Exp. Eye Res. (1992) **54**, 807–811

LETTER TO THE EDITORS

A Reassessment of Protein Synthesis by Lens Nuclear Fiber Cells

The natural history of the vertebrate lens includes many unusual differentiation events in the transition from epithelial to cortical fiber to nuclear fiber cell. One of the most dramatic phenomena is physical loss of the cell nucleus and most other organelles in the maturing fiber cell (Kuwabara, 1975). Yet, the fiber cells of the lens nucleus normally remain viable for the lifetime of the organism, maintaining their functional roles of optical transparency and refractility (Walls, 1942; Duke-Elder, 1958). The demonstration of protein synthetic capability for these cells would be a major biochemical index of this longevity, but the issue of protein synthesis there has been controversial at best (Spector and Kinoshita, 1964; Dilley and Van Heyningen, 1976). It has become generally accepted that this region of the lens is synthetically silent. However, Ozaki, Jap and Bloemendal (1985) have demonstrated by in vitro translation (using lens polysomes) and whole lens culture that there is [^{35}S]Met incorporation into adult bovine and human nuclear fiber cell proteins as revealed on one-dimensional SDS–PAGE fluorograms. In these fluorograms the polypeptide compositions of the nuclear profiles were essentially identical to those of the cortex with respect to the number of polypeptides, their M_r, and their proportionality to the total protein translated. This similarity included the presence of mid- to high-M_r polypeptides such as the cytoskeletal species actin and vimentin, and 68 and 92 kDa species. The material utilized in their study as nucleus consisted of a punched-out central cylinder of the lens (minus the polar ends of the punch) and represented approximately 25% of lens wet weight. The cortical material comprised the peripheral balance of the lens.

Our laboratory has been investigating the baseline levels of protein synthesis in various regions of the bovine lens in relation to the protein composition of the respective fresh lens regions. In doing so we have employed very stringent criteria different from those of Ozaki et al. (1985) for selecting nuclear and cortical samples. Our data indicate that there is a definable core of anucleate fiber cells containing poly(A)+ RNA. In contrast to findings of Ozaki et al. (1985), this purified mRNA is capable of translating a protein profile distinct from that of the nucleated cortical fiber cells. Moreover, this profile is comparable to the respective protein profile found in the fresh lens nucleus. Thus, the profiles of the nuclear regions (in vitro synthesized and fresh) comprise predominantly crystallin-M_r species (20–35 kDa), while the cortical profiles additionally include the cytoskeletal-M_r polypeptides (42–105 kDa). Lack of this distinction would imply the inclusion in both nuclear and cortical samples of transitional cortical fiber cells, leading to an homogenization of the two distinct polypeptide profiles and precluding determination of whether, in fact, there were any synthesis possible by the nuclear fiber cells.

Inasmuch as the size of the 'cold-precipitable' nuclear opacity [formed when the lens is placed at 4°C (Lasser and Balazs, 1972)] and the absence of cell nuclei are major delineators of the adult lens nucleus, these criteria were used in preparing the nuclei of adult bovine lenses for this study. After transporting the lenses from the abattoir on ice, the diameter of the 'cold' nuclear opacity was determined for each decapsulated lens (2 g wet weight). The cortex was then manually dissected in concentric layers down to the hard central core of fiber cells within the boundary of the 'cold' opacity. The resultant 6 mm diameter sphere weighed 0·32 g, representing 16% (by weight) of decapsulated lens fresh weight. Care was taken not to puncture the lens core with dissection instruments or otherwise to contaminate it with cortical material. The outermost 1-mm-thick layer of cortex was reserved for comparative studies. This comprised 32% (w/w) of fresh lens weight. These size and weight values were based upon determinations with 164 adult lenses.

Localization of cell nuclei within the lens was performed by indirect immunofluorescence microscopy. Methanol-fixed cryostat sections of the nuclear and cortical lens regions were incubated for 16 hr with a mouse monoclonal antibody to the nuclear lamina (generous gift from Dr R. D. Goldman) followed by a 2-hr incubation with FITC-conjugated goat anti-mouse secondary antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Methanol-fixed whole mounts of anterior capsule with attached epithelial cells were incubated for 30 min in each of the respective antibodies. Specimens were mounted in Fluoromount-6 (Fisher, Pittsburg, PA) and examined on a Zeiss Axiophot Photomicroscope with epi-fluorescence optics.

