ISOLATION AND CHARACTERIZATION OF NUCLEAR MATRIX PROTEINS FROM BRAIN CELL TYPES : POSSIBLE UTILITY IN CANCER CELL LINES

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DECLARATION

Dated 6 August, 1993

I here by declare that the work presented in this thesis has been carried out by me under the supervision of Dr. Mohan C. Vemuri and that this has not been submitted for a degree or diploma of any other University.

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N.NAGA RAJU

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Abbrevations

AMP	Adenosine monophosphate
cm	Centimeter
4CIN	4-Chloro-1-naphthol
DAPI	4, 6-diamidino-2-phenylindole
2DE	Two dimensional electrophoresis
DEAE	Diethylaminoethyl
DTT	Dithiothreitol
GFAP	Glial fibrillary acidic protein
HMG	High mobility group
hnRNA	**Heteronuclear** RNA
HPLC	High performence liquid chromatography
IEF	Isoelectrofocusing
IgG	Immunoglobulin G
kDa	Kilo daltons
kg	Kilo gram
KV	Kilo **volt**
LIS	**3,** 5-lithium diidosalicylate
mA	Milli Ampere
MAR	Matrix associated regions
MEM	Minimal essential medium
mg	Milli gram
µg	Micro gram
µl	Micro litre
µM	**Micro** molar
µ	Micron
ml	**Milli litre**
mM	Milli molar
mRNA	Messenger RNA
Mr	Relative molecular mass
MW	Molecular weight
NFT	Neurofilamental triplet protein
NHCP	Non **histone** chromosomal protein
nm	Nano **metre**
O.D	Optical Density
pI	Isoelecric point
rDNA	Ribosomal DNA
RNPs	Ribonuclear proteins
RPM	Rotations per minute
rRNA	Ribosomal RNA
SAR	Scaffold associated regions
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel elecrophoresis
SEM	Scanning electron microscope
SnRNA	Small nuclear RNA
ssDNA	Single strand deoxyribonucleic acid
TEM	Transmission electron microscope
V	Volts
v/v	Volume / volume
w/v	Weight / volume

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INTRODUCTION

Introduction

Classical ultrastructural studies on the cell nucleus have demonstrated an association of euchromatin and heterochromatin with an extensive non-chromatinous structure in the interior of the nucleus (Agutter, 1991 ; Hoffman, 1993). It was shown 50 years ago that a fraction of nuclear proteins resist extraction even with buffers of very high ionic strength (Mayer and Gulick, 1942). This subfraction was found to be made of a nucleoprotein fibrillary network. The term 'Nuclear Matrix' was assigned to this structure by Berezney and Coffey (1974). DNA associated with the nuclear matrix contained A-T rich sequences and therefore termed as matrix associated regions (MAR) or scaffold associated regions (SAR). The scaffold forms the base chromatin loops during DNA replication in organisms ranging from yeast to man. SARs were shown to be usually located at the borders of different genes, close to 5' or 3'-end cis-regulatory sequences or DNase I hypersensitive sites suggesting that SARs might participate in the transcriptional regulation through regulatory domains (Avramova and Paneva, 1992). The nuclear matrix is now considered to be a valid structure with a few defined functions and probably several yet to be identified functions. Some of the nuclear matrix functions supported by experimental proof are :

(a) organization of chromatin in the interphase nucleus of eukaryotes into loops of 30-100 kbp and each of these loops represents either a replicational or transcriptional single unit.

(b) the localization of replicational machinery (replisomes) in the nuclear matrix (Tubo *et* a/., 1985) which is considered to be the site of DNA replication.

(c) the nuclear matrix is enriched with actively transcribed genes (Zehnbauer and Vogelstein, 1985) and is associated with the processing of RNA.

(d) the nuclear matrix acts as a site of interaction for viral proteins (Covey *et al.*, 1984) and viral DNA (Rennie *et al.*, 1983).

(e) the nuclear matrix contains binding sites for some hormones, carcinogens (Gupta *et al.*, 1985), tumor promoters (Eisenman *et al.*, 1985), drugs and other substances (Kaufmann *et al.*, 1986 ; Bareack and Coffey, 1982).

Methodological options for nuclear matrix preparation

The *in situ* nuclear matrix was first isolated and characterized by Berezney and Coffey (1974) in liver. Subsequently many others (Faken and Hancock, 1974 ; Cook and Brazell, 1975 ; Paulson and Laemmli, 1977 ; Grasser and Laemmli, 1986b) have isolated and characterized nuclear matrices from a variety of organisms. Several of these isolation methods involved treatment of isolated nuclei with nucleases, non-ionic detergent and high salt buffers. This sequential extraction of nuclei finally yielded a nuclear matrix fraction usually containing the granular and fibrous internal matrix, which forms a web throughout the interior of the nucleus, the residual elements of the nuclear envelope (also termed as the pore complex lamina) and residual nucleoli. A major modification of the foregoing method was suggested by Mirkovitch *et al.* (1984) who used lithium 3, 5-diiodosalicylate (LIS) a chaotropic agent and a detergent instead of buffers with high ionic strength. The resultant preparation, termed as "nuclear scaffold" has been widely used to study the DNA sequences specifically associated with the matrix. The nuclear scaffold also

has been described as the nuclear skeleton, nuclear ghost or nuclear cage. The nuclear matrices are predominantly proteinaceous. Depending on the type of isolation methods employed, several studies showed minor variations in the biochemical constitution of the nuclear matrix, for example, if ribonuclease was omitted, the isolated nuclear matrix contained RNA as the second most abundant component. Matrix preparations examined by electron microscopy showed several common structural entities such as fibrillary and granular internal nuclear matrix, pore complex lamina and residual nucleoli.

Some studies suggested that structural integrity of the nuclear matrix involves metallo-protein interactions during matrix isolation, based on the inclusion of Ca^{++} or Cu^{++} (Lebkowski and Laemmli, 1982) or Mg^{++} (Bouvier *et al.,* 1985a). Matrix preparations using Mg^{++} were found to be enriched with residual RNP complexes which formed a salt-resistant intra-nuclear network. Digestion with RNase in the presence of low ionic strength EDTA was shown to alter the morphology of network, suggesting a biochemical role for Mg^{++} (Bouvier *et al.*, 1985b). The internal fibrous network of the nuclear matrix is more labile than that of the nuclear lamina. Therefore, most of the nuclear matrix protocols involve a stabilization step to avoid dissociation of the fibrous network. Various methods entail stabilization by fixation with acroline (de Graaf *et al.,* 1991), brief incubation at 37 or 42^0C, treatment with Cu^{++} (Razin *et al.*, 1985) and oxidation with sodium tetrathionate (NaTT) (Kaufmann and Shaper, 1984). The mechanisms involved in stabilization are not known excepting for the method of oxidation with NaTT. NaTT oxidizes sulfhydryl groups to disulfide bridges, which results in the stabilization of nuclear matrix (Kaufmann *et al.*, 1986). Basing on this data, it was subsequently shown that a reduction of disulfides is important in disassembly of the nucleus at prophase (Sturrman *et al.,* 1992).

Nuclear matrix and DNA replication

Wanka *et al.*, (1982) suggested that the nuclear matrix might be involved in unwinding the DNA double helix in a specific manner so that the daughter molecules can be separated easily from the parent template during DNA replication. But their study could not conclusively demonstrate the matrix binding region on the DNA molecule or specific attachment sites for DNA on the nuclear matrix. Tubo and Berezney (1985) provided evidence, using density shift experiments, for the replisome loop model in eukaryotic DNA replication. On the basis of the relation between loop sites and replication sites, the bases of the DNA loop behave as replication origins. Studies of Dijkwel *et al.* (1986) showed the position of replication origin relative to the nuclear matrix by autoradiographic analysis of nuclear matrix halo structures. Using synchronized BHK cells, a label at the beginning of S-phase remained matrix associated and later on migrated into the DNA halo, suggesting that replication origins remain matrix bound after the initiation of DNA synthesis. Tubo *et al.* (1985) provided enzymatic evidence that approximately 10 % of the total cellular DNA primase activity was associated with isolated nuclear matrix, suggesting that the nuclear matrix is an important entity in the eukaryotic replication of DNA in the cell nucleus. Further studies by Dave *et al.* (1989) showed terminal deoxyribonucleotidyl transferase(s), a class of DNA polymerase, is involved in the postembryonic DNA synthesis of Immunoglobulin gene recombination events in thymus nuclei and is associated with the nuclear matrix for its expression. This enzyme catalyses the addition of deoxyribonucleotides to the 3'-(OH) terminus of DNA without template direction. All these findings conclusively demonstrate the involvement of the nuclear matrix in DNA replication and DNA synthesis.

Enzymes of DNA, RNA metabolism and the nuclear matrix

Several enzymes involved in DNA and RNA metabolism have been shown to be associated with the nuclear matrix. These include DNA α- and β-polymerases (Foster and Collins, 1985), topoisomerases I and II (Berrios *et* a/., 1985), RNA polymerase II (Lewis *et al.,* 1984), poly (A) polymerase (Schroder *et al.,* 1984), DNA methylase (Burdon *et al.,* 1985) and DNA primase (Tubo and Berezney, 1987a). Though the function of these enzymes is known, it is not understood how and why these enzymes are associated with the nuclear matrix.

Topological states of DNA and the nuclear matrix

The nuclear matrix has also been implicated in the conversion of different topological states of DNA. DNA Topoisomerase II, mediates the interconversion of DNA through transient double strand breaks and rejoining. Topoisomerase II can relax positive and negative supercoiled DNA, catanate and decatanate DNA rings and unknot the knotted DNA (Wang, 1985). DNA Topoisomerase II has been shown to bind in a cooperative manner to SAR. This suggests a role for the nuclear matrix in confirming specific topology to DNA through DNA topoisomerase II. Studies by Tsutsui *et al.* (1988) suggested that the nuclear scaffold exhibits at least two classes of DNA binding sites of which one is specific to supercoiled DNA and does not bind relaxed or linear forms, while the other lacked this specificity. Cockerill and Garrard (1986a) suggested direct anchorage of topoisomerase II to the chromosomal loop domains. They mapped the binding sites in the mouse immunoglobulin-k gene and found common sequences in the binding site and in the corresponding region of other genes (Cockerill and Garrdard, 1986b ; Udvardy *et at,* 1985). The DNA binding sites on the nuclear scaffold exhibited a recognition mechanism which was

not based on nucleotide sequence but rather was conformation directed, in the sense that tortional stress generated in the looped domain served as a recognition signal. Similarly in another study (Hinzpeter and Depperet, 1987), it was shown that concatemerized oligonucleotides posses an unwinding nucleation site with strong affinity for the nuclear scaffold, and with augmented SV40 promoter activity. Mutated concatemerized oligonucleotides resisted unwinding, showed weak affinity and a lack of enhancement of promoter activity, suggesting that relaxation of the superhelical structure of DNA by topoisomerase II is important for SAR functions (Bode *et al.*, 1992).

Nuclear matrix and gene expression

Buttyan and Olsson (1986) using androgen dependent genes, demonstrated that actively transcribed genes are protected from nuclease digestion by their association with the nuclear matrix. They further used nuclear matrix protection assays to analyse the tissue specific expression in a highly related gene family and to predict transcriptional activity of this gene family in a specific tissue. In support of foregoing, Keppel (1986) provided evidence that transcribable human ribosomal RNA (rRNA) genes are attached to the matrix and that the tandom repeats of ribosomal DNA (rDNA) are not randomly associated with the matrix but are probably attached at transcriptional complexes. Studies on the visualization of mRNAs of fibronectin and neurotensin using fluoresence hybridization with cDNA and intron specific probes indicate that there is a highly ordered structural organization in association with the nuclear matrix (Xing *et al.*, Carter *et al.*, 1993). Also, it has been demonstrated that a receptor binding factor (RBF-1) for the avian oviduct progesterone receptor (PR) has high affinity binding sites on avian genomic DNA, which

are localized in the nuclear matrix. The direct action of progesterone results in the rapid expression of nuclear matrix protooncogenes *c-myc* and *c-jun* (Schuchard *et al.*, 1991).

Nuclear matrix association with heterogeneous nuclear RNA (hnRNA)

Herman *et al,* (1978) in a two-step extraction of chromatin were able to remove 99 % of the chromatin. The remainder of the RNA is associated with the nuclear matrix. This fraction of residual RNA was found to be chiefly heterogeneous nuclear RNA (hnRNA). They suggested that the integrity of the nuclear matrix is dependent on this RNA. There are pros and cons for this interpretation. For example, Miller e/ *al.* (1978a) showed that RNase treatment of the nuclear matrix does not alter its morphology, while electron microscopic studies by Fey *et al.* (1986a) showed a drastic alteration in the morphology of the nuclear matrix when RNase was included in the preparation of the nuclear matrix. The data reported by Smith *et al.* (1986) agrees with other studies showing that internal matrix structures are distorted when nuclear matrix associated RNA is degraded or metal ions are chelated. Their data further suggested that internal nuclear matrix assemblies are present *in situ* and their absence in biochemical preparations might be an artifact. Knowledge of the composition and organization of hnRNA in the granular and fibrous internal nuclear matrix structure is required to understand how RNA is associated with the nuclear matrix. Gallinaro *et al.* (1983) showed that pre-mRNA in the nuclear matrix and in the salt resistant complexes derived from hnRNP share a common constitutive unit, suggesting that hnRNP and mRNA are structurally similar.

Small nuclear ribonuclear particles (snRNP) and the nuclear matrix

Like hnRNA, small nuclear ribonuclear particles (snRNPs) were found associated with nuclear matrices prepared from chicken oviduct (Ciezek *et al.*, 1982). The snRNPs (also called U RNAs) play a role in the processing of pre-mRNA. Of these, U1 to U6 RNAs are major U RNAs and U7 to U10 are minor U RNAs in eukaryotes. Zieve and Penman (1976) showed that U2, U3, U4, U6 RNAs were associated with the nuclear matrix. Though Ul RNA is also associated with the nuclear matrix, it was lost during chomatin extraction. The function of snRNPs is not known ; but one hypothesis is that they can act like a backbone structure thereby facilitating packaging (Berezney, 1984), post-transcriptional modification (Herman *et al.*, 1978) and transport of RNA into the cytoplasm (Gallinaro *et al.,* 1983). A specific interaction between snRNPs and nuclear matrix structures has been indicated by a limited number of studies (Padgett *et al.,* 1986 ; Bringmann and Luhrmann, 1986). However, these fail to establish the functional significance of such an interaction.

RNA transport and the nuclear matrix

Different RNA species are transported at different rates from the nucleus, and in general, smaller RNAs are transported rapidly into the cytoplasm than larger mRNAs. Most of the mRNAs are polyadenylated while some are non-polyadenylated such as histone mRNA. Studies by Mariman *et al.*, (1982) showed that adenovirus PIX mRNA (about 9s polyadenylated and unspliced) reaches the cytoplasm within 4 minutes after the start of synthesis while late adenovirus mRNA of same the size reaches the cytoplasm only after 16 minutes. The latter mRNA was shown to be matrix bound. The rate of transcription of mRNA depends

on the rate of maturation. Reasons for the different rates of transport of matrix bound mRNAs remain to be elucidated.

Virus specific proteins and the nuclear matrix

Studies have shown that the nuclear matrix is an important site for viral interaction. Viral DNA and viral specific proteins have been found to be enriched in the nuclear matrix. Studies by Hinzpeter and Deppert (1987) showed that the interaction of viral antigens with chromatin and the nuclear matrix is mediated by protein-protein interactions rather than by protein–DNA interactions.

The viral genomes such as the bovine papilloma virus type 1 (BPV–1) has a stretch of sequences which are located immediately adjacent to the origin of DNA replication. These sequences (672 bp DNA fragment) have been reported to interact with the nuclear matrix which is ultimately responsible for the replication of virus in the nuclei of tumor and transformed cells (Adorn and Richard-Fay, 1991).

Phosphorylation of the nuclear matrix

Post–translational modification of proteins is one of the regulatory mechanisms in cellular responses. Phosphorylation reactions involving protein kinases and phosphatases are often involved. The interplay between these two enzyme activities, results in the phosphorylation and dephosphorylation of specific substrates which ultimately brings about signal transduction events. Ca^{++}/ Calmodulin dependent protein kinases mediate several neuronal events in the cytosol, at synaptosomes and in the nucleus. Most nuclear Ca^{++}/ Calmodulin protein kinases have been shown to be associated with the nuclear matrix. Such an association leads

to compartmentalization, which provides a mechanism for the regulation of the enzyme access to substrates critically involved in nuclear functions. Halikowska and Leiw (1987) characterized a highly phosphorylated nuclear protein (68 kDa ; pI 6.5-8.2) associated with mononucleosomal particles and also with the nuclear matrix. This protein showed a high degree of phosphorylation in regenerating liver after partial hepatectomy. Extensive phosphorylation of this protein and its association with the nuclear matrix suggests that it plays a key role in nuclear organization and function. Further, Zhelev *et al.* (1990) showed a marked increase of 125 kDa (pI 6.5) protein in mitotic mammalian cells. The protein was named "mitotin" and was shown to be associated with the nuclear matrix. Accumulation of mitotin in premitotic and mitotic cells was related to the phosphorylation of this protein and the metabolic stability of its phosphorylated forms. These events suggest a role for phosphorylation and for the nuclear matrix in the complex events of mitosis. In another study, differences in the nuclear matrix phosphoproteins in wild type and nitrogen mustard-resistant rat breast carcinoma cell lines have been reported (Moy and Tew, 1986). Though the antigens in the two tumor cell lines were similar, one of them, due to its hypophosphorylation, failed to bind cyclic-AMP (c-AMP) and thus differed from the other. Phosphorylation of matrix proteins leading to structural alterations in the nuclear matrix suggested that the nuclear matrix can change its configuration depending on the functional requirements of the cell.

Cytoskeletal proteins and the nuclear matrix

During transcription in eukaryotes, RNA polymerase II has to be activated by binding to promoter elements such as TATA box (Breathnach and Chambon, 1981). Eukaryotic cells contain multiple factors which

enable the binding of RNA polymerase II to this promoter element. One of these multiple factors has been purified, characterized and was found to be similar to nuclear actin (Egly *et al.*, 1984). Moreover, antibodies to nuclear actin when injected into oocytes, stopped transcription by RNA polymerase II, suggesting a possible role for nuclear actin in RNA metabolism. Nakayasu and Ueda (1984) have shown an interaction between pre mRNA and actin filaments in the nuclear matrix of mouse leukemia L5178Y cells. Other studies also detected actin as a major protein in nuclear matrices (Nakayasu and Ueda, 1986). These observations suggest that actin might be involved in a well defined function in RNA synthesis. However, the forgoing possibility requires further study.

Nuclear matrins are major nuclear matrix proteins

The protein composition of the nuclear matrix was recently analysed in detail (Nakayasu and Berezney, 1991). Approximately 12 major proteins were identified, of which nuclear lamins A, B and C and the nucleolar protein B-23, and residual hnRNPs constitute already identified proteins. The rest of the proteins termed nuclear matrins consist of matrin 3 (125 kDa slightly acidic), matrin 4 (105 kDa, basic), matrin D-G (60-75 kDa, basic) and matrins 12 and 13 (42-48 kDa, acidic). Peptide mapping studies showed no homology of these matrins to nuclear lamins. Matrin-3 had an extensive acidic domain with a nuclear targeting signal sequence, and is a highly conserved protein (Belgrader *et al.*, 1991a). Interestingly, matrins D-G comprise two pairs of related proteins (matrins D/E and F/G). The F/G matrin was found to be a DNA binding protein containing two putative zinc finger motifs (Hakes and Berezney, 1991a). A palindromic seven amino acid sequence (Ser-Ser-Thr-Asn-Thr-Ser-Ser) was discovered within one of the zinc finger DNA binding regions.

This sequence appears to be a potential site for phosphorylation and glycosylation and therefore might be involved in a regulatory role within the DNA binding domain.

In addition to F/G, **lamina** A and C (but not B), matrins D and E were reported to be specific DNA binding proteins having preference for single strand DNA (Hakes and Berezney, **1991b),** probably each with a separate sequence specificity. In DNA binding assays they showed preference for nuclear matrix DNA over total genomic DNA. These studies directly demonstrated that internal nuclear matrix proteins (D,E,F,G and 4), in addition to the **lamina** A and C, can bind to DNA, suggesting that loop attachment sites are internal as well as peripheral in the nucleus during DNA replication.

In another study, a DNA binding protein (SATB1) (human cDNA clone) from thymus having selective binding to MARs has been reported (Dickinson *et al.*, 1992). SATB1 showed an unusual binding site recognition in the sense that it binds to a ATC rich sequence, wherein one strand consists of mixed A's, T's and C's excluding G's. In mutants deficient **in** the ATC rich sequence, there was a significant reduction in binding, even when the direct contact sequence remained intact. This suggested that **SATB1** binding to MAR's is through the recognition of the ATC sequences by sugar-phosphate back bone structure of DNA. Luderus *et al.*, (1992), employed a heterogenous binding system using matrix preparations from rat liver and the MARs from the histone cluster of *Drosophila*, MAR binding nuclear proteins were identified. They measured MAR binding to lamins from calf thymus and *Drosophila.* They found a 67 kDa matrix protein, which was identified as **lamin B1** and also another minor protein lamin B2. They further suggested lamin B interacts with chromosome by

directly binding to MAR sequences leading to a decondensation of the chromatin, the repolymerization of lamina or both. This type of mechanism probably safeguards chromatin organization and argues for a key role of lamin B in certain cells that lack lamin A and C.

SCOPE OF THE PRESENT STUDY

Scope of the present study

A proteinaceous fibrillary and granular nucleoskeleton constitutes the major component of the nuclear matrix. Other morphological features of the nuclear matrix, such as nuclear pore complex, nuclear lamina (also called as fibrous lamina) are now generally accepted and immuno-cytochemical and immunochemical studies have identified some of their constituent molecules (Ris, 1991 ; Akey, 1991 ; Dingwall and Laskey, 1992 and Hinshaw *et al.*, 1992). However, models describing the association of the nuclear matrix with nuclear pore complex morphology are still evolving ; this aspect has been recently reviewed (Maqat, 1991). Recent advances suggest that the participation of the nuclear matrix in DNA replication and gene expression is through replicational assemblies (replisomes) which associate with the nuclear matrix in clusters forming special sites termed "clustersomes" (Berezney, 1991a). How exactly the DNA synthesis might be initiated on the clustersomes is yet to be determined. Also, the stimulation of gene expression associated at the SARs has to be further resolved, as SARs are usually found at the border between different genes which mark the boundaries of the chromatin domain. However there also have been several reports (Jarman and Higgs, 1988 ; Farache *et al.*, 1990) localizing the SARs within the transcribed sequences. The independent regulation of these domains might contribute significantly to gene expression.

However, studies are required particularly in clarifying the nature of nuclear matrix protein-DNA interactions, the composition of these proteins, and their function in eucaryotic cells. Structural and regulatory roles in the nuclear matrix and its protein composition in cancer cells has not been investigated thoroughly. A recent report indicates that a nuclear

matrix protein (Mr 56 kDa ; pI 6.58) detected in prostate cancer cells is not seen in normal prostate or benign prostatic hyperplacia (Partin *et al.*, 1993). As the nuclear matrix is involved in cellular regulation, via nuclear events (Xing *et al.*, 1993), there may well be characteristic nuclear lesion(s) in neoplastic cells.

The presence of nuclear matrix as an ubiquitous structure of the eukaryotic cell nucleus is now well established ; but the significance of the structure still remains to be resolved. Though it is known to be associated with nuclear functions such as transcription, replication and RNA processing, its involvement presumably in cell specific gene expression and cell cycle particularly in cancer cells attracts an investigation. This is because each type of cell exhibits a profile of the infinitesimal proteins in its nucleus, that is specific and unique to the cell type. When the cell becomes abnormal, the pattern of its nuclear matrix proteins changes. Since the *in situ* nuclear matrix is likely to be composed of a variety of components distinct for different cell types, and how these individual components assemble to integrate and drive the nuclear functions is not clear and constitutes a good avenue for the further research. Therefore, in the present study, the protein composition of nuclear matrix has been analyzed in different cell types of brain and their possibility diagnostic utility in abnormal cells.

MATERIALS AND METHODS

Materials and methods

The following list of chemicals, reagents and equipment were used in this study.

