogt *et al.*, 1989).

Prostate carcinomas display a high degree of biological diversity and can be present as localized disease within the prostate or become highly invasive and metastasize to regional lymph nodes and bone (Cussenot *et al.*, 1998). Although localized prostate carcinoma can be successfully treated (Gittes, 1991), the treatment success diminishes significantly when prostate tumor cells metastasize beyond the confines of the gland, mainly through perineural and stromal invasion (Villers *et al.*, 1989; Mc Neal *et al.*, 1990; Knox *et al.*, 1994). Several proto-oncogenes and tumor suppressor genes have been implicated in prostatic tumorigenesis.

(A) Oncogenes

Tumor Suppressor Genes

- (i) **p53** (Abnormalities of the *p53* tumor-suppressor gene are currently the most common genetic alterations associated with human malignancy. Although studies have demonstrated a much lower incidence of *p53* alteration in prostate cancer compared with other cancers, the association of *p53* mutations and aberrant protein expression with advanced stage of prostate cancer has been shown in a series of studies (Kubota *et al.*, 1995). In both primary and metastatic cancers, *p53* alterations play a role in a subset of advanced stages of prostate cancer (Brooks *et al.*, 1996).
- (ii) **Retinoblastoma gene Rb1** is the second most commonly altered tumor-suppressor gene in a variety of cancers. Mutations in *Rb* in prostate cancer are rare (Konishi *et al.*, 1996). It has been demonstrated that abnormal Rb protein expression has little prognostic significance in prostate cancer (Vasalainen and Lipponen; 1995).

(B) Other Molecules

Bcl-2

Bcl-2 is an oncogene that functions by overriding apoptosis. It is normally expressed in basal cells of the prostatic glandular epithelium. The presence of *bcl-2* staining of the tumor is highly correlated with the progression of prostate cancer and the androgen-independent phenotype, suggesting that the expression of *bcl-2* protein in prostate cancer is not a primary molecular event but rather is a secondary event associated with tumor progression and androgen independent. The *Bcl-2* protein appears to be an important biomarker that predicts recurrence in clinically localized prostate cancer (Dong *et al.*, 1997).

Androgen Receptor

Androgen receptor (AR) is the mediator of androgen to maintain the growth of both normal and cancerous prostate. It is also required for the progression of prostate cancer to a metastatic status, as its expression is visible in most metastasis of endocrine untreated tumors (Hobisch *et al.*, 1996). Lack of AR expression correlates with endocrine therapy resistance and poor survival of patients. Androgen deprivation remains the primary therapy for patients with metastatic prostate cancer and initial

response to hormone therapy is variable and often followed by the emergence of hormone-refractory disease (Long *et al.*, 1997).

Transforming growth factor- β and its receptors

Transforming growth factor- β is a potent inhibitor of epithelial cell growth. The effect of TGF- β is primarily mediated by a heteromeric complex of two kinases called receptor I and II. They are modulated by androgen growth factors and extracellular matrix (Kim *et al.*, 1996). Inactivation of either receptor can result in TGF- β resistance, a common and important step in human cancer development (Polyak, 1996). In normal prostatic tissue, both type I and II receptors are located in epithelial cells. A decline in the level of type I and II receptors is correlated with advancing histological aggressiveness of prostate cancer. Advanced prostate carcinomas become insensitive to the inhibitory effect of TGF- β because of the defect in its receptor (Kim *et al.*, 1996).

(C) Cell-cell and Cell-extracellular Matrix Interacting Molecules

1. C-CAM

Epithelial-cell adhesion molecules can be regulated by androgens and may act as growth repressors during differentiation of the prostatic epithelium. In human prostate cells, expression of C-CAM by transfection, reduced anchorage-independent growth *in vitro* and suppressed tumor formation *in vivo* (Hisieh *et al.*, 1995). Expression of C-CAM in cells transfected with *c-cam* containing adenovirus construct lasts quite long, suggesting that *c-cam* is a potential candidate for human prostate cancer therapy.

2. CD 44

CD44 is a transmembrane protein associated with cell-cell and cell-extracellular matrix interactions. Expression of both standard variant isoforms of CD44 proteins has been associated with aggressive behaviour and metastasis in various tumors. In one study, down regulation of CD44 standard form occurred in 70% of 109 prostate cancers and this reduction correlated with high tumor grade and aneuploid status. The CD44 isoforms was lost in 98% of the prostate cancers. The metastatic prostate cancers did not show preferential expression of either the standard form or any variant isoform (Jung *et al.*, 1996). Soluble CD44 isoform concentration was lower in the serum of prostate cancer and benign prostate hyperplasia patients compared with normal people (Jung *et al.*, 1996). However, several other studies demonstrated that increased expression of CD44 associates with higher tumor grade (Zhang *et al.*, 1996) and metastasis of prostate cancer (Bourguignon *et al.*, 1995). Functionally, a neutralizing antibody to CD44-inhibited cell proliferation and basement membrane invasion (Lokeshwar *et al.*, 1995). CD44 also mediates prostate cell adhesion to extracellular hyaluronic acid, suggesting the role of CD44 in prostate cancer growth and metastasis.