Total cellular RNA extraction was by the procedure of Chomczynski and Sacchi (1987) using 2 g each of liquid N_2-frozen cortex and nucleus. The final RNA pellet was suspended in sterile, diethylpyrocarbonate (DEPC; Sigma Chemical Co., St Louis, MO) treated water. In all subsequent steps involving RNA the solutions and utensils were DEPC treated and autoclaved (Blumberg, 1987). Poly(A)+ RNA was purified using poly(U) affinity paper (Hybond-mAP; Amersham, Arlington Heights, IL). In vitro translation

0014–4835/92/050807+05 $03.00/0

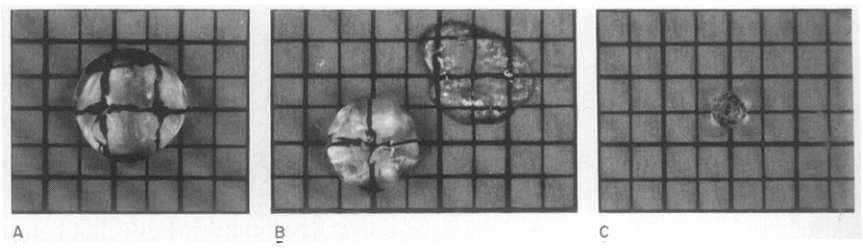

FIG. 1. Dissection of lens cortex and nucleus. A, Representative adult bovine lens following decapsulation. B, the same lens following removal of the approximately 1 mm thick, outermost layer of cortex; such a dissected cortex (shown to the side) comprised the source of cortical fiber cells. C, The dissected nucleus (approximately 6 mm in diameter) from the same lens as utilized in the study. Grid divisions are 5 mm.

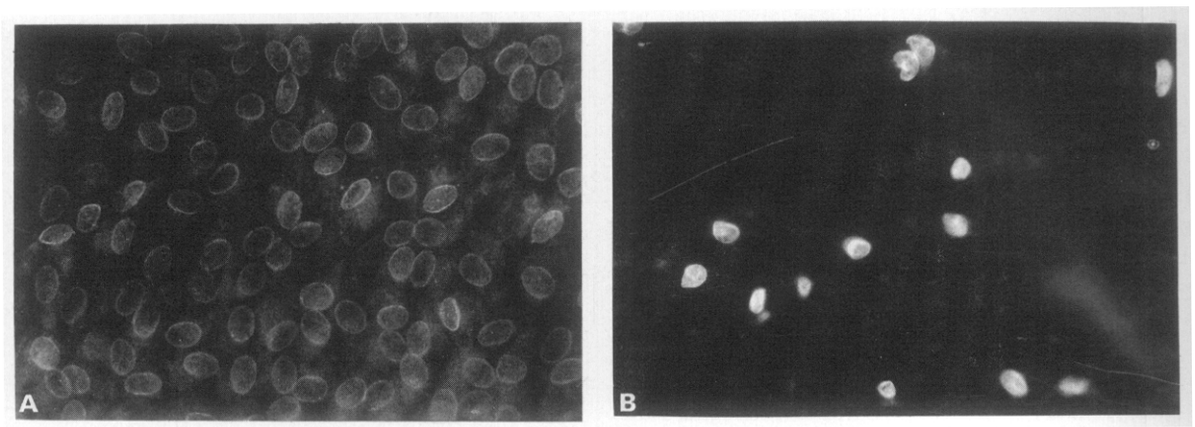

FIG. 2. Immunofluorescence microscopy to locate lens cell nuclei. A. Whole mount of lens epithelial cells on anterior capsule stained with monoclonal antibody to the nuclear lamina proteins. Cell nuclei were clearly identified. B, Cryostat section of mid-cortical lens region stained with the same antibody demonstrated cell nuclei as well. In contrast to the nuclei of the epithelial cells and superficial cortical fiber cells, these nuclei were in the process of pyknosis (note their smaller size and greater staining density). The fiber cells of the dissected lens core were unreactive with the antibody, confirming this region as the lens nucleus.