Aprotinin, DAPI, DNase I, Digitonin, 3, 5-diiodisalicylate (lithium salt), DTT, PMSF, RNase A, single strand DNA cellulose, spermidine, spermine, thiodiglycol, triton-X 100, tween-20, trypsin, trasylol, molecular weight markers such as bovine albumin, egg albumin, carbonic anhydrase, chymotrypsinogen, glyceraldehyde-3-phosphate dehydrogenase, α-lactalbumin, soyabean inhibitor, trypsinogen were purchased from Sigma chemical company, USA.

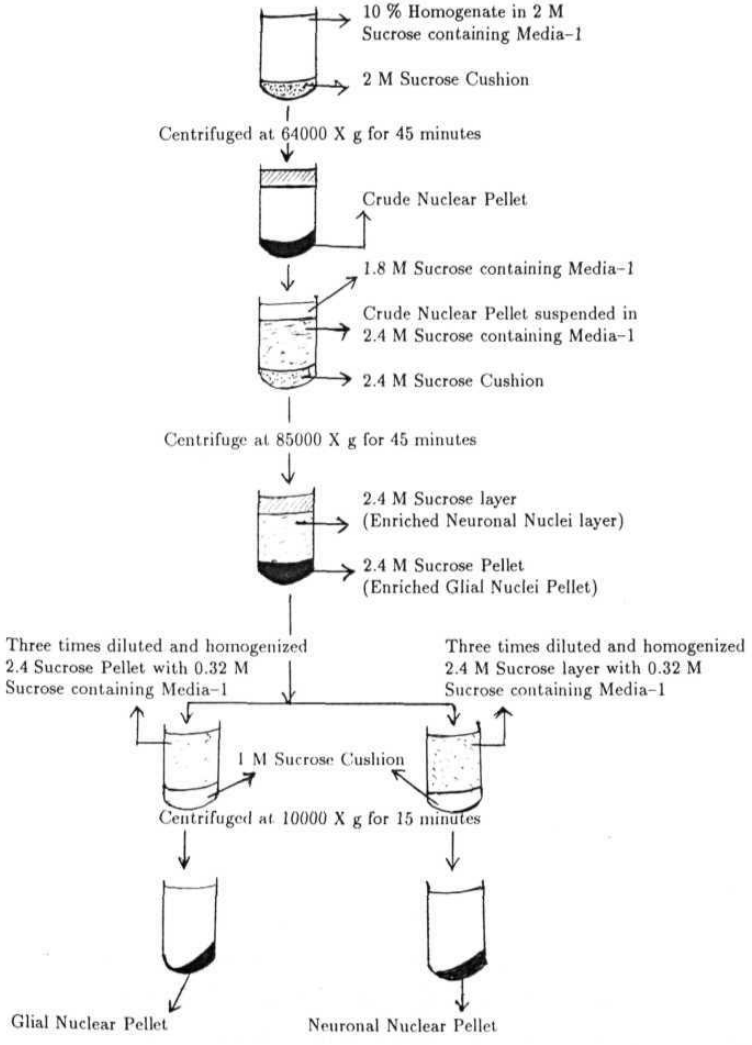
Acrylamide, agarose, coomassie brillient blue, β-meracaptoethanol, N, N-methylene-bis-acrylamide, SDS, sucrose, Tris were obtained from Sisico Research Laboratories, India.

Tissue culture media and chemicals such as Nutrient mixture F-10 HAM, Dulbecco's modified medium, MEM vitamins, pencillin, streptomycin, gentamycin, L-glutamine, sodium pyruvate, phenol red, horse serum, fetal bovine serum procured from Himedia Laboratories pvt. ltd, Bombay, India.

Nitrocellulose sheets, DEAE Cellulose were obtained from Whatman company. Ampholine, Surface pH electrode, High Voltage power supply, Fraction collector, Novablot unit were purchased from LKB, Bromma, USA. The 4-chloro-1-naphthol was obtained from Pierce.

All the other chemicals and reagents were used in this study were of analytical grade, locally availble.

ISOLATION OF NEURONAL AND *GLIAL* NUCLEI FROM THE BRAIN



Wistar strain albino rats were killed by decapitation, brain regions were rapidly removed and placed in chilled 0.32 M sucrose containing 1 mM $MgCl_2$, and 0.1 mM PMSF (Media-1). Nuclei were isolated by the method of Thompson (1973) with minor modifications. All the operations were performed at 4°C unless specified and all sucrose densities were prepared in media-1. Cerebral cortices were separated, homogenized to 20 % (w/v) in 2.0 M sucrose using Dounce tissue homogenizer with pestle 'B'. The homogenate was further diluted to 10 % with 2.0 M sucrose and filtered through one layer of muslin cloth. The filtrate was layered over a 2.0 M sucrose cushion and centrifuged at 64000 x g for 45 minutes using SW-28 rotor in Beckman ultracentrifuge. The pellet was suspended in 2.5 ml of 2.4 M sucrose and was layered on 1 ml cushion of 2.4 M sucrose (with this cushion, purity of glial nuclei will be increased). This gradient was further overlaid with 1.3 ml of 1.8 M sucrose in 4.8 ml tubes of SW-60 rotor and centrifuged at 85000 x g for 45 minutes, which resulted in three fractions

a) 1.8 M sucrose layer (contains lipids, broken nuclei and membranes) was removed with pasture pippet and discarded.

b) 2.4 M sucrose cushion overlay (enriched neuronal nuclei)

c) 2.4 M sucrose pellet (enriched glial nuclei)

Both the neuronal and glial enriched fractions were diluted separately with two volumes of 0.32 M sucrose, layered over 1.0 M sucrose cushion and centrifuged at 10000 x g for 15 minutes to obtain pure neuronal and glial nuclear pellets. Purity and integrity of nuclei was checked by 4, 6-diamidino-phenyl indole (DAPI) fluorescence and phase

contrast microscopy. The nuclei were stored at -20°C till further use in 0.32 M sucrose prepared in isolation buffer (IB) (3.75 raM Tris–HCl pH 7.4, 0.05 mM spermine, 0.0125 mM spermidine, 1 % thiodiglycol, 20 raM KC1 and 0.1 mM PMSF).

Cancer cell lines-cell culture

All the cell lines were obtained from NFATCC, Pune. The following cancer cell lines were grown in 25 cm^2 T–flasks with their respective medium and conditions described separately. All the cell lines were maintained in logarthmic growth in CO_2 incubator.

C6 glioma cells

Rat C6 glioma cells (39 th passage ; C6 glioma was cloned from a rat glial brain tumor induced by N–nitroso–methylurea) were maintained under standard tissue culture conditions (37°C, 5 % CO_2 and 95 % humid atmosphere) in 82.5 % nutrient mixture F–10 (HAM) medium supplemented with 15 % horse serum, 2.5 % fetal bovine serum and 0.015 mg / ml phenol red, 10 mM sodium pyruvate, 2 mM L–glutamine, 1 % (v/v) MEM Vitamins, 1 % (v/v) non-essential amino acids, 100 U / ml pencillin, 0.1 mg / ml streptomycin and 0.03 mg/ml gentamycin.

Neuro-2A

Neuronal cell types and small round stem cells containing neuroblastoma were established from a spontaneous tumor of strain A albino mouse. The 172 passage of this neuroblasoma was cultured in minimum essential medium with non-essential amino acids and BSS (90 %), fetal bovine serum (10 %) and 0.015 mg / ml phenol red, 10 mM sodium pyruvate,

2 mM L-glutamine, Vitamins, 100 U / ml pencillin, 0.1 mg / ml streptomycin and 0.03 mg / ml gentamycin under standard tissue culture conditions (37°C, 5 % CO_2 and 95 % humid atmosphere).

IMR-32

It is a mixture of two morphologically distinct cell types (a small neuroblast-like cell and a large hyaline fibroblast) and established from an abdominal mass occurring in a 13 month old male Caucasian. The 52 passage of this neuroblastoma was cultured in minimum essential medium with non-essential amino acids and BSS (90 %), fetal bovine serum (in-activated) 10 %, 0.015 mg / ml phenol red, 10 mM sodium pyruvate, 2 mM L-glutamine, 1 % (v/v) MEM vitamins, 100 U / ml pencillin, 0.1 mg / ml streptomycin and 0.03 mg / ml gentamycin under standard tissue culture conditions (37^0C, 5 % CO_2 and 95 % humid atmosphere).

SK-N-SH

Epithelial like human neuroblasoma, metastasis to bone marrow cell line, has neurogenic origin, exhibits a large doubling time and high levels of dopamine-β-hydroxylase. The cell line was maintained in Dulbecco's modified medium with non-essential amino acids and Earle's BSS (90 %), fetal bovine serum (10 %), 0.015 mg / ml phenol red, 1 mM sodium pyruvate, 2 mM L-glutamine, 1 % (v/v) MEM Vitamins, 100 U / ml pencillin, 0.1 mg / ml streptomycin and 0.03 mg / ml gentamycin under standard tissue culture conditions (37°C, 5 % CO_2 and 95 % humid atmosphere).

U-373MG

Epithelial like human glioblastoma, astrocytoma grade III cell line derived from a malignant glioma brain. The cell line was maintained in

Dulbecco's modified medium with non-essential amino acids and earle's BSS (90 %), fetal bovine serum (10 %), 0.015 mg / ml phenol red, 10 mM sodium pyruvate, 2 mM L-glutamine, 1 % (v/v) MEM vitamins, 100 U / ml pencillin, 0.1 mg / ml streptomycin and 0.03 mg/ml gentamycin under standard tissue culture conditions (37°C, 5 % CO_2 and 95 % humid atmosphere).

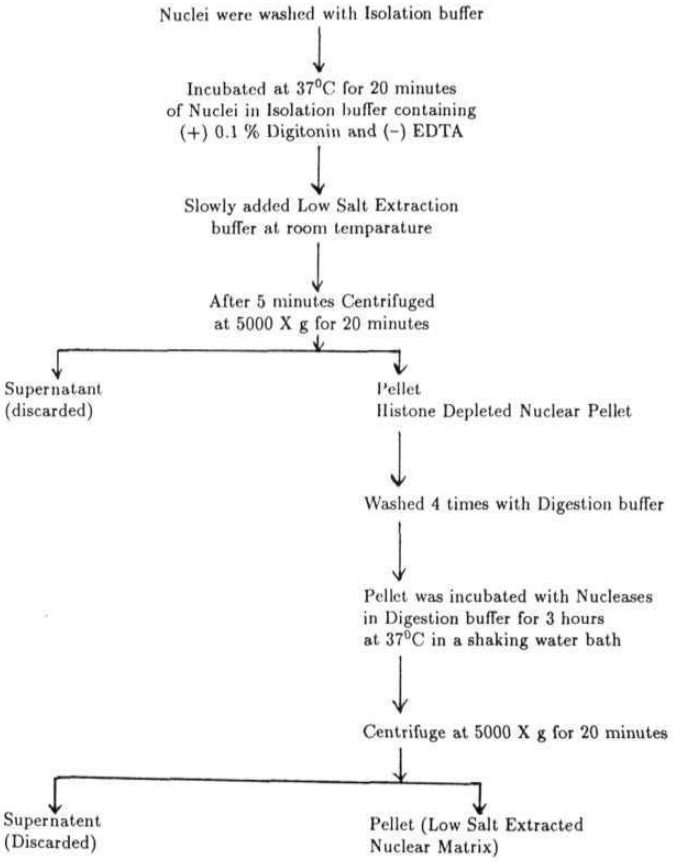
The cell lines, as soon as they reached confluency were passaged. The cells were given fresh medium for every three days. The monolayer cells were detached by trypsinization (0.1 % trypsin for 5 minutes). Following detachment, the trypsin was inactivated by adding 10 ml of medium containing serum. Cells were harvested by centrifugation at 1000 g for 10 minutes, followed by washing with medium. The cell pellet was suspended in less volume of medium and cell number was counted using a haemocytometer.

Isolation of nuclei from cancer cell lines

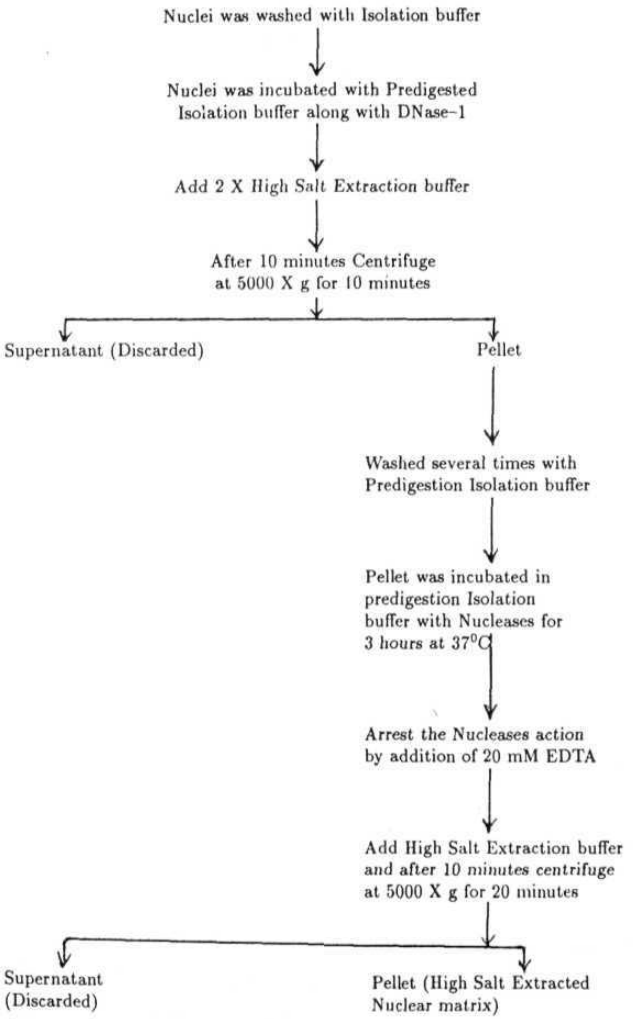
Cells were collected by centrifugation at 4000 x g for 10 minutes. The cell pellet was washed three times with IB and suspended in IB containing 0.1 % digitonin. The cells were homogenized manually in Dounce tissue homogenizer using pestle 'B' with 20 up and down strokes to get nuclei from the cells. The crude nuclei were collected by centrifugation at 3000 g for 10 minutes as a pellet. The crude nuclear pellet was resuspended in 2.0 M sucrose and the suspension was overlayed on 2.0 M sucrose cushion and centrifuged at 64,000 x g for 45 minutes in SW-60 rotor in Beckman Ultracentrifuge to get pure nuclei.

The neuronal, glial and cancer cell nuclear pellets were washed twice with IB separately and were suspended finally in IB. Optical density of the nuclei was measured at 260 nm.

NUCLEAR MATRIX EXTRACTED BY LOW SALT METHOD



NUCLEAR MATRIX PREPARATION BY HIGH SALT METHOD



Preparation of nuclear matrix

Nuclear matrices were prepared by two ways (1) Low salt method (LSM) (2) High salt method (HSM) following the method of Mirkovitch *et al.*, (1984) with minor modifications.

(1) Low salt method (LSM)

Ten O.D 260 units of nuclei were incubated at 37°C for 20 minutes in 100 μl of IB containing 0.1 % digitonin. To this 7 ml of low salt extraction buffer (LSEB) [(0.5 mM Hepes / NaOH pH 7.4, 0.25 mM spermidine, 2 mM EDTA / KOH pH 7.4, 2 mM KC1, 0.1 % Triton X–100, 25 mM 3-5 lithium diiodosalicylic acid] was slowly added. After 5 minutes, histone depleted nuclear pellets were recovered by centrifugation at 3000 x g for 20 minutes in a microfuge. The nuclear pellet was washed four times with digestion buffer (DB) (20 mM Tris–HCl pH 7.4, 0.05 mM spermine, 0.125 mM spermidine, 0.02 M KC1, 70 mM NaCl, 10 mM $MgCl_2$, 0.1 % Triton X-100, 100 KIU / ml trasylol and 0.1 mM PMSF). The final pellet was incubated at 37^0C for 3 hours in shaking water bath with DB containing both DNase 1 and RNase A (each 150 μg / ml concentration). The nuclease activity was terminated by adding EDTA to a final concentration of 20 mM and nuclear matrices were pelleted by centrifugation at 3000 x g for 10 minutes. The pellet was designated as nuclear matrix of low salt method (NM-LSM).

(2) High salt method (HSM)

Five O.D 260 units of nuclei were suspended in 300 ul of 4X predigested isolation buffer (PIB) (predigestion was done by adding of 10 mM $MgCl_2$, 50 mM NaCl and 250 μg / ml DNase 1 to the IB and incubated at 37°C for 4 hours) containing 0.1 % Triton X-100 and 0.1 % digitonin. After

10 minutes equal volume of 2X high salt extraction buffer (HSEB) (2.0 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM PMSF, 0.2 mM $MgCl_2$, 0.02 raM DTT and 0.4 % Triton X-100) was added and centrifuged at 3,000 x g for 10 minutes. The pellet was washed three times with PIB and was finally incubated for 3 hours at 37^0C in 2 ml PIB containing both DNase 1 and RNase A (each 150 /ig / ml). Nuclease action was terminated by adding EDTA to a final concentration of 20 mM in a equal volume of 1X HSEB. After 5 minutes, nuclear matrices were recovered by centrifuging at 3,000 g for 10 minutes. The pellet was designated as nuclear matrix of high salt method (NM-HSM).

Extraction of proteins from the nuclei and nuclear matrix

Ten O.D of 260 units of nuclei were incubated in 100 μl of the TMN buffer [10 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, DNase I (50 μg / ml), RNase A (50 μg / ml) and aprotinin (5 μg / ml)] for 2 hours at 37°C before extracting the proteins.

Total proteins were extracted from the nuclei (after incubation with TMN buffer) and nuclear matrix in 5 ml of TUMP buffer (50 mM Tris-HCl pH 7.5, 5 M urea, 5 mM $MgCl_2$ and 1 mM PMSF) by homogenizing with pestle 'A' in Dounce homogenizer. The supernatant containing proteins was recovered by spinnig at 10,000 x g for 10 minutes and was dialysed against the TMP buffer (10 mM Tris-Hcl pH 7.5, 7 mM β-meracaptoethanol and 1 mM PMSF). The dialysate was further centrifuged at 10000 x g for 10 minutes and the clear supernatant containing proteins was used for further analysis.

Scanning electron microscopic studies

Nuclei and nuclear matrix pellets were fixed in 4 % glutaraldehyde for 90 minutes. They were washed with phosphate buffer saline (10 mM sodium phosphate buffer pH 7.4 and 150 mM NaCl) twice (at 1000 x g for 5 minutes) and final pellets were suspended in 1 % ammonium molybdate solution. This suspension was used for scanning in scanning electron microscope Joel JSM–35 after gold vapor coating. Photographs were taken at 20 KV with a 60 μ objective aperture in a Joel JSM-35 scanning electron microscope equipped with a roll film camera.

Protein estimation

The concentration of protein in different samples was estimated by Lowry *et al.*, (1951) method using bovine serum albumin as standard.

DEAE–cellulose chromatography

The protein samples were dialysed extensively against equilibration buffer (20 mM Hepes pH 7.4, 1 mM $MgCl_2$, 0.5 mM EDTA, 5 % glycerol and 0.1 mM PMSF) containing 50 mM NaCl. Preswollen DEAE was suspended in the same buffer and a 10 ml column was packed, washed and equilibrated with the same buffer. The dialysed protein sample was loaded onto the column. The flow through (unbound proteins) was collected and the column was washed with minimum 6-8 bed volumes of equilibration buffer (or till the absorbance of the wash was zero). The bound proteins were eluted with a stepwise gradient of NaCl (0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0 M) in equilibration buffer and 2 ml fractions were collected using a LKB-fraction collector. The optical density of the fractions was measured at 280 nm and peak fractions were analyzed by electrophoresis (SDS-PAGE).

HPLC anlysis of the proteins

HPLC analysis was performed in Shimpac PA-DEAE column of Shimadzu SCL-6AV. The protein sample was dialysed extensively against Na_2HPO_4 - Citric acid buffer (9 : 1 mix). The dialysed samples were centrifuged (at 10,000 x g for 15 minutes) and the clear supernatant was used for HPLC anlysis. Before loading sample (0.2 ml) to the column, it was equilibrated with Na_2HPO_4 - Citric acid buffer. The bound proteins were eluted by binary linear gradient of Na_2HPO_4 - Citric acid buffer. The peak fractions were collected and analysed by electrophoresis.

single strand DNA–cellulose chromatography

The protein sample was dialysed against TEDP buffer (20 mM Tris-HC1 pH 7.4, 1 mM EDTA, 1 mM DTT and 0.1 mM PMSF) containing 50 mM NaCl. The ssDNA–cellulose was suspended in TEDP buffer and left for 2 hours, further equilibrated with TEDP buffer containing 50 mM NaCl. A 5 ml column was packed and equilibrated with the same buffer. The dialysed protein sample was loaded on to the column, flow through (unbound proteins) was collected and washed with minimum 6-8 bed volumes of TEDP buffere containing 50 mM NaCl. The DNA binding proteins were eluted by a stepwise gradient of NaCl (0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0 M) in TEDP buffer and 1 ml fractions were collected using LKB-fraction collector. The optical density of the fractions was measured at 280 nm and were analysed by electrophoresis.

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)

Sample preparation for slab electrophoresis

Nuclei and / or nuclear matrix proteins in TMP buffer were precipi-

tated by adding 5 volumes chilled acetone, kept overnight at -70° and centrifuged at 10,000 x g for 5 minutes in a microfuge. The protein pelles were dissolved in SDS sample buffer (8 % sucrose, 1 mM DTT, 0.015 % bromphenol blue and 1 % SDS) and heated at 100°C for 5 minutes, and allowed to cool for 5 minutes on ice. The samples were cleared by centrifugation at 10,000 x g for 5 minutes and the clear supernatant with known quantity of protein was loaded on to the gel.

Proteins were separated by electrophoresis in 18 % polyacrylamide gels as described by Thomas and Khornberg (1975) with minor modifications as suggested by Reddy and Suryanarayana (1989). In brief, the modifications include :

1. the bis-acrylamide concentration in the resolving gel was decreased from 0.4 M to 0.2 M.
2. Tris concentration in the resolving buffer was increased from 375 mM to 750 mM and the pH was also increased from 8.8 to 9.1.
3. glycine concentration in the running buffer was reduced to the half of the original concentration (from 380 mM to 190 mM).

Electrophoretic runs were carried out in the slab gel of 0.1 cm thick and 16 cm long at 170 Volts for approximately 4 hours or till the dye front reaches to the bottom.

Staining with coomassie blue

After electrophoresis the gels were removed carefully and fixed in 7.5 % glacial acetic acid for 20 minutes on a shaker. The proteins in the gel were stained with staining solution (7.5 % glacial acetic acid, 50 % methanol

and 0.01 % coomassie brilliant blue R-250) for 1 hour and destained with the destaining solution (7.5 % glacial acetic acid and 5 % methanol) with several changes on the shaker.

Two-dimensional electrophoresis (2DE) [IEF–SDS–PAGE

Sample preparation for isoelectrofocusing (IEF)

Nuclei and / or nuclear matrix proteins in TMP buffer were concentrated by acetone precipitation. The protein in the sample was solubilized in 40 μl of sample lysis buffer [9.5 M urea, 2 % ampholines (1.6 % of 5-7, 0.4 % of 3–10.5), 2 % NP-40 and 5 % β–mercaptoethanol and 2 % SDS] by swirling at room temperature, followed by one freezing and thawing cycle. The IEF protein mix was centrifuged at 10,000 x g for 5 minutes and the clear supernatant was loaded on to the IEF gel.