3. Integrins

Integrins are a large family of heterodimeric integral plasma membrane receptors that mediate cell-cell and cell-extracellular matrix interactions. Such interactions are involved in tumor growth and metastasis. In prostate cancer, reduction in the expression of most subunits of various integrins occurs during the progression of cancers (Cress *et al.*, 1995). Expression of the integrins $\alpha 2$, $\alpha 4$, $\alpha 5$, $\alpha 6$ and $\beta 4$ is lost in carcinoma and higher expression of $\alpha 6$ is associated with higher invasive activity (Nagle *et al.*, 1995). The $\alpha 6\beta 4$ is associated with hemidesmosome-like structures. The $\alpha 6\beta 1$ integrin mediates the adhesion of prostate cancer cells to bone matrix (Kostenuik *et al.*, 1996).

4. KAI1

KAI1 was identified for its ability to suppress metastasis of rat prostate cancer cells (Dong *et al.*, 1995). KAI1 protein is expressed by epithelial cells of human prostate, whereas its protein expression is downregulated during the progression of human prostate cancer (Dong *et al.*, 1996). Large scale studies are required to evaluate if KAI1 can be used as a marker for metastatic ability of prostate cancer cells.

5. E-cadherin

Cadherins are calcium dependent cell-cell adhesion molecules, and thus they are involved in the formation and maintenance of the histoarchitecture. Their activity is regulated by cytoplasmic interaction between cadherin, catenins and the actin-based cytoskeleton.

Cell-cell adhesion in tissues is mainly regulated by homotypic interaction of cadherin molecules, which are anchored to the cytoskeleton via cytoplasmic proteins including α , β , and λ catenins. Cadherin–catenin cell-cell adhesion complexes are believed to play a predominant role in carcinomas. Cell–cell adhesiveness is generally reduced in human cancers. Aberrant expression of these proteins has been found in many different human carcinomas. Alteration in the expression or function of molecules that affect cellular adhesion and proliferation are thought to be critical events for tumor progression. In general, inactivating mutations of the human E-cadherin gene are rare. In several cancers, including prostate cancer, loss of E-cadherin expression is correlated with a loss of the epithelial phenotype and gain of invasiveness.

Tumor cells are dissociated throughout the entire tumor mass in diffused type cancers, whereas the solid tumors with high metastatic potentials are often focally dissociated or differentiated at the invading fronts. Thus, both irreversible and reversible mechanisms for inactivating the cell adhesion system appear to exist.

Cadherin adhesion system is central in organizing epithelial structures and that tyrosine phosphorylation of catenins may modulate this organization process. Phosphorylation of β -catenin, possibly through interaction with EGFR and *c-erb B-2*, is thought to induce a disassembly of the E-cadherin–catenin complex from the actin filament network thus disruption of cell adhesion may in turn potentiate the neoplastic process (Shibamoto *et al.*, 1994). Epithelial cell layers exhibit an ordered polarized architecture. Such structures are however, disrupted during malignant transformation, which generally coincides with a loss to regulate cell growth.

The demonstration of an APC- β -catenin interaction raised much speculation as to the role of APC in regulating signal transduction through the β -catenin interaction with the cytoskeleton, thereby regulating some aspects of cell adhesion or cell growth (Rubinfeld *et al.*, 1993; Vogelstein and Clevers, 1993).

APC has been shown to be important in regulating cytoplasmic β -catenin level (Munemitsu *et al.*, 1995). The APC- β -catenin complex is translocated into the nucleus where it interacts with the nuclear transcription factors LEF-1 and hTCF-4 resulting in transcriptional activation. Truncation of the carboxy-terminal domain of the wild-type APC protein in tumor containing *apc* gene mutations results in accumulation of cytoplasmic β -catenin, which is thought to contribute to the inappropriate activation of target genes by the β -catenin-LEF-TCF complex (Munemitsu *et al.*, 1995).

Gamma–catenin (82KDa) bears homology to β -catenin and is identical to desmosomal plakoglobin (Jawhai *et al.*, 1999). Gamma–catenin is a member of adhesion system through binding to E-cadherin. Not much is known about the catenin.