(IVT) was performed using a rabbit reticulocyte lysate system as per manufacturer's instructions (Promega Corp., Madison, WI). Fifty-microliter reaction aliquots containing 6·25 μl lens mRNA (0·16 μg μl^{-1}) and 30 μl of Tran-^{35}S-label (mixture of methionine and cysteine; ICN, Inc., Costa Mesa, CA) were incubated at 25°C for 2 hr. Positive controls comprised substitution with Brome mosaic virus (BMV) mRNA, while the negative control constituted the omission of all mRNA. The fresh tissue proteins of lens cortex and nucleus were prepared for comparison with the IVT products either by extraction of 1 g of each specimen with Tris-buffered 8 M urea/NP-40 (for two-dimensional PAGE) or by separation with Tris buffer (pH 7·4) into the water-soluble (crystallin) and -insoluble (cytoskeleton/membrane) fractions (for SDS–PAGE). Proteins were analysed by SDS–12% PAGE (Laemmli, 1970) and two-dimensional PAGE (O'Farrell, 1975) using pH 3–10 IEF gels and SDS–12% gels. Immunoblot identification of specific proteins was performed by semi-dry protein transfer onto Immobilon-P (Millipore, Bedford, MA) and reaction with rabbit antisera to column-purified vimentin, actin, proteins of the beaded-chain filaments (46 and 105 kDa), αA-crystallin, and γ-crystallin, followed by detection with peroxidase-conjugated goat secondary antibody. Fixed gels of the IVT products were equilibrated in the fluor sodium salicylate (Chamberlain, 1979) prior to fluorography on X-Omat film (Kodak, Rochester, NY) at −80°C for 17 hr (some nuclear IVT fluorograms were exposed for 72 hr for detection of any low-level labeling).

A representative dissection of the adult bovine lens to yield the cortical and nuclear regions as utilized in this study is shown in Fig. 1. Using the monoclonal antibody to nuclear lamins as a probe, immunofluorescence microscopy failed to reveal any cell nuclei (pyknotic or otherwise) within the region here utilized as the lens nucleus. In contrast, cell nuclei were readily demonstrated in the epithelial cells, the outermost layers of fiber cells here used as cortex, and in the mid-cortical region (Fig. 2). This method provided a definitive means for confirming that the 16% (w/w) core sample was representative of nuclear fiber cells in the bovine lenses studied here.

The incorporation of radiolabel into polypeptides as revealed by SDS–PAGE fluorography demonstrated the presence of translatable mRNA in the core fiber cells of the lens (Fig. 3). There was a marked difference in the overall synthetic profiles between cortex and nucleus inasmuch as the nuclear IVT profile comprised mainly crystallin-M_r species (20–35 kDa), while the cortical IVT additionally included higher M_r polypeptides (42–105 kDa) principally reflective of the