Two-dimensional electrophoresis was done according to O'Farrel (1975) with minor modifications. In the first dimension, IEF tube gel was prefocussed by applying the voltage ; 200 V for 15 minutes, 300 V for 30 minutes and 400 V for 30 minutes. After prefocussing the samples were loaded and the gels were run for 9000 volt-hours [600 V for 12 hours, 700 V for one and half hour and 750 V for last one hour]. The IEF gels were removed and equlibrated in a equilibration buffer (1 % glycerol, 5 raM DTT, 2.3 % SDS and 0.625 M Tris–HCl pH 6.8) for half an hour to remove ampholines. The tube gels were transferred on to second dimension run (SDS-PAGE) with the stacking gel (4.75 % acrylamide and bis-acrylamide) over the resolving gel (11.27 % acrylamide and bis-acrylamide). The contact between IEF tube gel and second dimension slab gel was established by embedding with a hot solution of 1 % (w/v) agarose containing 0.0025 % (w/v) bromo–phenol blue (BPB). The second dimension run was performed at

15 mA till the tracking BPB dye enters into the separating gel, then at 20 mA till the dye reaches to the bottom. After completion of second dimension, gels were silver stained to identify the proteins.

Silver staining

Proteins were visualized by a sensitive silver staining procedure (Blum *et al.*, 1987) with minor modifications. After completion of the electrophoresis the stacking gel was removed and the separating gel was fixed in the fixing solution (50 % methanol, 12 % glacial acetic acid) for 1 hour on a shaker with gentle shaking and transferred into 50 % ethanol for 1 hour. The gels were treated with sodium thiosulphate (0.2 g / liter) for 1 minute and washed with distilled water for 1 minute, including three changes for every 20 seconds. Further, the gels were impregnated with silver nitrate (1 g / liter) containing 0.75 ml of 37 % formaldehyde for 30 minutes. After washing the gels with distilled water for 1 minute, the protein spots were developed with sodium carbonate (60 g / liter) containing 0.5 ml of 37 % formaldehyde per liter. After the protein spots developed, the gels were washed with distilled water and placed in fixing solution for 1 hour. The gels were immediately photographed and stored in 50 % ethanol.

Identification of **proteins**

In 2DE the proteins were identified basing on their molecular weight (Mr) and isoelectric point (p.I).

1. Determination of Molecular weight of the proteins

Molecular weight of the proteins was determined based on their relative co-migration along with known protein satandard markers (bovine albumin–66000 ; egg albumin–45000 ; glyceraldehyde–3– Phosphate

dehydrogenase-36000 ; carbonic anhydrase-29000 ; chymotrypsinogen-25000 ; trypsinogen-24000 ; soyabean trypsin inhibitor-20100 ; α-lact-albumin-14200).

2. Determination of the Isoeletric point of Protein

Isoelectric point of a specific protein was determined by comparison with pH gradient observed in the IEF tube gel with surface pH electrode (LKB-Producter) as well as gel extrusion into deionized water. The IEF gel was cut into the equal size (0.5 cm length) pieces and each gel piece was placed in 1 ml distilled water and allowed to stay overnight with gentle shaking. The pH extruded from the gel into the distilled water was measured with a pH meter. A standard graph was drawn by plotting gel length on X-axis and pH on the Y-axis.

Production of polyclonal antibodies

Polyclonal antibodies to Nuclear matrix proteins were raised according to Stoffler and Wittmann (1971) in albino rabbits. Nuclear matrix protein (1 mg / ml) present in TMP buffer was emulsified with equal volume of the Freund's complete adjuvant and injected subcutaneously into rabbit at multiple sites. After 4 weeks, booster injections each of 0.5 mg / ml of protein in Freund's incomplete adjuvant were given subcutaneously at 4 th, 5 th and 6 th weeks. Rabbits were bled after 3 rd booster injection through the pinna vein. Serum was collected after centrifugation of the clotted blood.

Immunodiffusion

Immunodiffusion (Ouchterlony) was performed as described by Stoffler and Wittmann (1971). Agarose (1.5 %) (w/v) was dissolved in 0.9 % NaCl and 19 mM sodium barbital buffer pH 8.4 by heating in boiling water bath

for 45 minutes. This was poured into immunodiffusion plates to a height of 0.3 cm and allowed to cool at room temperature. After cutting the wells, the test samples and antisera were placed in respective wells. The diffusion was allowed to take place for 12 hours at room temparature in moisture containing box. The immunodiffusion plates were photographed against dark background with scattering light. The immunodiffusion gels were extensively washed with 0.09 % NaCl and finally with water. The gels were dried and stained with coomassie brilliant blue as in SDS-PAGE gels.

Western blotting

Western blotting was performed according to Towbin *et al.*, (1979) with minor modifications. The nuclear matrix proteins were electrophoresed in the gel and transferred on to a nitrocellulose sheet (NC) (0.45 μ pore size) using TGM buffer (25 mM Tris-HCl pH 8.3, 0.192 M glycine and 20 % methanol) as a electrode buffer at 0.8 mA / cm^2 transunit (total gel length x breadth) in LKB-Multiphore II Nova blot for 2 hours. The electroblots were washed with TNT buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.1 % Tween-20) for 10 minutes and blocked with blocking buffer (5 % skimmed dry milk in TNT buffer) for 1 hour. The NC sheet was then incubated with 2 % primary antibody in blocking buffer for 2 hours with gentle shaking and washed six times each for 10 niinutes with TNT buffer. The blots were incubated with secondary antibody 1 : 2000 dilution of peroxidase conjugated anti-rabbit IgG in blocking buffer for 2 hours followed by washing six times each for 10 minutes with TNT buffer. For the colour reaction, the blots were soaked in TNHC buffer (10 mM Tris-HCl, 150 mM NaCl, 0.03 % hydrogen peroxide and 0.275 mg of 4-chloro-1-naphthol (4C1N). The reaction was terminated by washing the blot with water.

RESULTS

Results

In the present study the nuclear proteins were fractionated from whole brain (B), neuronal (N) and glial (G) nuclei using the procedure of Thompson (1973). The nuclei were subjected to nuclease digestion (DNase 1 and RNase A) for brief periods of time as indicated in the methods and were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Fig. 1). The pattern in general showed specific association of proteins with neuronal and glial fractions. In the glial fraction a protein with a molecular weight approximately 50 kDa (possibly GFAP) and a 32 kDa protein were found enriched, while another protein with 198 kDa was enriched in the neuronal fraction which could possibly be one of the members of neurofilamental triplet proteins (NFTs) basing on its molecular weight. The proteins enriched in neuronal and glial fractions could be observed together in whole brain fraction, which constitutes the nuclear protein fraction from the whole brain. The sodium dodecyl sulfate polyacrylamide gel electrophoretic pattern in overall suggests that, the total nuclear protein from whole brain fraction can be very clearly fractionated into neuron and glial fractions. The protein pattern in all these fractions matches with the pattern shown by Thompson (1973). Further, it indicates the presence of a heterogeneous type distribution of nuclear proteins in different cell types of brain.

The nuclear matrices were prepared from neuronal and glial nuclei by two different ways as described in the methods. Following the steps involved in the protocol the nuclear matrices were prepared using high salt method (HSM) and low salt method (LSM) as suggested and the matrices were subjected to the analysis by scanning electron microscopy (SEM) and biochemical characterization. The data from the scanning electron

microscopic studies (Fig. 2) suggest that nuclear matrix preparation whether by high salt method or low salt method do not show any morphological variation even though studies by others (Verheijen *et al.* 1988) using transmission electron microscopy (TEM) have shown wide variety of changes in the constitution of nuclear matrix preparation by high salt method and low salt method. The scanning electron microscopic pictures of nuclear matrix isolated by high salt method or low salt method showed that the shape of the nucleus was not altered even though most of its constituents were extracted while preparing the matrices, which agrees with earlier observations.

Fig. 3 shows the protein composition of nuclear matrix in neuronal and glial nuclei. The sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern showed slight variation which can mainly be attributed to the method of nuclear matrix preparation. In general, low salt method preparation yielded a matrix having relatively less protein composition than by high salt method. However, lamins were retained irrespective of high salt method or low salt method preparation of nuclear matrix. Since, the neuronal and glial fractions and the matrix protein composition in high salt and low salt methods suggested wide variations, a comparison of age dependent variations in nuclear proteins was done by isolating the nuclei from the brains of rats aged 10, 45 and 180 days. Further, the differences were not really obvious when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Therefore, the proteins were resolved on two dimensional electrophoresis (2DE) analysis, which involves separation of proteins by isoelectrofocussing (IEF) as well as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Earlier studies by others also showed that the high salt and low salt extracted matrices contain 6.5 and 1.9 % of protein respectively of the corresponding nuclear proteins suggesting that the nuclear matrices prepared by low salt method are relatively depleted structures compared to high salt extracted matrices (Smith *et al.* 1987). This is because of the methodological variations such as the presence of lithium 3, 5-diiodosalicylate, a chaotropic agent which allows stabilization of nuclear matrices. The data suggests that nuclear matrix preparation by low salt method (LSM) involving lithium diiodosalicylate (LIS) extraction efficiently depletes the non-histone chromosomal proteins (NHCPs) from the nucleus.

Neuronal and glial non-histone chromosomal proteins were analyzed from the brains of 10, 45 and 180 days aged rats. In Fig. 4. the top panels A, B and C show the pattern of non-histone chromosomal proteins from the neuronal cells. In over all the data suggest, that by the time the rat ages (development reaches) 45 days there are many proteins which seem to have been translocated into the nuclei. The 45 day samples accrue many number of peptides below the molecular weight range of 36 kDa. By the time the rat is 180 days old ; even though the low molecular weight proteins are retained, it is observed in general that 180 day neuronal nuclei contain relatively less number of proteins than 45 days sample. The panels D, E and F show the pattern of non-histone chromosomal proteins of glial nuclei. The non-histone chromosomal protein patterns of 10 and 45 days samples of glial nuclei do not show much variation, excepting for a slight quantitative increase in very few proteins. The number of non-histone chromosomal proteins between 10 and 45 days old samples remain almost unchanged. On the controversy, the 180 day old samples show up with many new proteins, which appear to be translocated into the glial nuclei of 180 days rat.

The nuclear matrices prepared from 10 and 45 day old neurons by low salt method and high salt method are shown in Fig. 5. At the age of 180 days most of the nuclear matrix proteins contained lamins A, B and C. More or less similar features are seen in case of 10 day old samples also. But the lamins were very faintly expressed in the nuclear matrix prepared by low salt method of 10 day old neurons. The non-lamin proteins associated with nuclear matrix were in general more at the age of 180 days, particularly in the nuclear matrix prepared by low salt method. It is rather intriguing to explain more number of nuclear proteins associated with matrix as the lithium diiodosalicylate extraction is known to deplete most of the proteins.

Fig. 6. represents nuclear matrix proteins from the glial cells of 45 and 10 day old rat. Similarly, the 180 day old rat neuronal and glial nuclear and nuclear matrix proteins pattern were shown in Fig. 7. In the nuclear matrix prepared by high salt method and low salt method the lamins A, B and C could be identified in 45 days as well as 10 day samples. However the lamin association with the nuclear matrix in glial cells appears to be rather selective. In the high salt method preparation of 180 day old glia, lamin B and C alone are seen but not A, while in low salt method sample appears to contain only lamin B. On the other hand the high salt method samples of 10 day glia contain all the lamins but the low salt method samples contain lamins A and C but not B. Apart from the selective association of lamins to the nuclear matrix the low salt method and high salt method preparations contain several other matrix associated proteins as can be noticed from the figure. In general the lamins were more associated with high salt extracted matrix when compared to the low salt extracted matrix of glia. Though in 10 day old preparation of high salt method lamin A, B and C could be noticed, in 45 days old sample only B and C could be identified. In low salt method preparations lamin A or B alone could be identified ; suggesting that low

salt method preparation might possibly deplete selective lamins. In addition to the alterations in the lamin composition, the non-lamin proteins associated with matrix appear to be differing in high salt method and low salt method, which is an interesting aspect in the present study. Since the matrix composition was shown to vary by the age, the method (high salt and low salt) and in different cell types (neurons and glia), only 45 days aged rats were chosen for further study. Since most of the proteins were in acidic range, the diethylaminoethyl (DEAE) cellulose column chromatography was employed to separate the matrix proteins.

In an attempt to fractionate nuclear matrix proteins, the nuclear matrix proteins were solubulized in 5 M urea and subjected to DEAE cellulose chromatography. The proteins were eluted with increasing concentrations of NaCl in a step gradient of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 M. The proteins in the eluates were precipitated with acetone after extensive dialysis. The protein fractions resolved from high salt matrix preparation of neurons after DEAE are shown in Fig. 8, while low salt matrix preparation of neurons subjected to DEAE chromatography under similar condition are shown in Fig. 9. The chromatograms at optical density at 280 nm peaks during step wise gradient elution shown in panel A. The peak fractions, when analyzed in sodium dodecyl sulfate polyacrylamide gel electrophoresis showed 2 to 3 bands ; but showed several bands on two dimensional electrophoretic analysis. The protein patterns from different fractions of high salt method showed mostly lamins, while the low salt method preparation yielded a better resolved protein separation in the sense that it had more of non-lamin proteins separated particularly in 0.4 M and 0.6 M elutes. Since most of these proteins were present in small amounts, the fractions were pooled and analyzed by two dimensional electrophoresis followed by silver staining. Though the nuclear matrix proteins particularly in low salt method preparation could be separated through DEAE

column, the step wise gradient elution failed to yield a fraction which is entirely homogeneous and made of a single protein. Consequently, most of the proteins were resolved in groups and could be identified in two dimensional gel electrophoretic gels in Fig. 8.

The high and low salt matrix proteins from glial cells were subjected to DEAE chromatography under similar conditions as described for neuronal matrix preparation. The DEAE chromatograms for glial high salt method (Fig. 10) and glial low salt method (Fig. 11) indicate that the 0.6 M fraction eluated from glial high salt method preparation contain lamins and very low molecular weight proteins which could possibly be some small ribonuclear proteins (RNPs) basing on their positions in a two dimensional electrophoresis (2DE). Though the small ribonuclear proteins were separated as early as 0.2 M, their separation was complete only in 0.6 M NaCl buffer. The 1.0 M eluate still showed up few lamins.

The low salt method preparation of glia during DEAE cellulose chromatography yielded fractions having a heterogeneous group of proteins which look like high mobility group (HMG) type, most of them were in high molecular weight range. The results from the DEAE cellulose chromatography of glial high salt and low salt matrix preparations indicate that like in the case of neuronal matrix preparation glial high salt method preparation yielded a protein separation, which is better resolved in low molecular weight range. Conversely, the glial low salt method preparation yielded chromatograms having high mobility group type of proteins associated with the nuclear matrix. These results indicate that the separation of nuclear matrix proteins by DEAE column chromatography differs depending on high salt and low salt preparation of matrices and the composition of proteins separated by DEAE chromatography differs quantitatively as well as qualitatively.

Since the DEAE chromatography was rather unsuccessful in terms of separating nuclear matrix proteins, the preparation was further subjected to high performance liquid chromatography (HPLC) on a Shimpak PA-DEAE column of Shimadzu SCL-6AV using binary linear gradient elution at a pH gradient of 2.6-7.0 with the buffer system citric acid and sodium phosphate. The peak fractions were collected and the protein was precipitated with acetone and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Patterns of high performance liquid chromatography fractions for high and low salt preparation have been shown in Fig. 12 (HPLC profiles of neuronal nuclear matrix proteins prepared by high and low salt method). The high performance liquid chromatography even though has yielded separation into peaks during binary linear gradient elution the sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of these fractions very clearly indicated that each of these peaks contain groups of proteins, most of them concentrated at high molecular weight range. An exactly similar kind of patterns were obtain from the high and low salt nuclear matrix preparation from glial cells as shown in Fig. 13. The high performance liquid chromatographic separation pattern also supports the results obtained in DEAE chromatography that the matrix proteins can not be resolved into individual protein by DEAE chromatography and therefore a combination of approaches have to be formulated in order to separate the matrix proteins.

Since most of the nuclear matrix proteins are implicated to be associated with replication forks during DNA replication, an attempt was made to identify single strand deoxyribonucleic acid (ssDNA) binding proteins on single strand DNA cellulose column. The single strand DNA binding of neuronal nuclear matrix proteins extracted by low salt method (Fig. 14) were eluted with increasing concentration of NaCl in a step

gradient of 0.1, 0.2, 0.4, 0.6 and 1.0 M and the proteins in the eluates were precipitated with acetone and solubulized in O'Farrel buffer and subjected to two dimensional electrophoresis. The 0.1 and 0.2 M fractions had groups of proteins while 0.4 M fraction did not show up any proteins. On the other hand 0.6 and 1.0 M fraction had several groups of proteins. Usually in a DNA cellulose chromatography, proteins eluted between 0.2 to 0.8 M are suspected to involve in regulatory function, while proteins eluted above 1.0 M might involve in structural organization of chromatin. In the present study presence of several groups of proteins in 0.6 and 1.0 M fractions of neuronal nuclear matrix proteins extracted by low salt method suggest that most of the nuclear matrix proteins might have a regulatory role.

Differing with this pattern, the DNA binding nuclear matrix proteins of glial cells showed (Fig. 15) almost no protein in fractions of above 0.4 M NaCl. The lamins were eluted at 0.2 M ionic strength itself and further elution yielded not many proteins unlike in the case of neuronal nuclear matrix prepared by low salt method with respect to single strand DNA binding protein. Though a clear function can not be attributed it can be speculated that neurons being non-replicating cells, most of the single strand DNA binding proteins of neuronal nuclear matrix prepared by low salt method observed at 0.6 and 1.0 M elutes might possibly involve in DNA repair or recombination and transcription. Under the condition of single strand DNA cellulose chromatography the results very clearly show that the neuronal nuclear matrix proteins prepared by low salt method were found to bound to single strand DNA, significantly better than the glial nuclear matrix proteins prepared by low salt method.

The immuno cross reactivity of different antibodies was checked by Ouchterlony (Fig. 16) and further by western analysis. The nuclear and matrix proteins have been separated on sodium dodecyl sulfate poly-

acrylamide gel electrophoresis and transferred on to nitrocellulose paper, which was subjected to western blotting analysis using different polyclonal antibodies as probes.

Anti serum to total nuclear matrix proteins of NHSM (neuronal nuclear matrix proteins extracted by high salt method), NLSM (neuronal nuclear matrix proteins extracted by low salt method), GHSM (glial nuclear matrix proteins extracted by high salt method) and GLSM (glial nuclear matrix proteins extracted by low salt method) was produced in rabbits by injecting total nuclear matrix protein. The cross reactivity of all the four antibodies (NHSM-antibody, NLSM-antibody, GHSM-antibody and GLSM-antibody) is shown in Fig. 17. The panel–A shows the immune reactivity of NHSM-antibody with all the nuclear and nuclear matrix preparations. In general, the antibodies raised for low salt extracted nuclear matrix preparations of neuronal and glia showed relatively intense immune reactivity. On the other hand, the high salt extracted nuclear matrix antibodies showed reactivity with polypeptides having a molecular weight range from 25 to 120 kDa. Further, an interesting feature was observed by the low salt matrix antibodies of neuronal as well as glia (Panel–B and D in lanes 4 and 5). Low salt matrix antibodies react with a polypeptide around 70 kDa, which could not be identified in the immunoblots treated with high salt matrix antibodies of neuronal and glia. The immunoblots further suggest the existence of a common group of nuclear matrix proteins irrespective of the cell type and method of matrix preparation. However, the identification of specific polypeptide reactivity with low salt matrix antibodies indicates a possibility for the presence of either cell or / and matrix method dependent enrichment of specific matrix proteins in the nuclei. This data agrees with the earlier reports in the literature (Stuurman *et al.*, 1990 ; Dworetzky *et al.*, 1990).

The tissue specific and brain region specific immune reactivity of the four antibodies is shown in Fig. 18. Considering the regional heterogeneity of the brain, the different regions of the brain (cerebellum, brain stem, cerebral cortex, hippocampus and mid brain), the brain stem showed very high reactivity, whereas mid brain showed least reactivity. In all the five different regions of the brain, three major NHSM reactive polypeptides with a molecular weight 105, 38 and 20 kDa were identified. Cerebral cortex, brain stem and hippocampus possessed another protein of 50 kDa. The NHSM antibodies were least reactive with non-neuronal tissues examined in the present study (such as liver, heart, kidney, muscle and lung). Kidney showed very faint immune reactivity particularly with a polypeptide around 80 kDa, while heart also showed a very minor protein around 70 kDa.

The NLSM antibody was reactive with a polypeptide around 38-40 kDa from all the regions of the brain. This polypeptide further showed intense reactivity with brain stem and hippocampus rather than other regions of the brain. However the NLSM antibodies did not react with any other polypeptides either from the brain or from other tissues examined.

The GHSM antibodies showed an immune reactive pattern which is almost similar to that of NHSM antibody with minor variations. The variations include the 20 kDa polypeptide seen in NHSM was not reactive with GHSM antibody. The 80 kDa polypeptide band was more intense in GHSM (in contrast to NHSM). The tissue pattern showed the presence of 90 kDa protein in heart more prominently than in NHSM. More than that appearence of 40 kDa protein in heart also observed in GHSM. Three different proteins (90, 80, 75 kDa) in muscle and 90 kDa protein in lung were noticed.

The GLSM antibodies showed an immune reactive pattern which is close to the pattern of NLSM antibodies. The 90 kDa protein band was reactive in brain stem, hippocampus and very faintly in cerebral cortex. There was no immune reactivity with the other tissue proteins.

The immunological data using NHSM and GHSM antibodies show that there is a regional heterogeneity of nuclear matrix protein distribution with in the brain mostly with two different categories of matrix proteins such as a large fraction of common nuclear matrix proteins and a small fraction of very specific nuclear matrix proteins. This diversity also is extended to non-neuronal tissues as very few polypeptides reactive to NHSM and GHSM antibodies are identified from other tissues such as heart, kidney and muscle. The NLSM and GLSM antibodies were more specific and limited in their distribution. They were reactive and present in almost all regions of brain but specific only to brain and these are either not reactive or atleast reactive with non-neural tissues, suggesting that the NLSM and GLSM antibodies are almost specific polyclonal antibodies to brain.

Using these polyclonal antibodies, the cross reactivity was checked with different cancer cell lines of neuronal as well as non-neuronal origin. The sodium dodecyl sulfate polyacrylamide gel electrophoretic pattern of neuronal nuclear, C6, SK-N-SH, U-373MG, IMR-32 and Neuro-2A is shown in Fig. 19. The C6 and U-373MG are of glioblastoma in origin while SK-N-SH and Neuro-2A are Neuroblastoma, IMR-32 is a neuroblastoma mixed with fibroblast. The sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis showed a different pattern in IMR-32 compared to other cell lines. The nuclear proteins from all these cell lines were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis and transferred on to an immunoblot in quadruplicates. These blots were probed with all the four different antibodies

(NHSM-Ab, NLSM-Ab, GHSM-Ab and GLSM-Ab) (Fig. 20). The NHSM and NLSM antibodies showed no reactive species of polypeptides in all the tumor cell lines. However, GHSM and GLSM antibodies showed a immune reactive specific pattern with different tumor cell lines. The GHSM antibodies showed reactive polypeptides of 66, 45 and 36 kDa proteins in C6, SK-N-SH and U-373MG tumor cells. Of the three proteins the 36 kDa peptide showed an intense staining. The tumor cell lines IMR-32, Neuro-2A showed no cross reactivity with these antibodies. Interestingly the GLSM antibodies showed immune reactivity with same tumor cell types but it was with a 29 kDa protein but not with 66, 45 and 36 kDa reactive species. The GHSM antibody recognized a 45-50 kDa protein in Neuro-2A tumor cell line, which can be recognized by GLSM anti-bodies. On the whole the data suggests two polypeptides 36 and 29 kDa are very prominently recognized by the GHSM and GLSM antibodies, in neuroblastoma and astrocytomas, suggesting differential expression of nuclear matrix proteins in different tumor cell line which could be of basic importance in identification and classification of tumors.

Fig. 1 : Protein patterns of the nuclear fractions. Nuclear fractions were electro-phoresed on SDS-PAGE gels as described in materials & methods and stained with silver nitrate.

Mr = Molecular weight markers (bovine albumin–66000 ; egg albumin–45000 ; glyceraldehyde–3–phosphate dehydrogenase-36000 ; carbonic anhydrase-29000 ; trypsinogen-24000 ; soyabean trypsin inhibitor-20100 ; α–lactalbumin–14200).