In addition to β and γ -catenins, $p120^{\text{ctn}}$ is another member of the catenin family (Daniel and Reynolds, 1995). It is phosphorylated by pp60^{V-Src}, a tyrosine kinase known to be associated with the

adherens junctions (Volberg *et al.*, 1992). It induces disruption of adherens junctions. The observations of Downing and Reynolds (1991) have suggested a role for p120^{ctn} in cell transformation and ligand-induced signaling (Daniel and Reynolds, 1995). There are at least four p120^{ctn} isoforms, which are thought to be generated by alternative splicing at its carboxy- and NH₂-terminal ends (Reynolds *et al.*, 1994; Mo and Reynolds, 1996; Keirsebilck *et al.*, 1998). The existence of multiple p120^{ctn} isoforms is thought to represent a means for modulating E-cadherin functions by selective expression (Staddon *et al.*, 1995). As p120^{ctn} modulates cadherin adhesion, the differences in isoform expression may be important in the regulation of adhesion and metastatic potential of tumors. Reynolds *et al.* (1996) demonstrated that p120^{ctn} was associated with all classical cadherin subtypes, and that overexpression of the p120 isoform in fibroblasts leads to change in cellular morphology with development of dendrite-like extension, with p120 isoform localized in these extensions. The tryrosic kinase substrated p120(cas), which is structurally similar to cell adhesion proteins β -catenin and γ -catenin/plakoglobin, has an arm domain that consists of 10–13 repeats of a 42-amino acid motif studied in the *Drosophila armadillo* protein. The p120^{ctn} binding site in E-cadherin is different from the β -catenin/plakoglobin binding site and it does not bind to α -catenin (Daniel and Reynolds, 1995; Jou *et al.*, 1995).

The first studies to examine E-cadherin in prostate cancer were performed by Bussemakers *et al.* (1992) in the Dunning rat prostate adenocarcinoma cell lines. These studies found a strong correlation between the lack of E-cadherin and metastatic and/or invasive potential. This correlation is strengthened by the direct observation of the progression of a non-invasive, E-cadherin positive tumor, to a E-cadherin negative, highly metastatic tumor.

Two other cadherin genes *i.e.*, M-and H-are located in the 16q24 region, and they are considered to be potentially involved in tumor metastasis (Kaupmann *et al.*, 1992; Lee *et al.*, 1996). In particular, reduction in H-cadherin expression has been observed frequently in breast cancer (Lee, 1996). Downregulation of catenins seems to be associated with dysfunction of the cadherin-mediated cell adhesion system involved in cancer invasion and metastasis (Kadowaki *et al.*, 1994; Matsui *et al.*, 1994; Ochiai *et al.*, 1994a; Pierceall *et al.*, 1995; Rimm *et al.*, 1995).

Morton *et al.* (1993) have shown that loss of normal E-cadherin function in prostate cancer lines can occur through mutational inactivation of the α -catenin. The loss of α -catenin gene may be casually related to impaired cadherin function (Hirano *et al.*, 1992). Genetic abnormality to β -catenin also is one of the mechanisms responsible for loosing cell-cell contact, and may be involved in enhancement of tumor invasion in human cancer.

Tyrosine phosphorylation of β -catenin regulated by c-erbB-2 protein may play an important role in the invasion, metastasis and morphogenesis of cancer cells, and that inhibition of the aberrant tyrosine phosphorylation of β -catenin effectively prevents invasion and metastasis of cancer cells. Loss of cell adhesiveness in cancer may be due to incomplete assembly of the cadherin-catenin complex at the junction. In addition, CpG methylation of the promoter region of E-cadherin is another possible cause of aberrant E-cadherin expression (Graff *et al.*, 1995).

A major mechanism leading to the decreased E-cadherin expression seems to be a decrease in transcription (Bussemakers *et al.*, 1993). Abnormalities in chromosomes 6, 7, 8, 9, 10, 11, 13, 16, 17 and 18 in prostate cancer have been frequently observed by LOH and CGH analyses (Suzuki *et al.*, 1996; Cher *et al.*, 1996). These results suggest that there are tumor suppressor or metastasis suppressor genes on these chromosomes that are involved in prostate carcinogenesis. In prostate cancer 54% of clinically localized and 100% of metastatic tumors showed allelic imbalances (AI) in at least one chromosome (Suzuki *et al.*, 1996).

Genetic imbalance of human chromosome 16q is most frequently and consistently observed in several cancers such as prostatic carcinoma (Carter *et al.*, 1990; Bergerheim *et al.*, 1991; Cher *et al.*,

1994; Phillips *et al.*, 1994; Sakr *et al.*, 1994) breast (Tsuda *et al.*, 1994), hepatocellular carcinoma and Wilms tumor. The results of CGH analysis indicate that > 55% of clinical samples of metastatic prostate tumors have deletions in the *q* arm of chromosome 16 (Cher *et al.*, 1996). Therefore, it has been hypothesized that there are tumor suppressor or metastasis suppressor genes on chromosome 16*q*.

Conclusion

Proportional hazard regression analysis showed that β -catenin, E-cadherin and α -catenin have strong predictive value, whereas γ -catenin/plakoglobin and *p120* CAS have a somewhat lower predictive value. Within patients with invasive tumors, those with a normal expression for either E-cadherin, α - or β catenin show a trend towards better survival, thus β , γ catenins and E-cadherin have similar prognostic values. The data suggest that these proteins are useful predictive markers for biological aggressiveness of prostate cancer.

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