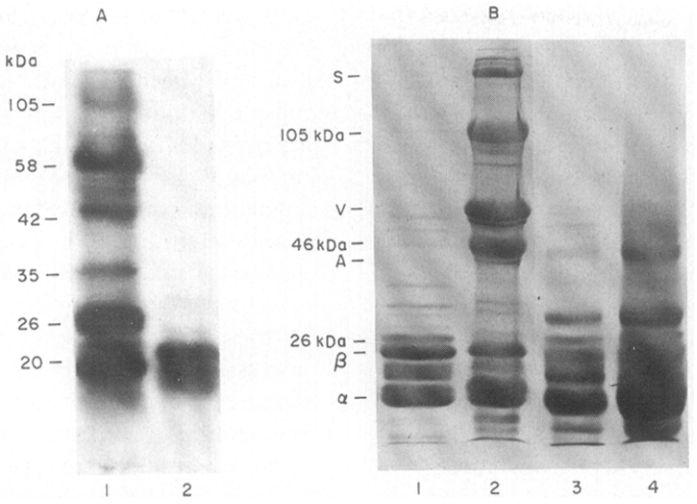

FIG. 3. Polypeptide profiles from in vitro translation and fresh lens. A, Fluorogram of IVT polypeptides separated by SDS–PAGE: lane 1, cortex; lane 2, nucleus. The capacity for protein synthesis by the nuclear preparation was indicated by the incorporation of label into polypeptides of the crystallin-M_r range (20–35 kDa). A major difference between the two profiles was the general lack of synthesis by the nuclear region mRNA of higher M_r species, which are predominantly cytoskeletal. These include vimentin (58 kDa) and beaded-chain filament components (46 and 105 kDa). B, Coomassie blue-stained SDS–PAGE profiles of proteins isolated from the respective regions of fresh lenses: lane 1, cortical water-soluble fraction; lane 2, cortical water-insoluble; lane 3, nuclear water-soluble; lane 4, nuclear water-insoluble. The separate fractions were utilized in order to maximize visualization of the component polypeptides of each. The nuclear IVT profile was similar to that of the fresh nuclear fractions in its lack of higher M_r cytoskeletal polypeptides but presence of crystallin-M_r species. Likewise, the cortical IVT and combined fresh profiles were very similar. S, Spectirin (235/240 kDa); 105 kDa, beaded-chain filament component; V, vimentin (58 kDa); 46 kDa, beaded-chain filament component; A, actin (42 kDa); 26 kDa, membrane intrinsic protein; 20 kDa, α- and γ-crystallins.

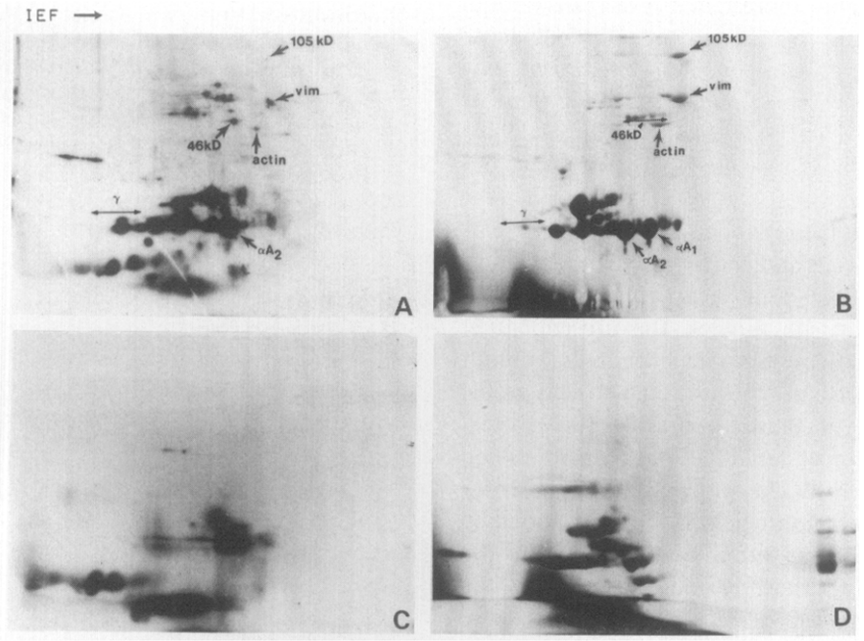

FIG. 4. 2-D PAGE analysis of IVT and fresh lens polypeptides. A and C, Fluorograms of 2-D PAGE of cortical (A) and nuclear (C) IVT products. B and D, Coomassie blue-stained gels of proteins isolated from fresh lens cortex (B) and nucleus (D). The presence of protein synthesis was demonstrated by the nuclear IVT. Its similarity to the fresh nuclear protein profile but distinction from the IVT and fresh cortical profiles was due primarily to the absence of higher M_r cytoskeletal polypeptides. Some of the differences between the IVT and fresh profiles, especially in cortex, can be explained by known post-translational modifications (e.g. 46 kDa and αA-crystallin).

cytoskeleton. In this respect the nuclear IVT pattern was generally comparable to the Coomassie blue-stained pattern of fresh nuclear proteins, while the differing cortical IVT pattern resembled the fresh cortical protein profile (Fig. 3). This finding lent validity to the IVT profiles as being complete and representative. The overall absence of the higher M_r polypeptides from the nuclear profiles was in agreement with an earlier report regarding the polypeptide composition of the fresh lens nucleus (Nasser et al., 1980). Further support was provided by immunofluorescence microscopy which demonstrated no re-

activity in the lens nuclear region when probed with a rabbit anti-lens vimentin serum (data not shown) and a monoclonal antibody to intermediate filament-associated protein (IFAP)-300 kDa (Lieska, Yang and Goldman, 1985; Yang, Lieska and Goldman, 1990) a vimentin-linked cytoskeletal component (Lieska et al., 1991).