B = Whole brain nuclear proteins

N = Neuronal nuclear proteins

G = Glial nuclear proteins.

a = 50 kDa (GFAP)

b = 190 kDa (NFP)

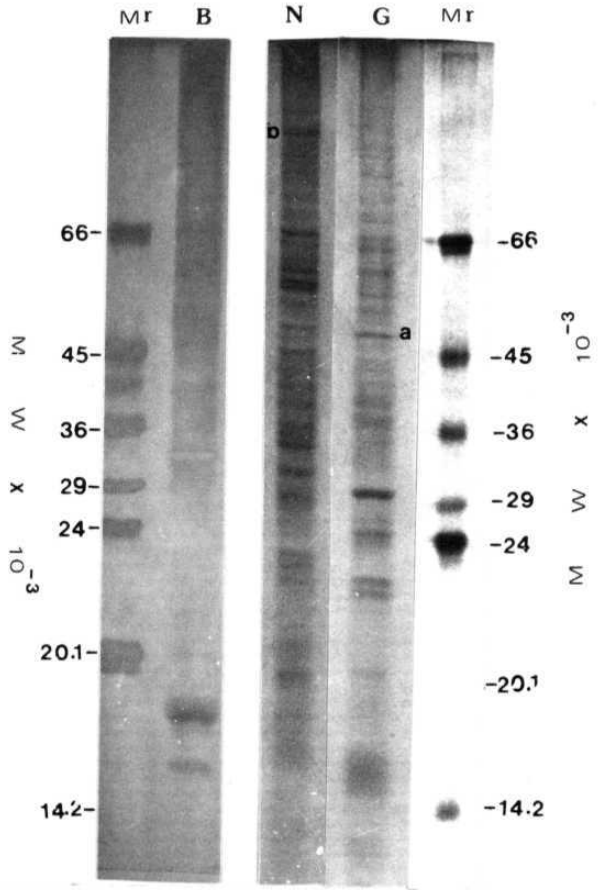

Mr
B
N
G
Mr
b
a
66-
45-
36-
29-
24-
20.1-
14.2-
M W x 10-3
-66
-45
-36
-29
-24
-20.1
-14.2
M W x 10-3

Fig. 2 : Scanning electron micrographs of nuclei and nuclear matrices. Nuclear matrices were isolated and prepared as described in experimental procedures.

Panel A = Nuclei from neurons

Panel B = Nuclear matrix from neurons prepared using high salt extraction method

Panel C = Nuclear matrix from neurons prepared using low salt extraction method

Panel D = Nuclei from glial cells

Panel E = Nuclear matrix from glia prepared by high salt extraction method

Panel F = Nuclear matrix from glia prepared using low salt extraction method.

Magnification on SEM 6000 X. (Note that the nuclear shape is retained despite high salt and low salt extraction methods to isolate nuclear matrices)

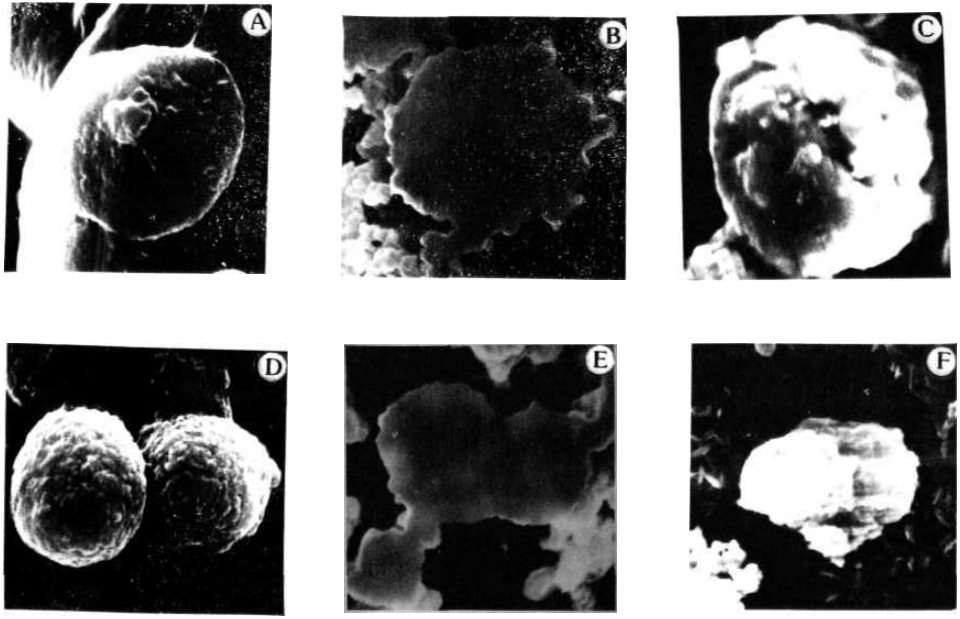

A
B
C
D
E
F

Fig. 3 : SDS-PAGE analysis of the nuclei and nuclear matrix associated proteins in neurons and glia

NN = Neuronal nuclear proteins

NHSM = Neuronal nuclear matrix prepared by high salt extraction method

NLSM = Neuronal nuclear matrix prepared using low salt extraction method

GN = Glial nuclear proteins

GHSM = Glial nuclear matrix prepared by high salt extraction method

GLSM = Glial nuclear matrix prepared using low salt extraction method.

Mr = Molecular weight markers (X 10^{-3} daltons)

Panel A = in 18 % gel

Panel B = 12.5 % gel

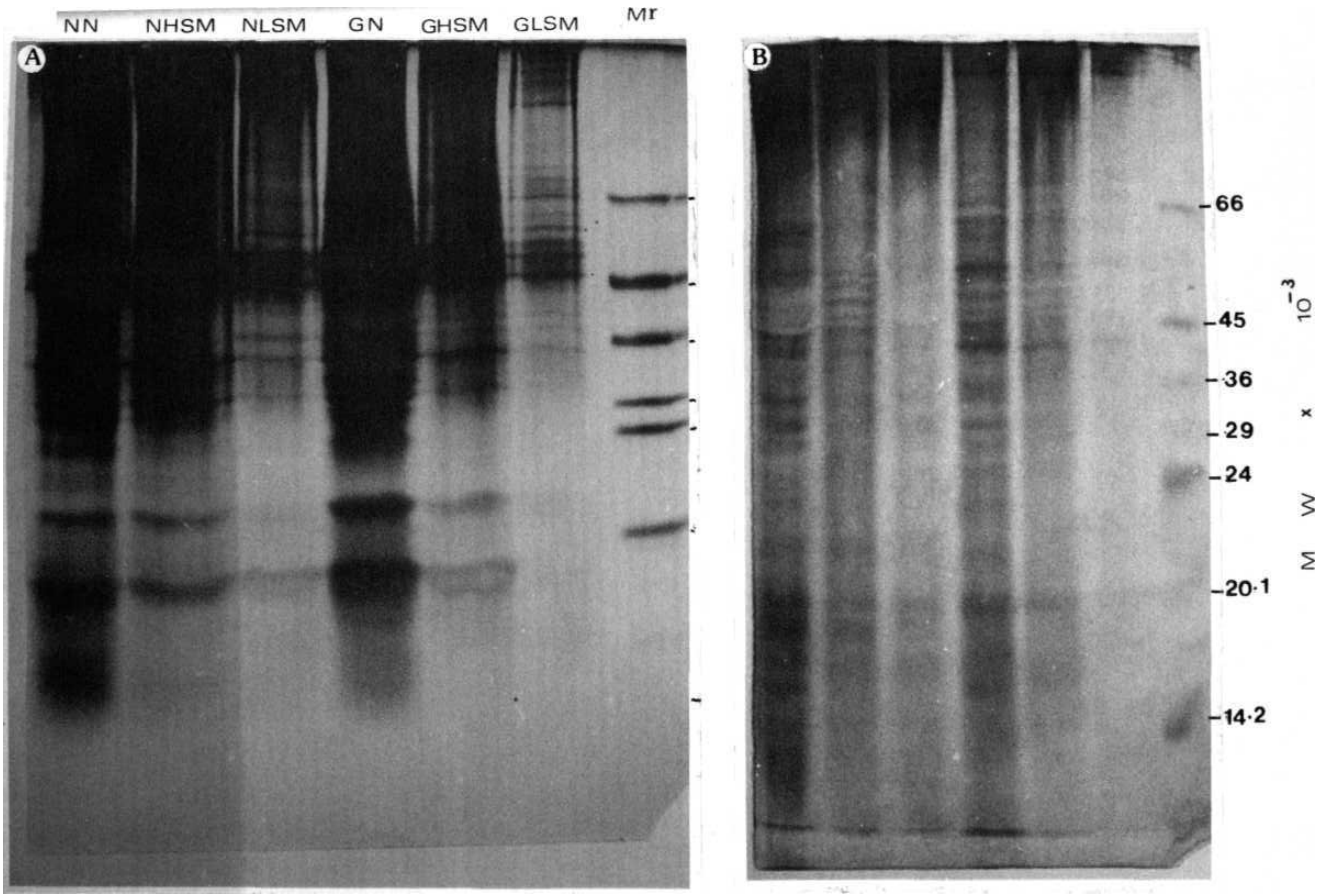

NN
NHSM
NLSM
GN
GHSM
GLSM
Mr
A
B
66
45
36
29
24
20·1
14·2
M W x 10⁻³

Fig. 4 : Developmental expression of neuronal and glial nuclear proteins. Silver stained two dimensional polyacrylamide gel electrophoretic patterns of proteins from

A = Neuronal nuclei of rat brain (10 days old)

B = Neuronal nuclei of rat brain (45 days old)

C — Neuronal nuclei of rat brain (180 days old)

D = Glial nuclei of rat brain (10 days)

E = Glial nuclei of rat brain (45 days old)

F = Glial nuclei of rat brain (180 days old)

Molecular weight (MW X 10^{-3} daltons) shown on the right side of the figure while IEF pattern runs acidic to basic from left to right on the abcissa of each panel.

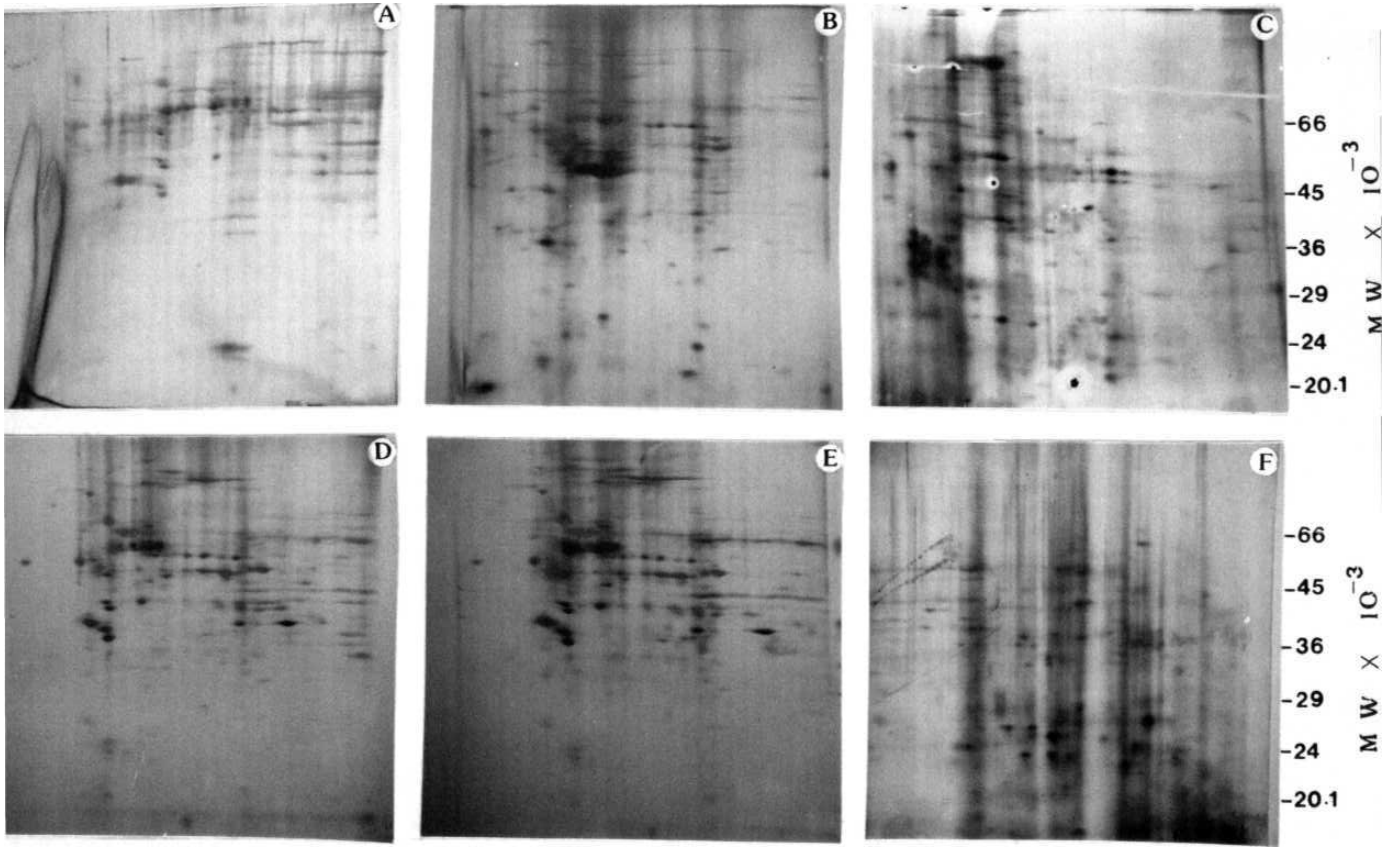

A
B
C
D
E
F
66
45
36
29
24
20.1
MW × 10⁻³

Fig. 5 : Developmental expression of neuronal nuclear matrix proteins. Silver stained two-dimensional polyacrylamide gel electrophoretic patterns of proteins from :

A = Neuronal nuclei of rat brain (45 days)

B = Neuronal nuclear matrix isolated by HSM (45 days old)

C = Neuronal nuclear matrix isolated by LSM (45 days old)

D = Neuronal nuclei of rat brain (10 days)

E = Neuronal nuclear matrix isolated by HSM (10 days old)

F = Neuronal nuclear matrix isolated by LSM (10 days old)

a = Lamin A

b = Lamin B

c = Lamin C

Molecular weight (MW X 10^{-3} daltons) shown on the right side of the figure while IEF pattern runs acidic to basic from left to right on the abcissa of each panel.

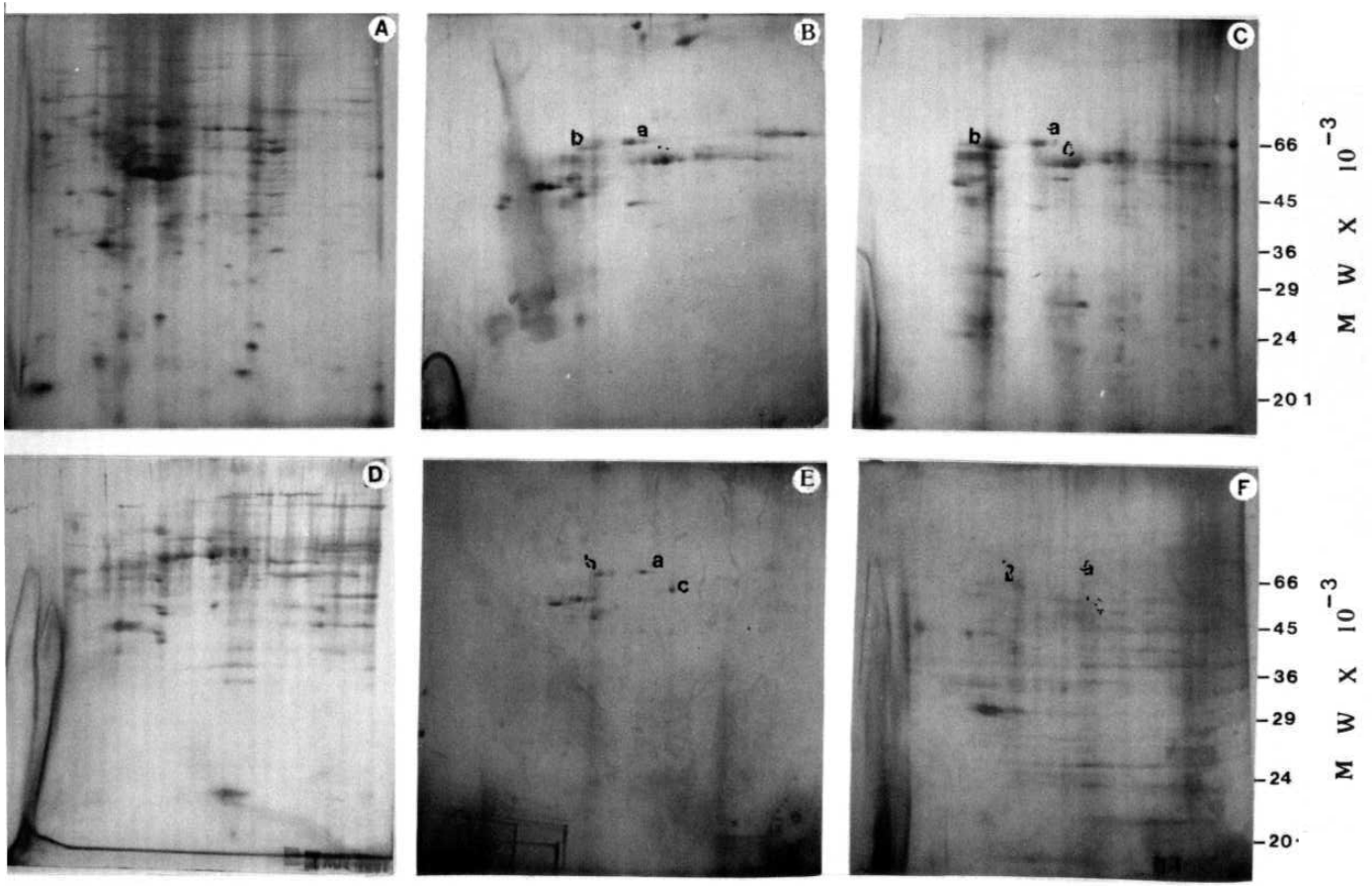

A
B
b
a
C
b
a
c
66
45
36
29
24
20 1
M W X 10 -3
D
E
a
c
F
66
45
36
29
24
20
M W X 10 -3

Fig. 6 : Developmental expression of glial nuclear matrix proteins : silver stained two dimensional polyacrylamide gel electrophoretic patterns of proteins from

A = Glial nuclei of rat brain (45 days)

B = Glial nuclear matrix isolated by HSM (45 days old)

C = Glial nuclear matrix isolated by LSM (45 days old)

D = Glial nuclei of rat brain (10 days)

E = Glial nuclear matrix isolated by HSM (10 days old)

F = Glial nuclear matrix isolated by LSM (10 days old)

a = **Lamin** A

b = Lamin **B**

c = **Lamin** C

Molecular weight (MW X 10^{-3} daltons) is shown on the right side of the figure while IEF pattern runs acidic to basic from left to right on the abcissa of each panel.

A
B
C
D
E
F
a
b
c
66
45
36
29
24
20·1
M W × 10⁻³

Fig. 7 : Developmental expression of neuronal and glial nuclear and nuclear matrix proteins : silver stained two dimensional polyacrylamide gel electrophoretic patterns of proteins from

A = Neuronal nuclei of rat brain (180 days old)

B = Neuronal nuclear matrix isolated by HSM (180 days old)

C = Neuronal nuclear matrix isolated by LSM (180 days old)

D = Glial nuclei of rat brain (180 days)

E = Glial nuclear matrix isolated by HSM (180 days old)

F = Glial nuclear matrix isolated by LSM (180 days old)

Molecular weight (MW X 10^{-3} daltons) is shown on the right side of the figure while IEF pattern runs acidic to basic from left to right on the abcissa of each panel.

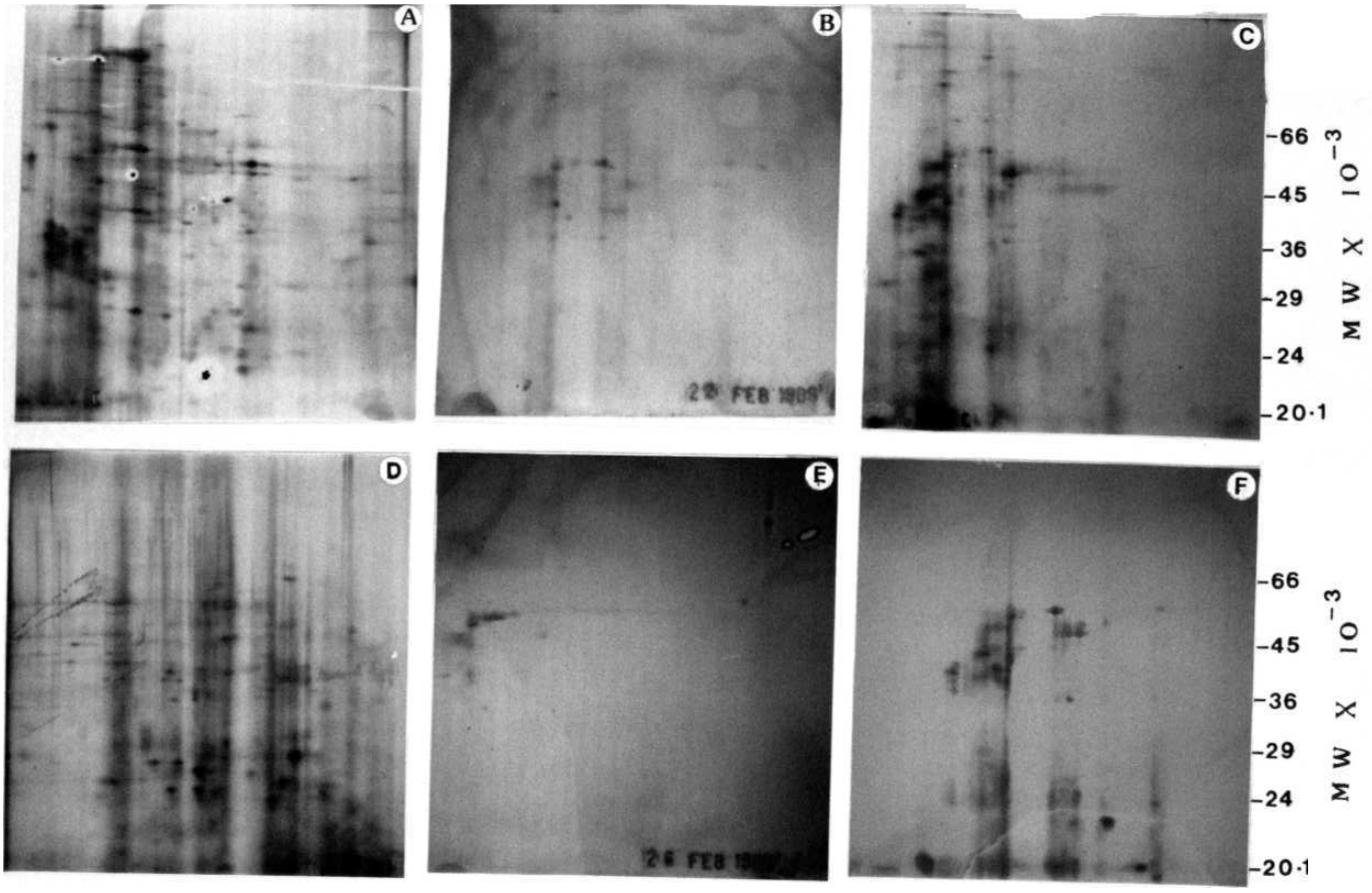

A
B
C
D
E
F
-66
-45
-36
-29
-24
-20·1
MW X 10-3
-66
-45
-36
-29
-24
-20·1
MW X 10-3

Fig. 8 : Isolation and fractionation of proteins from neuronal nuclear matrix extracted by high salt method from 45 days old rat brain. The nuclear matrix protein sample was applied on to a pre-equilibrated DEAE column with equilibration buffer and bound proteins were eluted in a non-linear gradient fractionation with increasing ionic strength buffers (0.1 to 1.0 M). Elutes were collected in 1 ml fractions and the absorbance profile at A 280 nm is shown in panel A.

Other panels show two dimensional gel analysis of 0.2, 0.4, 0.6, 0.8 and 1.0 M peak fractions as denoted in the panels.