Higher resolution analysis of the IVT products by two-dimensional (2-D) PAGE emphasized the major difference observed with one-dimensional SDS–PAGE between nuclear and cortical profiles: the virtual absence of synthesis by the nuclear preparation of polypeptides higher in M_r than the crystallins, including vimentin, 46 kDa, and 105 kDa (Fig. 4). Moreover, the nuclear and cortical translations appeared to produce different crystallin-M_r profiles. In general (e.g. considering M_r values, the proportionality between polypeptides, and the absence of high M_r species in the nuclear products), the IVT profiles were similar to the respective Coomassie blue-stained fresh lens protein profiles (Fig. 4), again validating the mRNA composition of the nuclear sample. The differences between respective IVT and fresh profiles, especially cortical, were related primarily to the presence of multiple charge forms in the fresh patterns. On the basis of identification by immunoblot analyses most of these probably represented post-translational modifications of the primary gene products. For example, αA_2 exists in the phosphorylated form, αA_1 (Spector et al., 1985), and the beaded-chain filament proteins (cf. bovine 46 kDa) (Lieska and Maisel, 1986) exist in multiple, phosphorylated forms (Ireland and Maisel, 1984).

Using criteria for unequivocally defining representative nuclear fiber cells and employing high resolution 2-D PAGE/immunoblot analysis in conjunction with comparative fresh tissue profiles, the present study has shown that bovine nuclear fiber cells contain mRNA capable in vitro of synthesizing a protein profile and that this profile is distinct from that of the cortex. Moreover, the profile resembles the accumulated protein profile of fresh lens nucleus. The similarity between the cortical and nuclear IVT profiles found by Ozaki et al. (1985) may thus have been due to the inclusion of transitional cortical material in the nuclear region preparation (25% of lens fresh weight vs. 16% in the present study). Another procedural difference between that study and ours was that Ozaki et al. (1985) utilized free and membrane-bound lens polysomes for cell-free translation, whereas we employed affinity-purified poly(A)+ mRNA. Their adult system generated major polypeptides of 68 and 92 kDa, while their calf cortical profile differed greatly from their adult patterns by not synthesizing these polypeptides. In contrast, our calf cortical IVT profile (not shown) was comparable to their calf and to our adult cortical profiles.

The concept of active protein synthesis by the lens nucleus is supported by in situ hybridization studies using labeled anti-sense probes which demonstrate in the nuclear region of rat lens the presence of mRNA sequences specific for MP26 (Bekhor, 1988) and α-, β- and γ-crystallins (Wen et al., 1991). It remains to be determined whether all such transcripts are intact and functional in situ. Nonetheless, protein synthetic capabilities would allow for a more normal functioning of these terminally differentiated cells than would otherwise be possible in cells lacking a nucleus. At the same time this capacity would introduce a new liability for these fiber cells with respect to cataractogenic processes inasmuch as the rejuvenating function of ongoing transcription appears to be absent. The demonstrated reduction in crystallin mRNA translation in murine hereditary cataracts (Shinohara and Piatigorsky, 1980) and the selective depression of crystallin mRNA levels in terminally differentiating fiber cells in rat sugar cataracts (Wen et al., 1991) suggest that altered protein synthesis in the lens nuclear fiber cells may be an important step in the development of certain types of cataracts.

Acknowledgements

The authors thank Dr Clint Magill of Texas A&M University, College Station, TX, U.S.A., for instruction in cell-free translation techniques and Dr Robert D. Goldman of Northwestern University School of Medicine, Chicago, IL, U.S.A., for generously providing the monoclonal antibody to nuclear lamina proteins. Dr Deren Shao of our institution is to be thanked for preparing the immunostained cryostat sections of lens. Virginia Kriho and Diane Johnson-Seaton are acknowledged for their technical expertise.

This study was supported by grants EY06804 (N.L.) and NS26395 (H.Y.Y.) from the National Institutes of Health, Bethesda, MD, U.S.A.