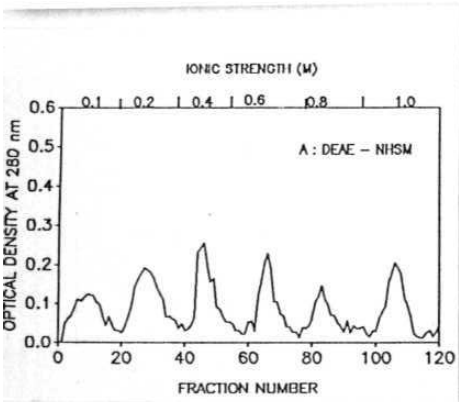
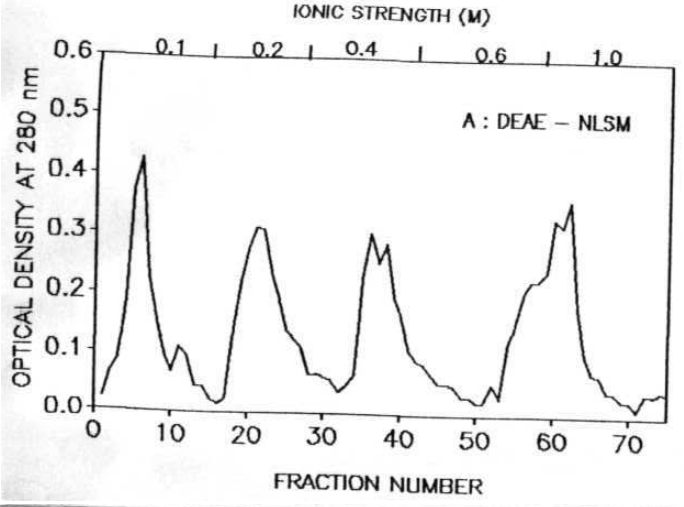
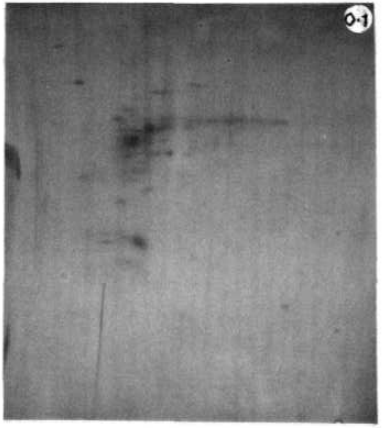
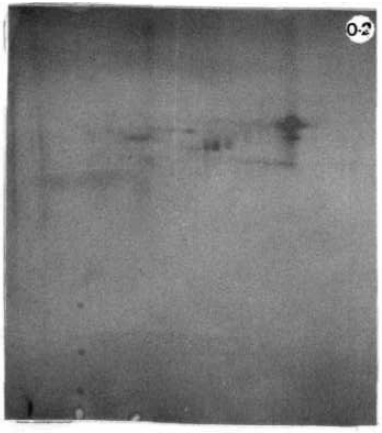

IONIC STRENGTH (M)
0.1
0.2
0.4
0.6
0.8
1.0
A : DEAE – NHSM
OPTICAL DENSITY AT 280 nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
20
40
60
80
100
120
FRACTION NUMBER

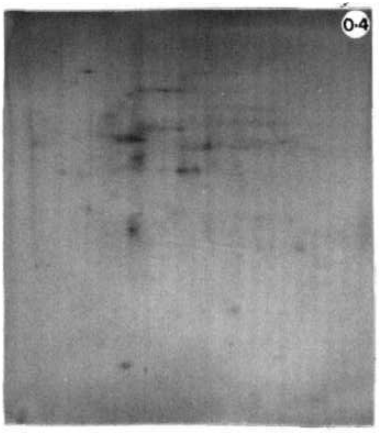
Fig. 9 : Isolation and fractionation of proteins from neuronal nuclear matrix extracted by low salt method from 45 days old rat brain. The nuclear matrix protein sample was applied on to a pre-equilibrated DEAE column with equilibration buffer and bound proteins were eluted in a non-linear gradient fractionation with increasing ionic strength buffers (0.1 to 1.0 M). Elutes were collected in 1 ml fractions and the absorbance profile at A 280 nm is shown in panel A.

Other Panels show two dimensional gel analysis of 0.1, 0.2, 0.4 and 0.6 M peak fractions as denoted in the panels.


IONIC STRENGTH (M)
0.1
0.2
0.4
0.6
1.0
A : DEAE – NLSM
OPTICAL DENSITY AT 280 nm
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
70
FRACTION NUMBER


0·1


0·2


0·4

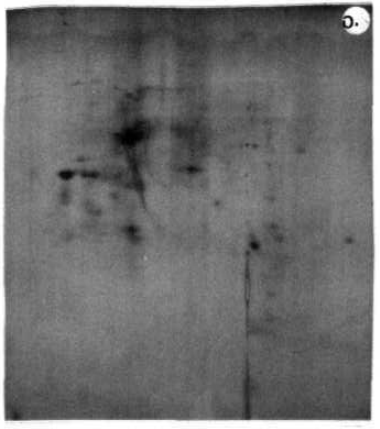
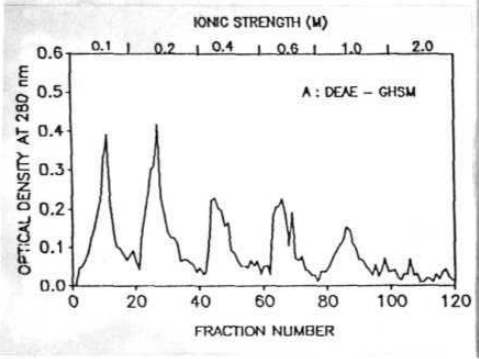
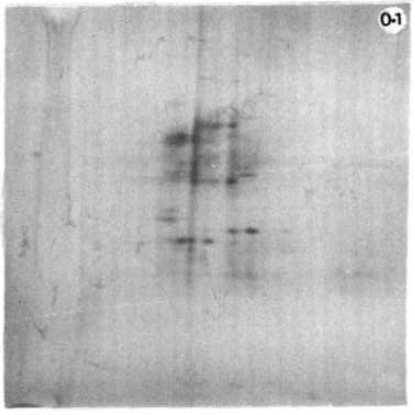
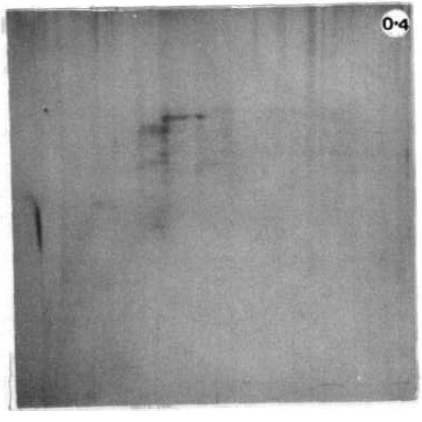

0·

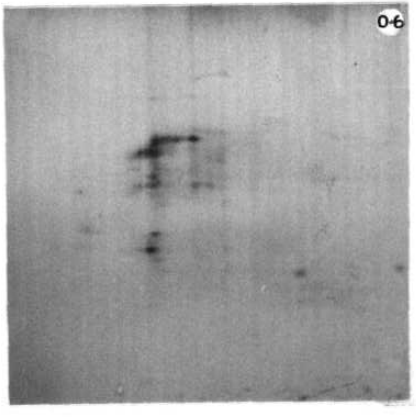
Fig. 10 : Isolation and fractionation of proteins from glial nuclear matrix extracted by high salt method from 45 days old rat brain. The nuclear matrix protein sample was applied on to a pre-equilibrated DEAE column with equilibration buffer and bound proteins were eluted in a non-linear gradient fractionation with increasing ionic strength buffers (0.1 to 1.0 M). Elutes were collected in 1 ml fractions and the absorbance profile at A 280 nm is shown in panel A.

Other panels show two dimensional gel analysis of 0.1, 0.2, 0.4, 0.6 and 1.0 M peak fractions as denoted in the panels.


IONIC STRENGTH (M)
0.1
0.2
0.4
0.6
1.0
2.0
A : DEAE – GHSM
OPTICAL DENSITY AT 280 nm
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
20
40
60
80
100
120
FRACTION NUMBER


0·1


0·4


0·6

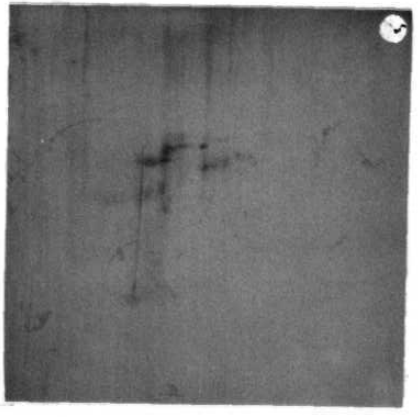


Fig. 11 : Isolation and fractionation of proteins from glial nuclear matrix extracted by low salt method from 45 days old Rat brain. The nuclear matrix protein sample was applied on to a pre-equlibrated DEAE column with equilibration buffer and bound proteins were eluted in a non-linear gradient fractionation with increasing ionic strength buffers (0.1 to 1.0 M). Elutes were collected in 1 ml fractions and the absorbance profile at A 280 nm is shown in panel A.

Panel B = shows SDS-PAGE analysis of the elutes in different fractions.

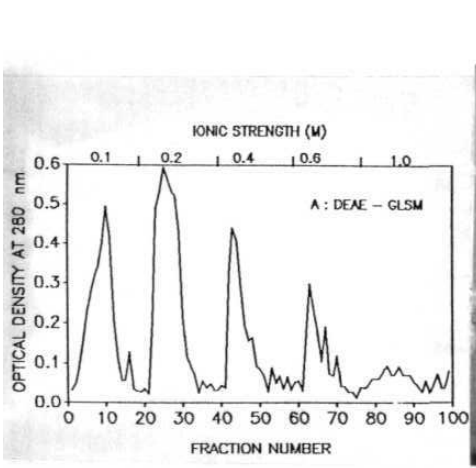
BLC = Before loading on to the column

A = After washing with equilibration buffer

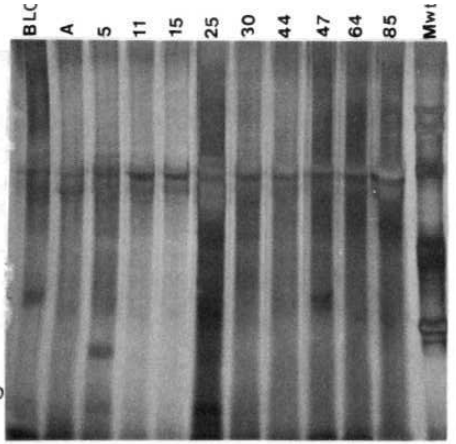
5-85 = correspond to fraction numbers

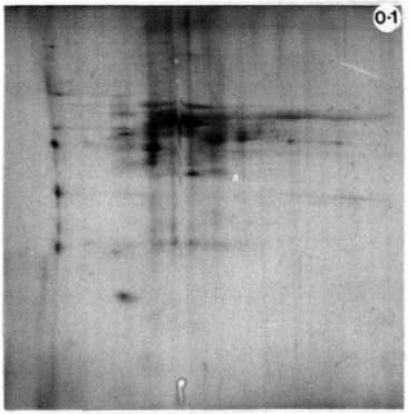
M.wt = Molecular weight markers (bovine albumin-66000 ; egg albumin-45000 ; glyceraldehyde-3-phosphate dehydrogenase-36000 ; trypsinogen-24000).

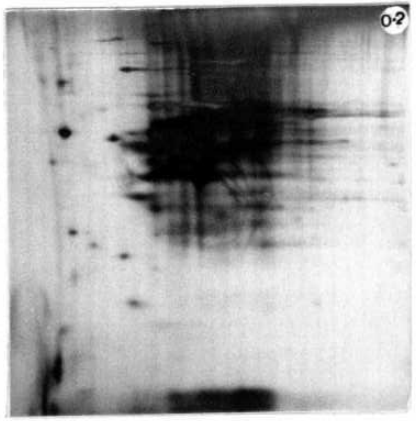
Other Panels show two dimensional gel analysis of 0.1, 0.2, 0.4 and 0.6 M peak fractions as denoted in the panels.

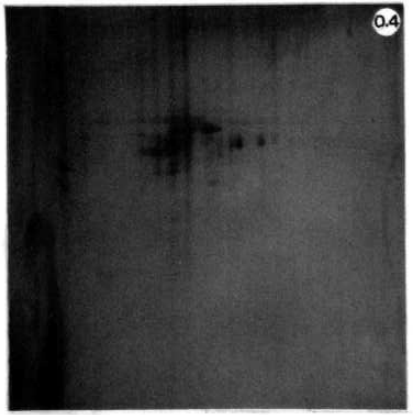

IONIC STRENGTH (M)
0.1
0.2
0.4
0.6
1.0
A : DEAE – GLSM
OPTICAL DENSITY AT 280 nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0 10 20 30 40 50 60 70 80 90 100
FRACTION NUMBER

B


BLC
A
5
11
15
25
30
44
47
64
85
Mwt


0-1


0-2


0.4

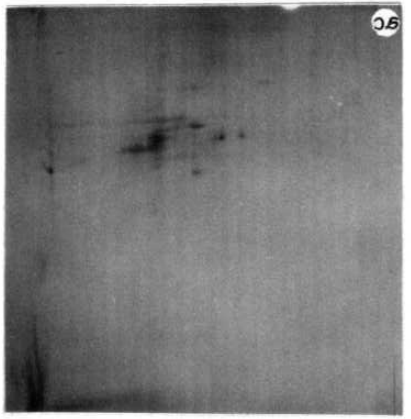

0.6

Fig. 12 : Nuclear matrix proteins were prepared and analysed by high performance liquid chromatography employing Shimpack PA-DEAE column. The proteins were eluted in binary linear gradient mode with citric acid-phosphate buffer system.

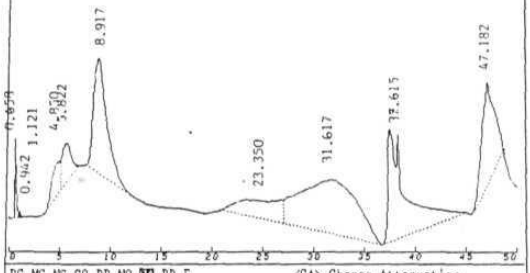
Panel A = Chromatogram of proteins associated with neuronal nuclear matrix prepared by high salt method.

Panel B = SDS-PAGE analysis of peak fractions. The number corresponds to the peak fraction in chromatogram of Panel A.

Panel C = Chromatogram of proteins associated with neuronal nuclear matrix prepared by low salt method.

Panel D = SDS-PAGE analysis of peak fractions. The number corresponds to the peak fraction in chromatogram of panel C.

A

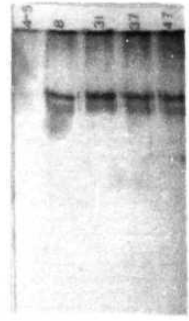


PC MC MS SO PP MO M2 PR E <CA> Change Attenuation

** CALCULATION REPORT **

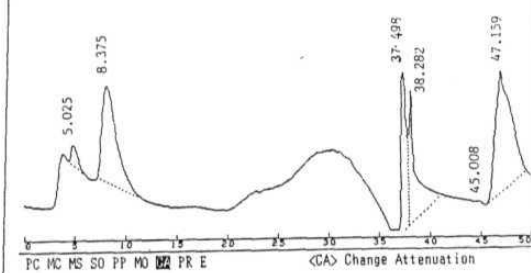
CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC
1	1	0.658	1245	174			1.0815
	2	0.942	76	17	V		0.0657
	3	1.121	48	12	V		0.0416
	4	4.85	3118	55			2.9688
	5	5.822	4106	64	V		3.5664
	6	8.917	18235	199			15.8383
	7	23.35	9756	28			8.4744
	8	31.617	38065	96	V		33.0612
	9	37.615	24918	183			21.6429
	10	47.182	15267	163			13.2602
		TOTAL	115134	990			100

B



COLUMN : SHIMPACK PA DEAE (F-9054)
SOLVENT : 0.1 M CITRIC ACID AND
0.2 M Na_2HPO_4, pH GRADIENT
FROM 2.6 TO 7.0
DETECTOR : 280 nm
HPLC : SHIMADZU LC-6AD
FLOW RATE : 1ml per minute
SAMPLE : NEURONAL NUCLEAR MATRIX
BY HIGH-SALT METHOD

C



PC MC MS SO PP MO M2 PR E <CA> Change Attenuation

** CALCULATION REPORT **

CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC
1	1	5.025	1709	36			2.2186
	2	8.375	17426	174			22.6191
	3	37.498	10875	304			14.1164
	4	38.282	23095	247	V		29.9771
	5	45.008	481	10	V		0.6247
	6	47.159	23454	216			30.4439
		TOTAL	77041	988			100

D

COLUMN : SHIMPACK PA DEAE (F-9054)
SOLVENT : 0.1 M CITRIC ACID AND
0.2 M Na_2HPO_4, pH GRADIENT
FROM 2.6 TO 7.0
DETECTOR : 280 nm
HPLC : SHIMADZU LC-6AD
FLOW RATE : 1ml per minute
SAMPLE : NEURONAL NUCLEAR MATRIX
BY LOW-SALT METHOD

Fig. 13 : Nuclear matrix proteins were prepared and analysed by high performance liquid chromatography employing Shimpack PA-DEAE column. The proteins were eluted in binary linear gradient mode with citric acid-phosphate buffer system.

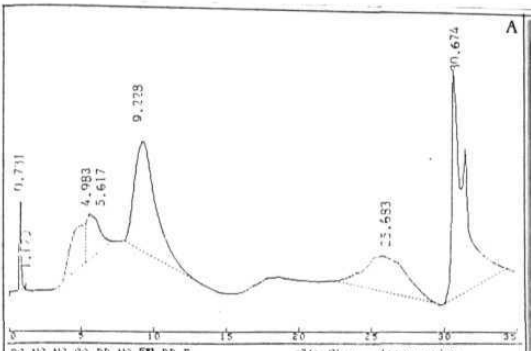
Panel A = Chromatogram of proteins associated with glial nuclear matrix prepared by high salt method.

Panel B = SDS-PAGE analysis of peak fractions. The number corresponds to the peak fraction in chromatogram of panel A.

Panel C = Chromatogram of proteins associated with glial nuclear matrix prepared by low salt method.

Panel D = SDS-PAGE analysis of peak fractions. The number corresponds to the peak fraction in chromatogram of panel C.

A



PC MC MS SO PP MO PR E <CA> Change Attenuation

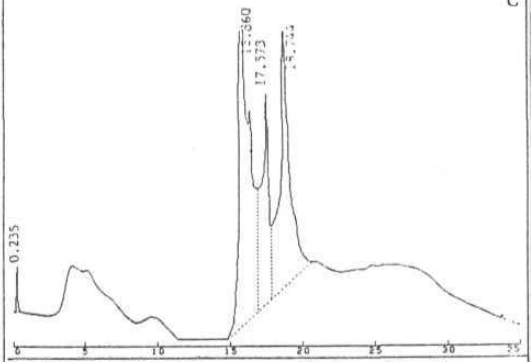
** CALCULATION REPORT **

CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC
1	1	0.731	1314	188	S		1.8786
	2	1.12	43	12	T		0.0613
	3	4.983	5013	73			7.1675
	4	5.617	4772	79	V		6.8226
	5	9.228	20076	194			28.7041
	6	25.683	13604	65			19.4505
	7	30.674	25119	372			35.9153
	TOTAL		69941	983			100

B

COLUMN : SHIMPACK PA DEAE (F-9054)
SOLVENT : 0.1 M CITRIC ACID AND
0.2 M Na_2HPO_4, pH GRADIENT
FROM 2.6 TO 7.0
DETECTOR : 280 nm
HPLC : SHIMADZU LC-6AD
FLOW RATE : 1ml per minute
SAMPLE : GLIA NUCLEAR MATRIX
BY HIGH-SALT METHOD

C

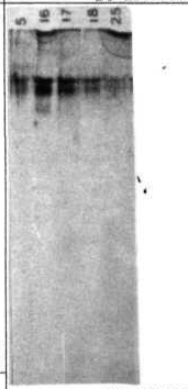


PC MC MS SO PP MO PR E <CA> Change Attenuation

** CALCULATION REPORT **

CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC
1	1	0.235	440	104			0.6473
	2	15.86	31192	575			45.9405
	3	17.573	13118	379	V		19.3212
	4	18.744	23147	476	V		34.0910
	TOTAL		67896	1534			100

D

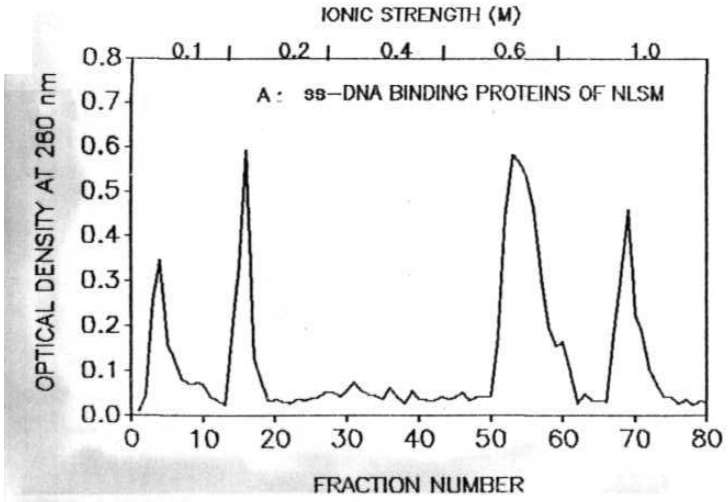


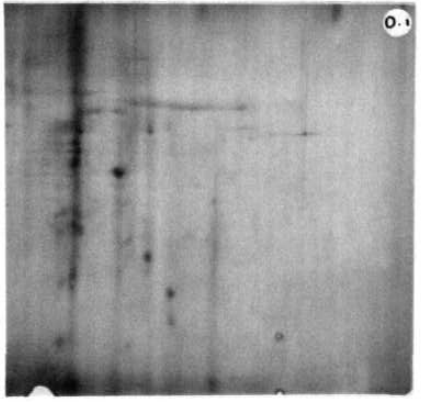
COLUMN : SHIMPACK PA DEAE (F-9054)
SOLVENT : 0.1 M CITRIC ACID AND
0.2 M Na_2HPO_4, pH GRADIENT
FROM 2.6 TO 7.0
DETECTOR : 280 nm
HPLC : SHIMADZU LC-6AD
FLOW RATE : 1ml per minute
SAMPLE : GLIA NUCLEAR MATRIX
BY LOW-SALT METHOD

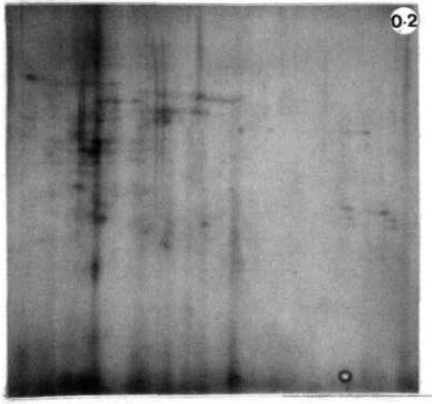
Fig. 14 : single strand DNA–cellulose chromatography of proteins from neuronal nuclear matrix extracted by low salt method from 45 days old rat brain. The nuclear matrix protein sample was applied on to a pre–equilibrated ssDNA cellulose column with equilibration buffer and bound proteins were eluted in a non-linear gradient fractionation with increasing ionic strength buffers (0.1 to 1.0 M). Elutes were collected in 1 ml fractions and the absorbance profile at A 280 nm is shown in Panel A.

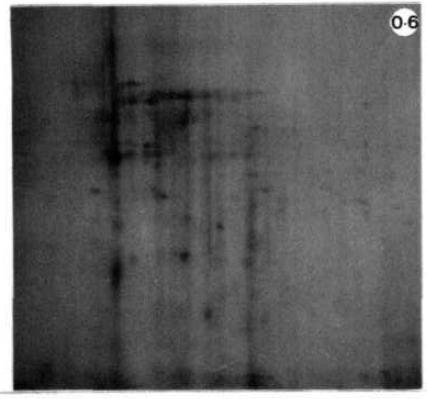
Other Panels represent two dimensional gel analysis of the 0.1, 0.2, 0.6 and 1.0 M peak fractions.

Mr represents molecular weight markers


IONIC STRENGTH (M)
0.1
0.2
0.4
0.6
1.0
A : ss–DNA BINDING PROTEINS OF NLSM
OPTICAL DENSITY AT 280 nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
70
80
FRACTION NUMBER


0.1


0.2


0.6

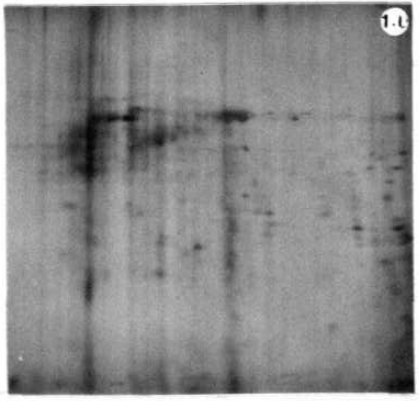
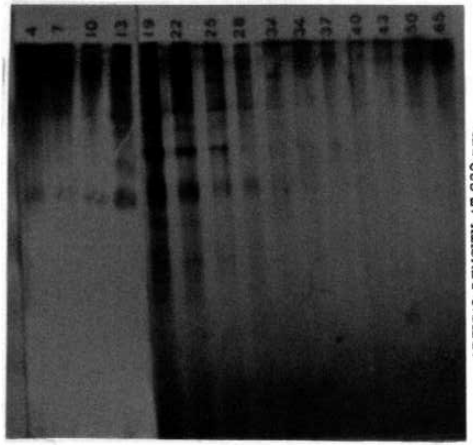

1.0

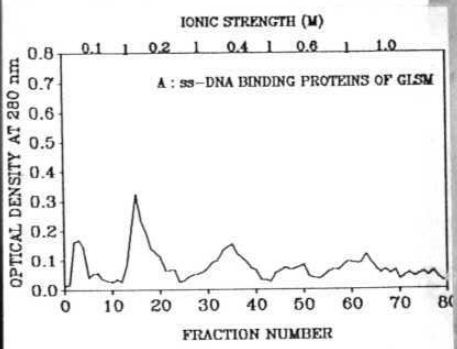
Fig. 15 : single strand DNA–cellulose chromatography of proteins from glial nuclear matrix extracted by low salt method from 45 days old rat brain. The nuclear matrix protein sample was applied on to a pre–equilibrated ssDNA cellulose column with equilibration buffer and bound proteins were eluted in a non-linear gradient fractionation with increasing ionic strength buffers (0.1 to 1.0 M). Elutes were collected in 1 ml fractions and the absorbance profile A 280 nm is shown in Panel A.

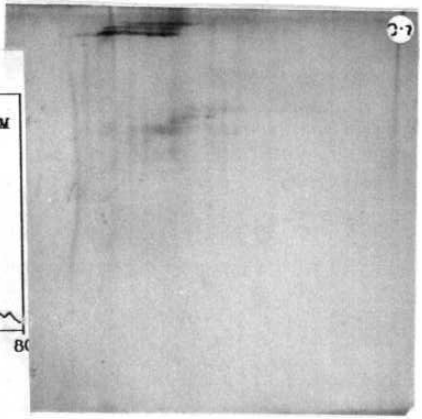
Panel B shows the SDS-PAGE analysis of the elutes in different fractions. Numbers 4-65 correspond to the respective fractions.

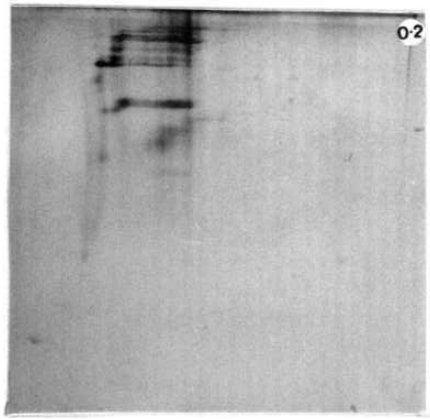
Other Panels represent two dimensional gel analysis of the 0.1, 0.2, 0.4 and 0.6 M peak fractions.

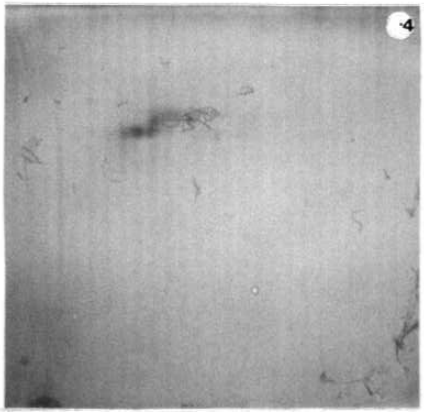
B




IONIC STRENGTH (M)
0.1 0.2 0.4 0.6 1.0
A : ss-DNA BINDING PROTEINS OF GLSM
OPTICAL DENSITY AT 280 nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 10 20 30 40 50 60 70
FRACTION NUMBER







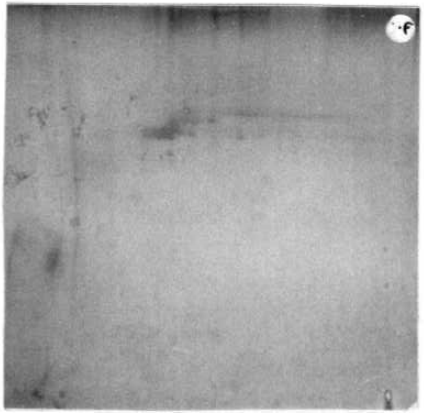


Fig. 16 : **Immunodiffusion**

Panel A = NHSM antibody

Panel B = NLSM antibody

Panel C = GHSM antibody

Panel D = GLSM antibody

1 = Neuronal nuclear proteins

2 = Neuronal nuclear matrix proteins extracted by high salt method

3 = Neuronal nuclear matrix proteins extracted by low salt method

4 = Glial nuclear proteins

5 = Glial nuclear matrix proteins extracted by high salt method

6 = Glial nuclear matrix proteins extracted by low salt method

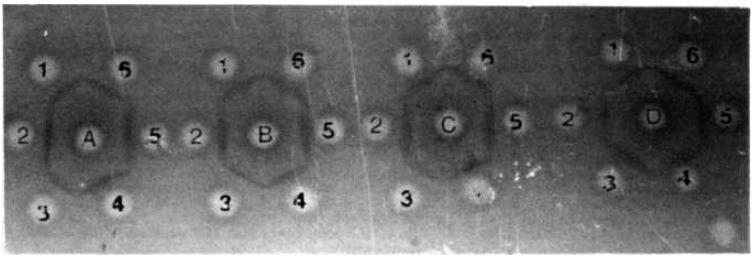

1
5
1
6
1
5
1
6
2
A
5
2
B
5
2
C
5
2
D
5
3
4
3
4
3
3
4

Fig. 17 : Western blot analysis of Nuclear and Nuclear matrix associated proteins with the following antibodies.

Panel A = Samples treated with NHSM antibody

Panel B = Samples treated with NLSM antibody

Panel C = Samples treated with GHSM antibody

Panel D = Samples treated with GLSM antibody

In each Panel the numbers pertain to :

1 = Neuronal nuclear proteins (NN)

2 = Neuronal nuclear matrix prepared by High salt extraction method (NHSM)

3 = Neuronal nuclear matrix prepared using Low salt extraction method (NLSM)

4 = Glial nuclear proteins (GN)

5 = Glial nuclear matrix prepared by High salt extraction method (GHSM)

6 = Glial nuclear matrix prepared using Low salt extraction method (GLSM).

MW = Molecular weight markers (bovine albumin–66000 ; egg albumin–45000 ; chymotrypsinogen-25000 ; soyabean trypsin inhibitor–20100 ; cytochrome–C–12300).

a = 70 kDa protein

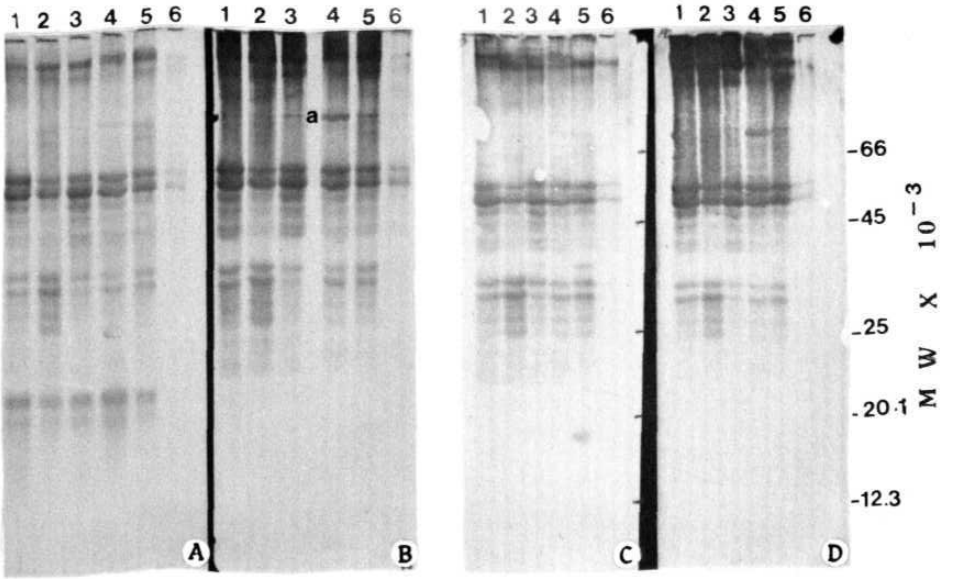

1 2 3 4 5 6
1 2 3 4 5 6
a
A
B
1 2 3 4 5 6
1 2 3 4 5 6
C
D
-66
-45
-25
-20.1
-12.3
M W X 10⁻³

Fig. 18 : Tissue specificity of Nuclear matrix proteins. Western blot analysis. Nuclear proteins were isolated from the tissues :

1 = Cerebellum

2 s Brain stem

3 = Cerebral cortex

4 = Hippocampus

5 = Mid brain

6 = Liver

7 = Heart

8 = Kidney

9 = Muscle

10 = Lung

and were separated on SDS-PAGE gels, electroblotted and immunostained with antibodies in :

Panel A = NHSM antibody treated blot

Panel B s NLSM antibody treated blot

Panel C = GHSM antibody treated blot

Panel D = GLSM antibody treated blot

MW = Molecular weight markers

a = 105 kDa protein

b = 38 kDa protein

c = 20 kDa protein

d = 90 kDa protein

e = 80 kDa protein

f = 75 kDa protein

g = 68 kDa protein

h = 50 kDa protein

i = 40 kDa protein

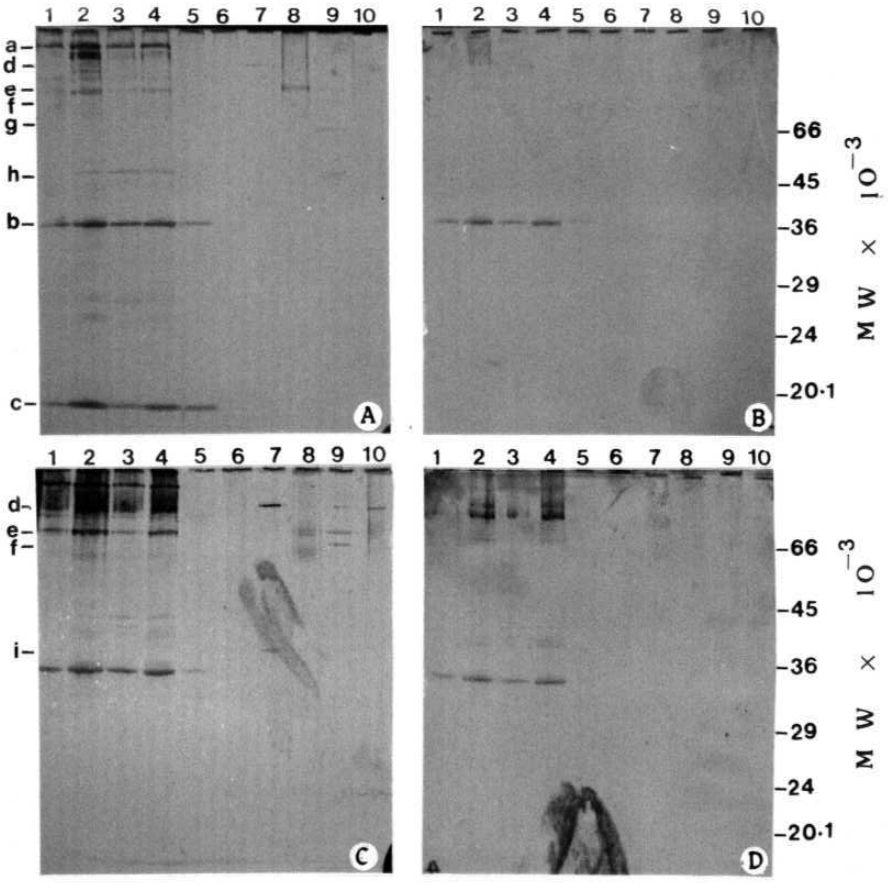

1 2 3 4 5 6 7 8 9 10
a
d
e
f
g
h
b
c
A
B
66
45
36
29
24
20·1
MW × 10⁻³
d
e
f
i
C
D

Fig. 19 : Nuclear protein patterns from normal cells and tumor cell lines. Nuclear proteins were electrophoressed on SDS-PAGE gels as described in materials & methods and stained with silver nitrate.

NN = Rat Neuronal nuclei

GN = Rat Glial nuclei

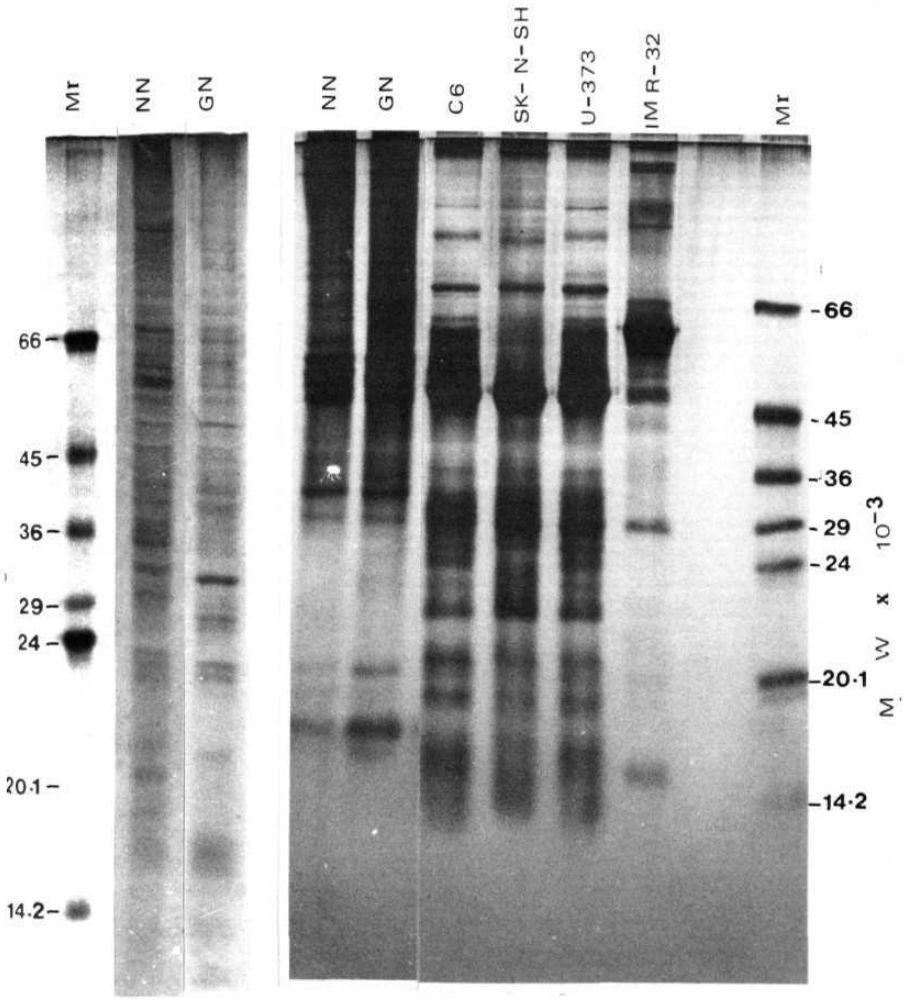
C6 = Rat C6 glioma cell line

SK-N-SH = Epithelial like human neuroblastoma (neuroblastoma / metastasis to bpne)

U-373MG = Epithelial like human glioblastoma (glioblastoma / astrocytoma)

IMR-32 = Human Neuroblastoma (Fibroblast mixture cell type)

Mr = Molecular weight markers (MW)


Mr
NN
GN
NN
GN
C6
SK-N-SH
U-373
IMR-32
Mr
66
45
36
29
24
20·1
14·2
66
45
36
29
24
20·1
14·2
M W x 10⁻³

Pig. 20 : Immuno reativity of the four antibodies with tumor cell line

Panel A = Treated with NHSM antibody

Panel B = Treated with NLSM antibody

Panel C = Treated with GHSM antibody

Panel D = Treated with GLSM antibody

GN = Glial nuclear proteins

C6 = Rat C6 glioma nuclear proteins

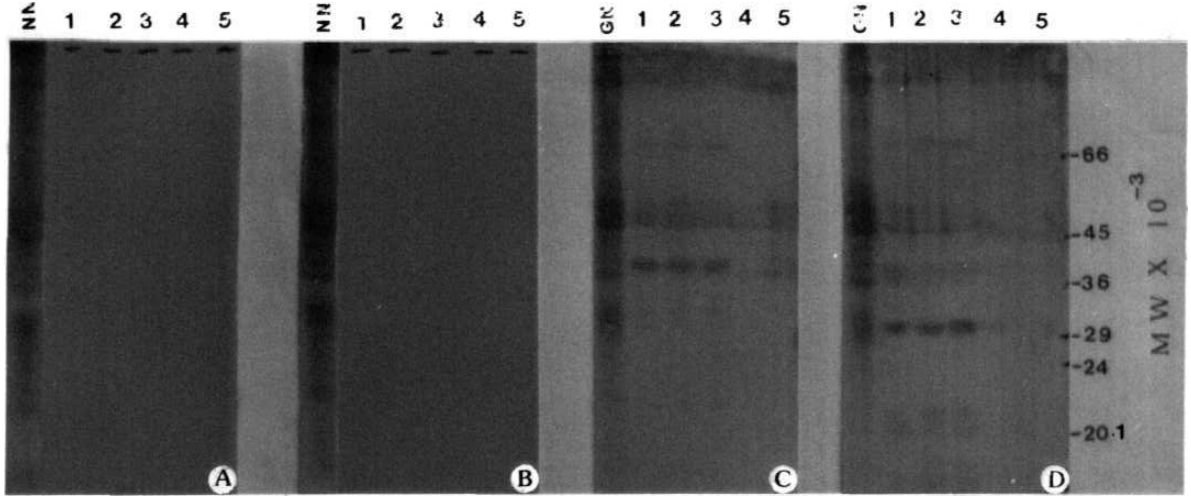
SK-N-SH = Epithelial like human neuroblastoma nuclear proteins

U-373MG = Epithelial like human glioblastoma nuclear proteins

IMR-32 = Neuroblastoma and Fibroblast mixture cell type nuclear proteins

Neuro-2A = Neuroblastoma nuclear proteins

Mr = Molecular weight markers (bovine albumin–66000 ; egg albumin-45000 ; glyceraldehyde–3–phosphate dehydrogenase-36000 ; carbonic anhydrase-29000 ; trypsinogen-24000 ; soyabean trypsin inhibitor-20100 ; β–lactalbumin-14200).


NN 1 2 3 4 5
NN 1 2 3 4 5
GN 1 2 3 4 5
GN 1 2 3 4 5
66
45
36
29
24
20.1
MW X 10-3
A
B
C
D

DISCUSSION

Discussion

The regulatory functions of chromatin such as transcription, replication and recombination occur at two levels (von Kries *et al.*, 1991). The first level involves the binding of regulatory proteins to specific sequences of DNA (example : transcription factors, promoter / enhancer sequences) constituting the control region. The second level involves the formation of DNA looping constituting the formation of topologically sequestered loop domains (Cook and Brazell, 1978 ; Lebkowsky and Laemmli, 1982). The DNA looping has been demonstrated as important regulatory feature and this structure is known to be involved in all aspects of genetic expression (such as DNA replication, transcription and recombination). Primarily the loop domains are generated by binding of specific stretch of DNA sequences to the internal nuclear structure called as nuclear matrix, or Chromosome scaffold. The DNA sequences having preferential binding to the nuclear matrix are often referred as matrix associated regions (MARs) (Cockerill and Garrard., 1986b) or scaffold associated regions (SARs) (Gasser and Laemmli., 1986), which have been further characterized to possess clusters of A-T rich sequence motifs and encompass approximately 0.3 to several kilo base pairs of DNA (Mirkovitch *et al.,* 1984 ; Gasser and Laemmli, 1986a ; Cockerill and Garrard, 1986a and Gasser and Laemmli, 1986b).

The DNA elements involved in DNA looping and nuclear matrix sequences, have been fairly characterized. On the other hand, the picture on protein composition and the nature of proteins involved in nuclear matrix is emerging only of late. The existence of a nuclear matrix with descrete protein composition has been now well established by several studies (Berezney, 1991b ; Luderus *et al.*, 1992 ; Nakayasu and Berezney,

1991) and few of these nuclear matrix proteins have been identified and functionally characterized (Romig *et al.*, 1992). The nuclear matrix has been implicated in tissue and cell specific gene expression as the nuclear matrix protein composition was found to be distinct in different tissues (Fey and Penman, 1988 ; Stuurman *et al.,* 1989 and Dworetzky *et al.*, 1990). Conversely, some studies have shown for the presence of common set of matrix proteins, as nuclear matrix has a similar basal structure in all most all mammalian cells (Stuurman *et al.*, 1990). Further, protein composition of nuclear matrix was shown to be differentiation state dependent (Fey and Penman., 1988 ; Stuurman *et al.*, 1989). A change in the expression of nuclear matrix associated proteins during cellular transformation and growth cycle has been correlated (Brancolini and Schneider., 1991). As it stands now, all these possibilities stand valid and further scope exists for the presence of nuclear matrix proteins in differentiation state and species specific dependent manner. Differential gene expression in mammalian cells involves gross as well as subtle changes in nuclear organization such as differential endonuclease hypersensitivity leading to a more and better exposure of transcriptionally competent genes than suppressed or silent genes (Hutchison and Weintraub., 1985). Cell type specific gene expression is also associated with modification (such as phosphorylation) of nuclear proteins known to directly bind to DNA or involve in an indirect role in DNA organization by influencing nuclear matrix assembly.

The nuclear matrix is now presumed to be instrumental in the control and coordination of gene expression during differentiation. The nuclear matrix has been implicated in cell and tissue specific gene expression, which is primarily rendered by the distinct set of proteins (regulatory) associated with nuclear matrix in different tissues and cell types. In the present study, an attempt is made to characterize the nuclear matrix

associated proteins in neurons and glial cells, with an idea that the nuclear matrix protein composition in these cell types will reflect on the role of nuclear matrix in post mitotically non-dividing cells (neurons) and normally dividing cells (glial cells). This in turn might help understanding brain specific gene expression.

Different non-histone nuclear matrix proteins are present in the neuronal and glial nuclei and even though both the cell types share major number of proteins and their composition, there are yet few number of proteins (such as the 50 kDa protein in glial nuclei), which are rather specific to cell type and proteins of such category are yet to be clearly identified and functionally characterized. It is also possible by this method to harvest nuclei from different cells of brain and therefore a further study into the diversity of nuclear protein composition in different cell types of brain such as neurons, astrocytes, oligodendrocytes and microglia would contribute for a better understanding of the role of non-histone nuclear proteins in cell type specific gene expression (Heizmann *et* a/., 1980).