NORMAN LIESKA*
KATHY KROTZER[a]
HSI-YUAN YANG
Departments of Anatomy and Cell Biology, University of Illinois College of Medicine, Chicago, IL 60612, and [a]Texas College of Osteopathic Medicine, Fort Worth, TX 76107, U.S.A.

* For correspondence at: Department of Anatomy and Cell Biology, University of Illinois (M/C 512), 808 S. Wood St, Chicago, IL 60612, U.S.A.

References

Bekhor, I. (1988). MP26 messenger RNA sequences in normal and cataractous lens: a probe for abundance and distribution of a fiber cell-specific gene product. *Invest. Ophthalmol. Vis. Sci.* **29**, 802–13.

Blumberg, D. D. (1987). Creating a ribonuclease-free environment. *Methods Enzymol.* **152**, 20–4.

Chamberlain, J. P. (1979). Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal. Biochem.* **98**, 132–5.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162**, 156–9.

Dilley, K. and Van Heyningen, R. (1976). Some aspects of human lens metabolism: glycolysis and protein synthesis. *Doc. Ophthalmol. Proc. Ser.* **8**, 171–8.

Duke-Elder, S. (1958). *System of Ophthalmology*. Vol. I. *The Eye in Evolution*. Mosby: St Louis, MO.

Ireland, M. and Maisel, H. (1984). Phosphorylation of chick lens proteins. *Curr. Eye Res.* **3**, 961–8.

Kuwabara, T. (1975). The maturation of the lens cells: a morphologic study. *Exp. Eye Res.* **20**, 427–43.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature*, **227**, 680–5.

Lasser, A. and Balazs, E. (1972). Biochemical and fine structure studies on the water-insoluble components of calf lens. *Exp. Eye Res.* **13**, 292–306.

Lieska, N. and Maisel, H. (1986). A novel 5 nm filament purified and reconstituted from lens. *Invest. Ophthalmol. Vis. Sci.* **27** (Suppl.), 286.

Lieska, N., Shao, D., Johnson-Seaton, D. and Yang, H.-Y. (1991). Intermediate filament-associated protein (IFAP)-300 kD expression in the adult bovine ocular lens. *Anatom. Rec.* **229**, 53a.

Lieska, N., Yang, H.-Y. and Goldman, R. D. (1985). Purification of the 300 K intermediate filament-associated protein and its in vitro recombination with intermediate filaments. *J. Cell Biol.* **101**, 802–13.

Nasser, S., Bradley, R., Alcala, J. and Maisel, H. (1980). Regional differences in the composition of the bovine lens urea-soluble proteins. *Exp. Eye Res.* **30**, 109–13.

O'Farrell, P. H. (1975). High-resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007–21.

Ozaki, L., Jap, P. and Bloemendal, H. (1985). Protein synthesis in bovine and human nuclear fiber cells. *Exp. Eye Res.* **41**, 569–75.

Shinohara, T. and Piatigorsky, J. (1980). Persistence of crystallin messenger RNA's with reduced translation in hereditary cataracts in mice. *Science* **210**, 914–17.

Spector, A., Chiesa, R., Sredy, J. and Garner, W. (1985). cAMP-dependent phosphorylation of bovine lens α-crystallin. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4712–16.

Spector, A. and Kinoshita, J. (1964). The incorporation of labeled amino acids into lens protein. *Invest. Ophthalmol.* **3**, 517–22.

Walls, G. L. (1942). *The Vertebrate Eye and its Adaptive Radiation*. Cranbrook Institute of Science: Bloomfield, MI.

Wen, Y., Shi, S., Unakar, N. J. and Bekhor, I. (1991). Crystallin mRNA concentrations and distribution in lens of normal and galactosemic rats: implications in development of sugar cataracts. *Invest. Ophthalmol. Vis. Sci.* **32**, 1638–49.

Yang, H.-Y., Lieska, N. and Goldman, R. D. (1990). Intermediate filament-associated proteins. In *Cellular and Molecular Biology of Intermediate Filaments*. (Eds Goldman, R. D. and Steinert, P. M.). Pp. 371–91. Plenum Press: New York.

(Received 23 May 1991 and accepted 28 August 1991)