To characterize the nuclear matrix associated proteins from neurons and glia, two methods were employed in which the high salt was used in one case and a chaotropic agent (lithium diiodosalicylate) was used in another case. Both the methods were employed in the present study since, unphysiologically high salt contractions have been employed in many studies to isolate the matrix, as preparation of matrix under isotonic or physiological conditions tends to aggregate the chromatin (Cook, 1991). To overcome this difficulty, lithium diiodosalicylate was employed, which acts as a chaotropic agent under low salt conditions and overcomes the problem of chromatin aggregation. Earlier reports have shown morphological variation in the nuclear matrices isolated under high salt extraction

(Mirkovitch *et* a/., 1984). Most of the studies have shown that the integrity of nuclear matrix was better retained in the method where the chaotropic agent was employed (Mirkovitch *et al.*, 1984 ; Smith *et al.*, 1987 and reviewed by Verheijen *et al.*, 1988). In this study, the nuclear matrices were characterized by scanning electron microscopy (SEM) studies and not much of a morphological variation is observed. This aspect has a limita–tion in the sense that it was only a scanning elctron microscopic (SEM) study but not a transmission electron microscopic (TEM) study and therefore most of the internal details are not revealed in the scanning electron micrographs.

The one dimensional gel electrophoretic analysis of nuclear matrix proteins isolated from neurons and glia by high salt method and low salt method revealed distinct differences in their protein composition. To characterize these alterations in nuclear matrix protein composition, high resolution two dimensional electrophoresis was used to determine qualitative changes occurring within the neurons and glia at different time periods of development such as 10 and 45 days. Further in a similar manner the non-histone chromosomal protein composition of neurons and glia from 10, 45 and 180 day old rats was also analyzed by two dimensional electrophoresis, as described in the results section. The data in over all indicates a number of proteins present at the day 10 which continue to be associated throughout the developmental stages, while a subset of non-histone chromosomal protein fraction kept dynamically changing in a development dependent manner. Some of these proteins could be tracked down in nuclear matrices indicating that they continue to be associated with the nuclear matrix. Profiles of such nuclear matrix proteins which continue to be associated with nuclear matrix (with the lamins) and the specific fractions of non-histone chromosomal proteins associated with the

nuclear matrix indicated a possible commitment of these proteins in cell specific gene expression. The two dimensional gel electrophoresis analysis of nuclear matrix proteins from neurons and glia (by both methods) indicate that the protein composition of nuclear matrix varies dramatically and might involve in supporting and regulating cell specific gene expression. Though the specific mechanisms by which nuclear matrix may mediate cell and tissue specific gene expression remains to be established, the data in the present study indicates indirectly that there is a possibility for the presence of two types of nuclear matrix protein composition. One type possibly belonging to "common minimal matrix" proteins whose synthesis persist throughout the developmental stages of specific cell types and continuous association with the nuclear matrix (Stuurman, 1990). The other type involves a dynamic set of fluctuating protein composition which is rather specific to a given stage of cellular differentiation. Further, the nuclear matrix may even be composed of proteins restricted to cellular differentiation and those required for general cellular metabolic process.

Majority of the nuclear matrix proteins (80 %) behaved as acidic proteins by showing an acidic p.I. (Berezney and Coffey., 1977 ; Berezney, 1979). In an attempt to purify the specific nuclear matrix proteins, the DEAE chromatography and HPLC analysis yielded protein fractions which are further heterogeneous than homogeneous. As a consequence the nuclear matrix proteins purification requires a combinational approach of protein purification methods rather than an approach by single method. The large number of non-histone chromosomal proteins present in nucleus and further isolation of a structural entity like that of a nuclear matrix and purification of specific nuclear matrix proteins constitutes in itself a rather narrowed possibilities for purification of a specific protein. Nevertheless it is not an impossibility and therefore alternative methods have to be employed.

Since the nuclear matrix proteins have been implicated in the DNA replication, with the organization of specific genes and also promoter binding factors of genes that are actually transcribed, the nuclear matrix proteins in this study were tested for their interaction with DNA by analyzing single strand DNA binding nuclear matrix proteins. The single strand DNA cellulose chromatography experiments indicated the presence of a very large number of nuclear matrix proteins in minor groups to be associated with single strand DNA. This data can be correlated with the studies where several matrix associated proteins (called SARs) are known to bind to A-T rich single copy fragments of 250-1500 base pairs (Romig *et al.,* 1992). The function of these SAR elements is unknown but some studies have indicated that SAR elements were able to stimulate transcription, and also protect a transcribed region from position effects of neighbouring consequences (Phi-Van *et al.*, 1990 ; Klehr *et al.*, 1991). SAR elements are identified in intron sequences (Cockrill and Garrard, 1986a ; Kas and Chasin, 1987). Though the clear functions are yet to be identified it is generally agreed that these elements are involved in DNA replication as SAR elements have been found close to origin of loops during DNA replication (Cook, 1991).

It has been reported that two classes of DNA binding proteins appear to be associated with the nuclear matrix. One class displays the characteristic, consistent of the formation of DNA loop domain having a defined sequence. This aspect has been examplified by (von Kries *et al.*, 1991) attachment region binding protein (ARBP) which binds to a 200-350 base pairs of MARs and serves primarily a structure role in matrix–chromatin interaction (Gasser and Laemmli, 1986b ; Cockerill and Garrard, 1986a and 1986b ; Mielke *et al.*, 1990). Another protein exhibiting similar role is the Adenovirus terminal protein. This protein also has been

shown to serve primarily a structural role in matrix association. This is a terminal protein which is covalently attached to the 5'-end of the Adenovirus DNA and mediates tight binding of the DNA to the nuclear matrix. The second type of DNA binding nuclear matrix represent factors directly involved in transcriptional control which exhibit sequence specific interactions with specific promotor regulatory elements contained within a MAR. The DNA binding properties of nuclear matrix proteins suggest that the nuclear matrix could serve as site of assembly of factors involved DNA replication as well as transcription.

Though the major nuclear matrix proteins such as lamins have been reported by several studies (Fey *et ah,* 1984a ; Berezney and Coffey, 1974), the minor proteins of nuclear matrix have not yet been characterized. In an attempt to identify the low abundance nuclear matrix protein an analysis was made in this study using polyclonal antibodies produced for total nuclear matrix antigens. Analysis by western blots provided several unanticipated findings. The immunological cross reactive experiments as well as the search made for the tissue specific presence of nuclear matrix proteins showed that there are marked differences in the protein composition of the nuclear matrix among different tissues. At the same time a set of matrix proteins which are common to many of the tissues also have been identified. These observations agree with the earlier reports for the existence of a common set of matrix protein in cells of different origin (Struuman *et al.*, 1990), as well as the observations made by Fey and Penmann (1988) that the nuclear matrix protein composition varies in a cell type specific manner. However, a close inspection in our study further shows that the proteins identified in this particular study could almost be designated as brain specific nuclear matrix proteins as marked differences in the protein composition were observed in the immunoblots between

brain regions and other tissues (such as liver, heart, kidney, muscle and lung). Only a subset of matrix proteins were common and that too in heart and muscle from the brain.

Since the , nuclear matrix proteins from neurons and **glia** showed considerable differences, the western blot analysis was extended to transformed cell lines of brain origin as few studies earlier have indicated that protein components of the nuclear matrix could also be tumor specific (Fey and Penman., 1988). The result showed an interesting observation that the antibodies raised for neuronal matrix were not reactive with any of the transformed cells. On the other hand the antibodies raised against **glial** nuclear matrix proteins reacted in a differential manner with the tumor cell lines basing on their cellular origin. These differences could possibly be due to several factors including alterations caused by transformation. However, these differences in nuclear matrix proteins reactive to specific antibodies between the normal cells and transformed cell lines could be demonstrative of cell alterations which have occurred during cell transformation. Similar type of observations were made by the study of Getzenberg and Coffey., 1991). Thus the changes in the nuclear matrix proteins in the transformed cell lines may also correspond to altered DNA organization, replication pattern in the phenotypically transformed cell lines. This also might explain why cancer cells are able to express different nuclear proteins than the normal cell counterpart. Therefore a detailed analysis of nuclear matrix associated proteins in different tumor cell lines. If cancer could possibly be able to explain the changed pathology and diagnosis in cancers and consequently might prove a valuble tool in the diagnosis of cancers as well as in the understanding of DNA organization controlled by nuclear matrix particularly in normal nuclear function and in tumor transformation.

SUMMARY

Summary

The nuclear matrix is a non-histone proteinaceous skeletal structure in the interior of the nucleus, having attachment sites for DNA replication. The term 'nuclear matrix' was assigned to this nucleoprotein fibrillary network by Berezney and Coffey (1974). DNA tightly associated with the nuclear matrix contained A-T rich sequences and therefore termed as matrix associated regions (MARs) or scaffold associated regions (SARs). In eukaryotes the nuclear matrix has been implicated in DNA replication, trascription, regulation of gene expression, post-transcriptional processing, RNA splicing, carcinogen binding, oncogene proteins and binding to certain hormone receptors. The nuclear matrix is now considered as a definitive structure with few defined functions and several yet to be identified functions.

The nuclear matrix can be structurally compartmentalized into nuclear envelope, internal chromatin matrix and residual nucleoli. In terms of its composition, the nuclear matrix contains protein (≈ 10 %), RNA (≈ 2 %), DNA (≈ 1 %) and phospholipids (≈ 2 %). Though the quantitative composition of the nuclear matrix components is approximately identical among all most all cells, qualitatively and functionally the nuclear matrix protein composition has been shown to vary. Further, the knowledge on proteins associated with the nuclear matrix apart from Ianiins is very limited and in view of their functional importance, characterization of the nuclear matrix associated proteins from brain has been attempted in this study.

Brain is a complex tissue having regional, temporal and cellular heterogenity. Of all the variations, cell heterogenity is further intricate due to the co-localization of non-dividing neurons and dividing glial cells.

The characterization of proteins associated with the nuclear matrix is not fully analyzed excepting for lamins A, B and C in liver. Whether or not the nuclear matrix protein composition has any tissue specific pattern is also not known. Since, the nuclear matrix has been implicated in DNA replication, does this structure has something to do with cell division ?, If so, whether there are changes in the matrix protein composition in non-dividing and dividing cells such as neurons and glia. When this protein composition data is generated from dividing and non-dividing cells, will it give any clues (or can it be used) in identifying or to understand the molecular mechanisms in abnormally dividing cells such as Cancer cells.

Attempts are made in this study to separate the nuclear matrix proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and two dimensional electrophoresis (2DE) involving Iso-electrofocussing (IEF) and SDS-PAGE, purification by diethylaminoethyl cellulose (DEAE) & high performance liquid chromatography (HPLC) and characterization by single strand deoxyribonucleic acid (ssDNA) binding activity. Polyclonal antibodies were developed against the nuclear matrix proteins and the antibodies were used for immunological characterization of the nuclear matrix proteins interms of a) regional heterogenity in brain, b) tissue specific expression, c) development specific expression and d) screening against nuclear extracts of chosen cancer cell lines having same cellular origin.

The nuclear and nuclear matrix protein composition was analyzed in 1. neurons (postnatally after 15 days of birth the differentiation stops) 2. glial cells (differentiating cell type) of different age groups of rats (10 day, 45 days, 180 days 3. cancer cell lines such as C6 glioma (induced by nitroso-methyl urea), Neuro-2A (spontaneous neuroblastoma), IMR-32

(mixture of neuroblastoma and hyline fibroblast), SK-N-SH (bone marrow epithelial like neuroblastoma having neurogenic origin) and U-373MG (epithelial like glioblastoma, astrocytoma grade III, from a malignant glioma brain).

The isolation of neuronal and glial nuclei was done according to Thompson (1973) from cerebral cortex of rat with minor modifications. The purity of neuronal and glial nuclei was improved by using sucrose cushions in every centrifugation. Clear cut differences were observed in SDS-PAGE and two dimensional elctrophoresis (2DE) patterns of nuclear protein composition in neuronal and glial nuclei. There have been considerable differences in the composition of nuclear proteins in 10 day, 45 days and 180 days samples of neuronal and glial cells, suggesting that nuclear protein composition is dependent on developmental and cell type heterogenity in brain. However, in the nuclei from 45 days old rat brain, maximum number of proteins are present when compared to 10 days and 180 days rat. But for this, difference in protein composition between 45 days to 90 days was minor. So, 45 days old rat brain has been selected for further experiments.

The nuclear matrix was prepared by two methods, the high salt extraction method (HSM) and low salt extraction method (LSM) using lithium diiodosalicylate according to Mirkovitch *et al.*, (1984). The protein composition of nuclear matrix in neurons and glia showed slight variations, which mainly depends on the methods of nuclear matrix preparations (HSM and LSM). Scanning elctron microscopic (SEM) studies showed that nuclear matrix preparation whether by HSM or LSM do not show any morphological variation.

The low salt method preparation yields a matrix having relatively less protein composition than by high salt method. However, lamins are retained irrespective of high salt or low salt preparation. The purification of matrix associated proteins by DEAE chromatography or by HPLC are only partially successful. The elute fractions obtained in chromatography apparently show homogenity on SDS-PAGE gels, but when resolved on two dimensional electrophoresis show very high polypeptide heterogenity. Some of the nuclear matrix proteins other than lamins showed binding to single strand DNA, which were eluted at 0.2 M and 0.4 M ionic strength suggesting regulatory functional importance.

In view of difficulties in purification, polyclonal antibodies were developed for total matrix associated proteins in neurons and glia. The four antibodies, NHSM-Ab (antibodies were raised against the neuronal nuclear matrix proteins extracted by high salt method), NLSM-Ab (antibodies were raised against the neuronal nuclear matrix extracted by low salt method), GHSM-Ab (antibodies were raised against the glial nuclear matrix proteins extracted by high salt method), GLSM-Ab (antibodies were raised against glial nuclear matrix proteins extracted by low salt method) in general, showed immune reactivity having several similarities. But the antibodies prepared by low salt method gave a better immune reactivity in both neurons and glia.

Two proteins $\approx$ 71 kDa and 60 kDa showed immune response in glial cells by LSM antibodies, which are yet to be characterized. The regional heterogenity studies showed that brain stem had a high immune reactivity while mid brain showed least.

The data for tissue specific pattern suggests that some of the nuclear matrix proteins are brain specific. However, two minor proteins are

noticed to be shared by kidney and heart. In particular the LSM antibodies are highly specific for brain and other tissues showed least reactivity.

When the antibodies were used for screening nuclear proteins from tumors by employing nuclear extracts of tumor cell lines (such as C6 glioma, Neuro-2A, U-373MG, SK-N-SH and IMR-32) immune responses were obtained in tumors having identical cellular origin. The neuronal nuclear matrix antibodies were not of much use in terms of their immune reactivity with the tumor cell types.

The GHSM-Abs showed 66 kDa, 45 kDa and 36 kDa reactive species in tumor cell lines having identical cellular origin like neuroblastoma and glioblastoma (C6 glioma). The GLSM-Abs showed a 29 kDa band in all these tumors having identical cellular origin. A tumor cell type with different cellular ancestory like that of IMR-32 did not react with these antibodies. The immune responses with specific proteins in tumors of identical cellular origin suggest possible utility of these antibodies in early tumor development.

In summary, this study showed that nuclear matrix protein composition is not dependent on cell division status, such as non-dividing and dividing cells. But some of the nuclear matrix proteins could be possibly tissue specific, while selective nuclear matrix proteins could still be possibly used in cancer detection, as cancer cell lines of similar cellular origin show up specific nuclear matrix patterns.

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ABSTRACTS

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A POSSIBLE ROLE OF ENDOGENOUS LECTIN BINDING PROTEIN IN RICE BEAN SEED.

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Pradip K. Datta, Pranab S. Basu and Tapash K. Datta, Indian Institute of Chemical Biology, Calcutta 700 032.

Hydrophobic binding could be an important factor for stabilizing the protein-protein interaction. In the present investigation we have observed that the binding of rice bean lectin-I binding protein (LBP) decreased the flourescence emission of 1,8-anilinonaphenesulfonic acid (ANS) AND 2-6-toloidinylnaphthalenesulfonic acid (TNS) after interaction with rice bean lcctin-I. For this reason, we decided to investigate the hydrophobic binding properties of the lectin-I and lo determine the conformation of lectin-I-LBP interaction. The flourescence of ANS, TNS and an unchaged analogue N-phenyl-1-naphthylamine (NA) were greatly enhanced in the presence of lcctin-I. The opposite result was observed after addition of LBP in the whole system respectively. Fluorescence titrations with ANS, TNS and NA yielded affinity constants of 7.58 x $10^3 M^{-1}$, 3.29 x $10^3 M^{-1}$ and 5.7 x $10^4 M^{-1}$ respectively. Lcctin-I contained 42-78% ß -conformation, 10-33% alpha conformation along wilh random coil at pH 7.2 depending upon the analytical methods used. The percentage of ß-conformation increased with the addition of N-acetyl D-Galactosamine. LBP contained 4-12% alpha conformation 9-17% ß-conformation along with random coil at pI I 7.2 depending upon the analytical methods used. The ß-conformation of Lectin-I was decreased in presence of LBP.

A SIALIC ACID SPECIFIC LECTIN FROM Pila globsa :

Partha Sarathi chowdhury, Debasish Mitra and Manju Sarkar, Indian Institute of Chemical Biology, Jadavpur, Calculla 700032

The hcmolymph of the apple snail. Pila globsa, shows the presence of lectin sinc it agglutinates crythrocytes from various animals like rabbit, rat, mouse, pig and human. The Agglutination of rabbit crylhrocylcs by the hcmolymph lectin is strongly inhibited by sialic acids and various sialoglycoproteins. Since among the glycoproteins bovine submaxillary mucin (BSM) proved lo be the most potent inhibitor of agglutination BSM-Scpharosc-4B is employed for the purification of the lectin. The purified lectin is active over a wide temperature range of 10" - 37°C and is most active al pI I 6-7. The sialic acid specificity of the lectin is confirmed by the abolition of ils hemagglutinating activity Following treatment of crythrocytes by neuraminidase and trypsin, which arc known lo cleane sialic acid and sialoglycopeptides respectively from crythrocyte surface. Inhibition study indicates that N-glycolylneuraminic acid, N-acetylneuraminic acid, N-acetylneuramin lactose, BSM, sheep submaxillary mucin, fetuin, thyroglobulin, glycophorin, human chorionic gonadotrophin and ovalbumin arc polenl inhibitors of the lectin activity. Analysis of the inhibition data indicates that it is not the percentage of sialic acid in lhc glycoproleins but the type of glycosidic linkage lo the proteins, the subterminal sugar and the nature of linkage between sialic acid and the subterminal sugar which is important for binding.

STUDIES ON LECTIN FROM CAJANUS CAJAN:

Miss Seema Hasan, Interdisciplinary Biotechnology Unit, and Department of Biochemistry, JNMC, Aligarh Muslim University Aligarh,

Glucose/mannose specific lectin was earlier identified and isolated from Cajanus cajan pulse in microgram quantities. The yield was poor because of the poor solubility in aqueous buffer. We therefore studied different conditions for optimal solubilization of lectins from pulse. From 20 gms of pulse about 2 gms of protein including leclin could be sohibili/.cd by water or aqueous buffer containing NaCl (<0.3M) above which the amount of protein solubilized increased by 50% at pI I 7.5. The effect of pI 1 on solubilization was studied in the pI I range 3-C al 0.4M NaCl. After prolonged standing precipitation occurred at all the pI I values but the protein was maximally precipitated at pI I 4.0. However the leclin aclivily could be detected only in the supernatant. The effect of specific ligand i.c. 0.1 M glucose on leclin solubility was investigated. Glucose had little effect on lhc solubility. The homogenate delipidated with ether was clear and active. Its 50% ammonium sulphate fraction showing lectin activity was fractionated lo DEAE cellulose column (2.3x11cms) and the protein was eluted by discontinuous NaCl gradient (0.0 to 0.SM) in 10 mM phosphate buffer pI I 7.4. The protein eluted with 0.05 NaCl in lhc buffer showed lectin aclivily and ils yield was 15% which is substantially higher than that obtained by affinity chromatography.

CHARACTERIZATION OF A 50 Kd NUCLEAR MATRIX PROTEIN IN NEURONS

499

N, Nagaraju and Mohan C. Vemuri, School of Life Sciences, Univcrsily of Hyderabad, Hyderabad-134.

Ncuronal and glial nuclei were isolated from rat cerebral cortex. Nuclear matrices were prepared from these nuclei employing low and high salt extraction procedures. Nuclear matrix preparations from neurons contain a highly enriched fraction of a polypeptide with a molecular weight of 50 Kd. This polypcpiidc is further characterised by two dimensional iso-electrofocussing/SDS-PAGE. The functional significance of this prolcin in terms of ncuronal function and its involvement in lhc po.st nalal cessation of ncuronal cell division is discussed.

Neurochemical Research, Vol. 16, No. 1, 1991 pp. 23-27

Identification of Two Novel **Cerebrospinal** Fluid Proteins in Non Specific Mental Retardation

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(Accepted November 6, 1990)

Cerebrospinal fluid (CSF) from twenty three patients with non specific mental retardation and fourteen age matched normal samples was subjected for qualitative analysis of protein profiles by two-dimensional gel electrophoresis (2-DE) and the proteins were visualised by ultra sensitive silver staining. Two proteins designated as mental retardation associated proteins (MRAP-I and MRAP-II) were identified in six male patients out of twenty three patients CSF samples. MRAP-I had an isoelectric point of 7.4 with a relative molecular weight 16.5 kDa, while MRAP-II had an iso-electric point of 7.2 with a relative molecular weight 16.8 kDa. The two proteins ate presumed to be originated from brain, as they could not be traced in the serum of patients, nor due to proteolytic degradation. Despite unknown origin and identity, their presence in the CSF of a specific group of mentally retarded male patients suggest their possible clinical utility and to define protein alterations in mental retardation.

KEY WORDS: CSF; proteins; mental retardation; 2-DE; disease associated proteins.

INTRODUCTION

In more than one-third of the cases with mental retardation the cause is not known and they are termed as idiopathic or non specific (1, 2). In view of the appreciable incidence of **idiopathic** mental retardation, efforts have been made to delineate it further. **The** identification of the X-chromosome marker associated with mental retardation is the result of such a delineation (3). As yet, there still is a large group which is not associated with the marker X-chromosome. In (his study an attempt has been made to delineate it further by analysing the CSF.

CSF is appropriately viewed as the clinician's access to the brain and is capable of reflecting the pathophysiological status of brain function (4). CSF protein abnormalities in disease can now be better resolved by employing 2-DE (isoelectrofocusing-sodium dodccyl sulfate-polyacrylamide gel electrophoresis). Further, sensitivity of this method can still be improved by silver staining the 2-DE gels as silver stain can detect very low amount of proteins, yielding a better diagnostic approach (5) Hence we analysed the CSF protein patterns in non specific mental retardation cases, with the view to identify variations in protein profiles of CSF. We describe here the consistent finding of two abnormal CSF proteins only in a group of mental retarded patients.

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EXPERIMENTAL PROCEDURE

We studied the CSF protein patterns in 23 cases with non specific mental retardation and 14 age matched controls in whom mental retardation and neurological deficits were excluded. The cases were referred to **Institute** of Genetics for genetic studies. These cases were

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designated as non-specific menial retardation by a) eliciting history for antenatal, natal and post-natal causes likely to produce mental retardation for eg: infection, brain trauma etc. b) clinical examination c) clinical investigations like fundus examination, X-rays d) investigations like urine for reducing substances, amino acid profiles, chromosomal analysis. Oilier evaluations included family incidence, pedigree and intelligent quotient estimates.

It is observed that the patients with the new proteins had certain common physical features like a narrow forehead and/or bitemporal narrowing which was consistently present besides other minor associated features like wide car lobules, large mouth, hyper extensible finger joints which were present in some of them. Five of the patients had moderate mental retardation, while one had mild retardation. None of these children had seizures, they were not on medication and their births were normal. Some of them had minimal neurological deficits. The clinical details of these six patients arc given below:

Case No. I. A 16 year old male with a narrow forehead, short nose and a large mouth. He had exaggerated knee jerks on the left side. He was moderately retarded.

Case No. 2. A 13 year old male with a long facies, bilcmporal narrowing a large mouth, long tapering fingers. He had mild retardation.

Case No. 3. A 10 year old male with a long facies, a narrow forehead and hyper extensible finger joints. He was moderately retarded.

Case No. 4. A 14 year old male with stunted growth, a narrow forehead a long facics, synophyrs. He was moderately retarded.

Case No. 5. A male aged 12 year wilh stunted growth, a long facics and a narrow forehead. He had moderate retardation.

Case No. 6. A 6 year old male wilh a hypotonic facics. He had a slight broad forehead and bi-temporal narrowing. He had moderate mental retardation. The CSF was collected by lumbar puncture and was frozen immediately till use.

Clinical Examination. Dy clinical examination, condilions like activism, glycogen storage disorders, gangliosidoses and mucopolysaccharidoses were ruled out.

Clinical Investigation. Clinical investigations included, assays for blood protein, urine examination, X-ray of skull and spine and fundus examinations. None of the cases showed any abnormality, inlracranial calcification, fundus examination was found to be normal, thereby ruling out Tay-Sach's disease, Niemann-Pick disease and disorders with cataract or Kayser-Ileischarings.

Electrophoresis. SDS-Polyacrylamide gel electrophoresis was performed in 18% acrylamide according lo Laemmli (6) and by Thomas and Kornberg (7) wilh few modifications. The CSF sample was precipitated by 5 volumes of acetone in -20°C and the protein is solubilized in SDS sample buffer (1% SDS, 1.5% B-mercaptoethanol, 8% sucrose and 0.001% bromophenol blue) and heated at 100°C for 5 minutes just before loading on lo the gel. The gels were run at 170 volts for 4 hours till the dye front reaches bottom. The gels were silver stained for visualization of peptides and the 1-D protein profiles were analysed by automated laser scanning using the software program of Biomed Instruments, 1988.

Two-dimensional nonequilibrium pH gradient gel electrophoresis (NEPIIGE) was performed as described by O'Farrel et al (8). CSF sample was acetone precipitated over night, solubilizcd in 40 µl of sample buffer (9.5 M urea, 2% 3-10 ampholines and 5% B-mercaptoethanol) and was loaded on lo NEPHGE gel containing 2% ampholincs (pll 3-10) for isoelectrofocusing. The gels were electrophoresed with out prefocusing having cathode on bottom and anode on lop for 2000 volt hours. The gels were removed al the end of the run and subjected lo second dimension (SDS-PAGE). Silver staining of proteins in the gels was done as described by Blum et al (9).

Polypeptides were identified by their relative molecular mass (Mr) during coelectrophoresis with purified standards (Sigma) and the relative isoelectric point (p.I) was determined by comparison wilh pll gradient observed in the IEF gel wilh surface pll electrode (LKB-Produkter) as well as gel extrusion into deionised water (10). Assessment of alterations in normal and mental retarded sample protein patterns in 2-DE was by direct visual scanning.

Total protein levels in all the CSF samples were analysed following the method of Scopes (11).

Proteolytic Activation. In order lo check whether these two new proteins arose from protein degradation, we incubated CSF of normal and MR patients at 25°C in the absence of proteolytic inhibitors for up to 90 days lo assess if the protease activity in the CSF samples would result in these two new proteins. Samples from the experiments were then subjected lo elcclrophoresis, silver slaining and scanning as described above.

RESULTS

Changes in CSF proteins in mental retarded patients were identified by comparison with protein profiles of CSF samples from normal individuals. The CSF protein profiles were compared from the following groups.

23 patients wilh typical idiopathic mental retardation symptoms.

14 normal age matched individuals who were clinically examined particularly to exclude any abnormality of nervous system especially mental retardation.

The total protein concentration in CSF samples from normal and mentally retarded group is presented in Table I. Very high protein levels were noticed in mental retarded samples in comparison wilh the normals. Neurological disorders with similar increased CSF total protein content l ave been repotted earlier (12).

The qualitative one dimensional SDS-PAGE profiles showed that protein pattern from the normal and mentally retarded samples was identical. However, a new protein with a relative molecular weight 16 kDa designated as mental retardation associated protein (MRAP) was observed in 6 out of 23 mentally retarded patient samples (Figure 1). The six patients showing the novel

Table I. Total Protein Levels in the Cerebrospinal Fluid of Normal and Mental Retarded Individuals

Subjects	Number of samples	Protein concentration (in grams per litre)
Normals	14	0.240 ± 0.093
MR cases	23	0.579 ± 0.168
MRAP absent cases	17	0.608 ± 0.167
MRAP cases	6	0.469 ± 0.132

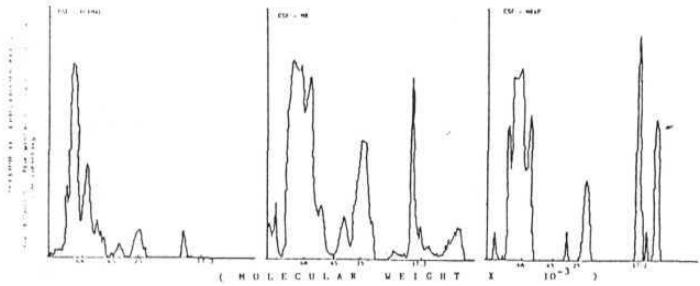

Fig. 1. Automated Laser Scan SDS-PAGE profiles of cerebrospinal fluid proteins from Normal (CSF-NORMAL), Mental retarded (CSF-MR) and menial retarded individuals in whom some abnormal prolcins are associated (CSF-MRAP). Relative Molecular weight is shown on X-axis. Arrow indicalcs the peak pertaining lo Menial Rclardalion Associated prolcins (MRAP).

hand were all males aged between six to sixteen years as described earlier.

In view of the appearance of this new protein in mental retarded patient samples, we tried to resolve this protein on two dimensional gel clcctrophoresis involving isoelectrofocusing. It was identified from this analysis, that this is not a single protein but the band is made of two proteins having two different p.I tending towards basic isoelectric point. Therefore we tried NEPHGE analysis for better resolution which revealed (hat these proteins have a close relative molecular mass and were found to differ in their isoelectric points. They could be resolved into two individual proteins (MRAP-I with a p.I of 7.4 and M.Wt 16.5 kDa; MRAP-II with a p.I of 7.2 and M.Wt 16.8 kDa) as shown in NEPHGE gels (Figure 2B). Since NEPHGE gels can not be used to determine absolute isoelcctric point due to unstable pH gradient (8), the p.I was derived from IEF gels (10). However, the p.I derived from NEPHGE gels was also very close to the p.I derived from IEF gels.

Experiments were conducted to check the appearance of MRAPs due to proteolytic degradation by incubating the samples at 25°C in the absence of proteolytic inhibitors as indicated in methods; but no proteins migrated to the position occupied by MRAPs (Fig. 3) ruling out the possibility of MRAPs appearance by proteolytic activity.

DISCUSSION

This study of CSF protein profiles from mentally retarded patients indicates the clinical advantages of using high resolution 2-DE coupled with ultra sensitive silver staining to study proteins involved in neurological diseases. In CSF samples from six patients, we delected two proteins MRAP-I and MRAP-II having approximately similar molecular mass and differing p.I. The presence of these proteins is only in six cases of mental retardation; but their identification may be of diagnostic value in distinguishing pathologically different mental retarded cases, though they show apparent identical common features. The appearance of these two new proteins is neither age related, as age matching between normals and mental retarded was perfect; nor drug related, as the patients were not on therapy at the time of lumbar puncture.

Several proteins in CSF are known to be generally derived from serum by unknown mechanisms (13) and analysis of serum proteins from six patients revealed absence of these proteins, ruling out their origin from serum. In a further attempt, we ruled out the appearance of MRAP-I and II due to proteolytic degradation as long incubation periods of CSF samples did not produce new peptides close to MRAP-I and MRAP-II. We suspect the proteins to have been originated from the brain, as transependymal formation of CSF accounts for one third of total CSF (14) and once proteins have entered the interstitial fluid of brain, they are freely exchanged (15). An alternative explanation for the appearance of these new proteins is the post-translational modification of some normal CSF proteins. Post-translational modification such as phosphorylation is ruled out in this case as it will change the p.I of protein at least narrowly and can be detected on a 2-DE gel. The scope for other kinds of post-translational modifications such as acylation remains to be investigated and can not be ruled out. Fur-

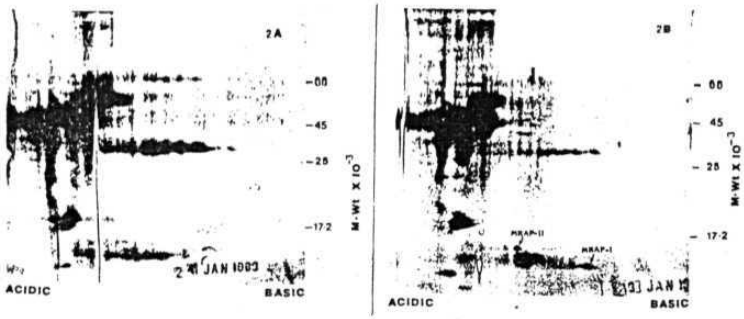

Fig. 2. Two-dimensional NEPHGE gels of the cerebrospinal fluid proteins from Normal (2A) and Mental retarded (2B) proteins. The amount of protein applied to the first dimension was 70 μg in 2A and 50 μg in 2B. MRAP stands for Mental Retardation associated proteins (shown by arrows).

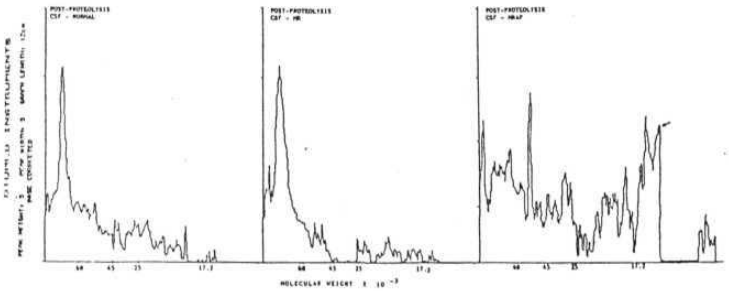

Fig. 3. Same as for Figure 1, but samples are subjected for proteolysis for 90 days.

ther the p.I of MRAP-I and II suggests that they might have preferably originated from brain itself, rather than protein degradation or post-translational modification. It has been reported that proteins with greater p.I and small sire can enter the CSF more easily (15) and the basic p.I and smaller relative molecular mass of MRAP-I and II denotes their possible entry into CSF from brain. Greater abundance of proteins in brain will not allow separation of tissue proteins clearly (16) and therefore it is necessary to develop antibody probes for MRAP-I and II to investigate further.

Despite the unknown origin of MRAP-I and II, these **preliminary** findings of apparent specificity of MRAP-I and II in the CSF of mentally retarded patients, with specific features suggests that they might be of considerable help in assigning some specificity lo non specific mental retardation. Since the patients having MRAP-I and II were all males, with similar phenotype, one can easily assign this group to fall into the X-linked mental retardation category. The newly identified CSF proteins, MRAP-1 and II may possibly serve as genetic markers in **the** future for this particular group with specific phenotype (further confirmatory studies needed) and this observation will also serve as a basis for future efforts to define mental retardation related protein alterations.

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2-D PAGE '91

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MULTIPLE GELS IN 2DE - BETTER RESOLUTION

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1 INTRODUCTION

Identification and characterization of proteins was rantlo easier by high resolution two-dimensional electrophoresis developed by O'Farrell [1]. This popular technique involves iso-electrofocussing (IEF) in the first dimension and SDS-PAGE in the second dimension. Visualization of polypeptides resolved by 2-DE by ultra sensitive silver staining [2], has a considerable contribution in improving the sensitivity of 2-DE technique, allowing screening and detection of rare proteins in diseases of clinical and biological samples. There have also been many modifications such as addition of SDS in IEF [3], Non-equilibrium pH gradient gel electrophoresis (NEPHGE) involving a refinement in the resolution of basic proteins [4], development of "giant gels" to analyse expression of protein-gene products [5] and computerised scanning of 2-DE gels to make protein catalogs [6], Host of these modifications are in the first dimension run or take advantage of the excessive protein loading as in "giant gels" which significantly increased the resolution and utility of 2-DE gels. Despite these improvements, there are still minor draw backs, in the second dimension such as the limitation of running only two gels at a time, leading to batch-to-batch and run-to-run variations especially when large number of samples have to be subjected to 2-DE. Simple visual protein pattern recognition itself could be confusing when more than two samples have to be analysed for protein changes using 2-DE. In order to overcome this difficulty, we have attempted to improve the second dimension SDS-PAGE run by transferring at least eight IEF tube gels at a time on to the second dimension slab gels allowing resolution of eight 2-DE gels, which we call "multiple gels".

This paper is an attempt to describe the simple fabrication of "multiple gel" unit **and** to document the utility of multiple gels in protein pattern analysis.

2 MATERIALS AND METHODS

ISO ELECTROFOCUSSING:

Protein **samples from plasma** membranes of neurons and **glia** of rat brain were subjected to **IEF** in tube gels as described earlier [7].

MULTIGEL UNIT:

The apparatus "Multigel unit" for running eight slabs at a time is essentially similar, but a **scaled-up** version of the unit used by Reid **& Kieleski** [8] and O'Farrel [1]. Apparatus dimensions are 54 x 7 x 17 cm (l x **w** x h). The lower perspex chamber is of 62 x 13 x 8 cm size (Fig.lC).

GEL CASTING AND ASSEMBLY:

Glass plate of 5 mm thickness, 54 x 17 cm was cut into rectangulars and used in making the slab gels. The outer plate was used without any further modification (Fig.1B), while the inner plate was notched 3.5 mm deep and 48 cm long leaving 3 cm

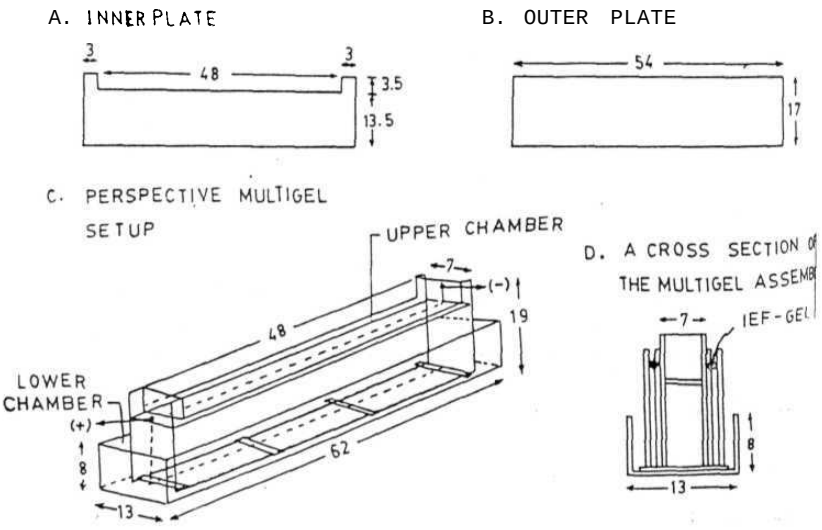

Fig 1: Multigel Unit and its components.

on either side of the plate (Fig 1A). The gels were cast in the same way as in conventional slabs. But during the polymerization of stacking gel the surface of each gel is overlaid with one clean glass rod of 2.5 mm diameter (48 cm) or four rods of 12 cm one beside the other. This results in the formation of a smooth gel surface on which the first dimension IEF gel can be placed.

IEF GEL TRANSFER AND SECOND-DIMENSION RUN:

When the gel casts were assembled and clamped on to the unit, it results in the formation of an upper buffer chamber(Fig.1D).The assembly is placed into lower chamber carefully from one end to avoid trapping of air bubbles beneath the gel. The glass rods on the top of the stacking gels were removed and IEF gels were transferred into the smooth groove made by the glass rod. The IEF gel was annealed to the stacking gel with hot agarose. By this method eight IEF gels can be placed, four on each side of the multigel unit. After the addition of upper chamber buffer, the gels were electrophoresed at 120 mA in the stacking gel region and 160 mA in the resolving gel region, for 4 to 5 hours till the dye front reaches bottom.The electrodes were disconnected after the completion of the run and the glass plates were pried apart with a kitchen knife. Since the IEF gel still remains stuck to the stacking gel, each gel was sliced vertically into four gels guided by the position of IEF gel. The gels were fixed as described earlier [7],

DETECTION BY SILVER STAINING:

Visualization of proteins was by the method of Merrill et.al.,[2) as modified by Blum et.al., [9].

3 RESULTS

2 DE pattern of plasma membrane proteins of neurons and glia obtained by means of separation on multigel unit are shown in Figs 2-3. An example of only two protein patterns is shown although similar protein separation pattern was achieved in all eight samples using multigel unit. By comparison of the separation pattern from samples A and B in each figure as indicated, the position of proteins (numbers) can be clearly

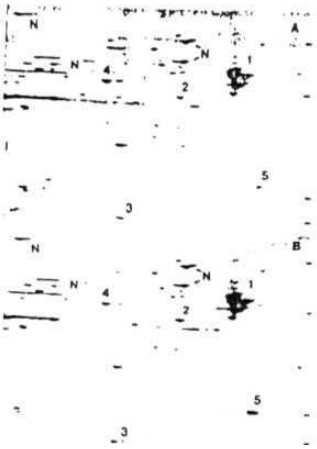

Fig 2: 2-DE of Neuronal membrane proteins. N indicates protein(s) specific to neurons.

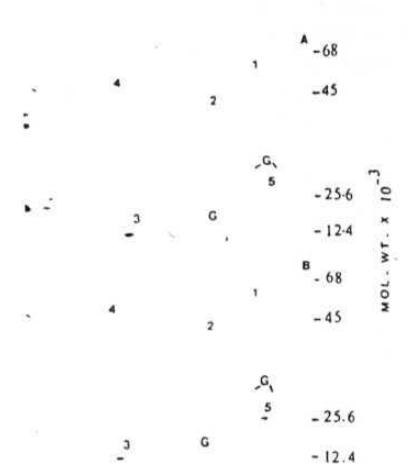

Fig 3: 2-DE of Glial membrane proteins. G indicates protein(s) specific to Glia.

identified. In all the eight gels, the proteins were identified at the same position of x-y coordinates, resulting in highly reproducible protein separation pattern.

4 DISCUSSION

Two dimensional gel electrophoresis is a most efficient analytical method for separating protein samples. The method described by O'Farrel [1] fifteen years ago has been taken over virtually unchanged in the following work, and with a minor modification in the second dimension, better and consistent resolution is achieved. The simple modification we made, is to fabricate an apparatus to run eight conventional slab gels (9 x 11 cms) as two large gels. The reproducibility and resolution of protein is shown in Fig.2-3. This modification allows certain advantages. Casting of four conventional gels as a single large gel is done at a time with the help of two lateral and one bottom

spacers. Thus the routine use of almost nine spacers can be omitted. Gel casting and assembly as individual slabs and electrophoresing them as separate runs consumes more time. In multigel unit, all this is reduced to one third with respect to 1 casting, assembly and man hours. The variations from batch-to-batch are absolute minimum, as all the gels are run simultaneously under same running conditions. The multigel unit facilitates multiple handling of IEF samples and allows very economical performance of the original procedure (1) with highly reproducible protein resolution pattern. This simple modification also increases the chances of getting comparable protein profile gels (publishing quality gels) rendering easy analysis of protein patterns.

5 ACKNOWLEDGEMENTS

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Differences in the Plasma Membrane Proteins of Chronic Alcoholic Rat Brain

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Abstract *Plasma membranes were isolated from the cerebral cortex of control and chronic ethanol-treated rat brains. Analysis of protein composition by SDS-PAGE and by two-dimensional gel electrophoresis (IEF-SDS-PAGE) revealed significant differences in the membrane protein patterns between control and ethanol-treated rat cerebral cortices, indicating the loss of several proteins in membranes from ethanol-treated rat brains. Plasma membrane-associated protein species are categorized into ethanol-sensitive and -insensitive proteins, based on their response to ethanol. This study reports that ethanol depletes certain intrinsic proteins of membranes that might be responsible for plasma membrane disruption by ethanol.*

Keywords Plasma membrane, membrane proteins, ethanol, brain, membrane disruption.

The neurochemical consequences of ethanol suggest physical disruption of plasma membrane in brain. The bulk of the evidence favors biophysical interaction of ethanol with membranes, and a decrease in the membrane order was concentration dependent on the addition of ethanol in vitro. The extent of membrane disorder was quantified by electron spin resonance (2), indicating that even low concentrations of ethanol can effectively fluidize membranes. Further, fluorescence anisotrophy studies suggested that plasma membranes could be disrupted by ethanol (6) and that the damage is more on the membrane core than on the surface of the phospholipid bilayer. Differential scanning calorimetry (8) and light-scattering studies (16) have confirmed the interference of ethanol with membrane components.

Increased membrane fluidity could alter receptor functions and neurotransmission process. However, the overall functional consequences of membrane disruption could be beyond the neurotransmission process. It has been proposed that membrane disorder is the actual cause of intoxication, and the membrane disruption is thought to be mediated

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Abbreviations: 2-DE, two-dimensional gel electrophoresis; EDTA, ethylenediamine tetraacetic acid; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethyl sulfonyl fluoride; SDS, sodium dodecyl sulfate.

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by proteins present in the membrane (5). Experimental evidence for direct action of ethanol on membrane proteins has been sparse. Our previous work has shown membrane protein defects in liver of chronic alcoholic rats (15). In the present study we report alterations in the protein profiles of membranes isolated from the cerebral cortex of rats in response to chronic ethanol intake in vivo. The loss of proteins in membranes of chronic alcoholic brain might have implications in the mechanism of membrane disorder by ethanol.

Materials and Methods

Ampholines and Ficoll-400 were purchased from LKB, Produkter, and other chemicals from Sigma. Silver nitrate and acrylamide were from Astra-IDL, India, and were analytical grade.

Chronic Ethanol Treatment of Experimental Animals

Wistar male and female rats of 80 ± 2 days of age, weighing approximately 200 ± 20 g, were used in this study. The animals were housed individually with access for food and water ad libitum for at least 7 days prior to experimentation. The environmental conditions in the animal house were adjusted for a constant temperature and a day-night cycle of 12 h with light from 7 a.m. to 7 p.m. The food given to animals was standard laboratory rat feed. Control animals were given tap water, while experimental animals received ethanol as 5% in tap water (v/v), prepared from absolute ethanol, as their only drinking fluid throughout the experimental period, which was up to their fifth month of age. Results of fluid consumption indicated that 7.5 g ethanol/kg body weight/day was consumed by each experimental rat. The protocol adapted to study the effect of chronic ethanol treatment is essentially followed from published procedures (4, 17). Another set of control animals was also maintained on tap water containing isocaloric sucrose substituted for ethanol. Following ~70 days of ethanol treatment, no significant differences were observed in the protein profiles of control and sucrose-fed rats. Therefore in subsequent studies comparison was made between control (tap water-fed rats) and ethanol liquid-fed rats only. Changes in the body weight, food and fluid intake were monitored every day between 10 a.m. and 11 a.m. Blood alcohol concentrations (BAC) were measured with alcohol dehydrogenase, as suggested in Sigma Chemical Company procedure 332-UV.

Preparation of Plasma Membrane

On the last day of the experimental period of chronic ethanol treatment, rats were decapitated and brains were dissected on an ice platform. Cerebral cortex was separated and homogenized using 10 volumes of ice-cold media-I [0.32 M sucrose prepared in 10 mM Tris-HCl (pH 8), 0.1 mM $MgCl_2$, 0.1 mM EDTA, and 0.2 raM PMSF]. Plasma membranes were prepared by slightly modifying the procedure of Pinkett and Anderson (14). All the steps were carried out at 4 °C, unless specified. The homogenate was centrifuged at 1000*g* for 7 min and the supernatant was saved. The pellet was rehomogenized in media-I, and centrifuged at 1000*g* for 7 min. The supernatants were combined and centrifuged at 16 000*g* for 15 min. The membrane pellet was suspended in 25.5% sucrose prepared in media-I and centrifuged at 100,000*g* for

60 min. The membrane pellet was resuspended in media-I and layered over a cushion of 10% Ficoll (w/v in media-I), and centrifuged at 100,000*g* for 60 min. The purified membranes banded above the Ficoll cushion and were diluted 10-fold with media-I and centrifuged at 12 000*g* for 20 min. Membranes were then resuspended in media-I and stored at −70°C till further analysis. The purity of the membrane fraction was checked by assaying the activities of Na^+,K^+-ATPase and adenylate cyclase in different fractions obtained during the preparation of membranes. The fraction obtained at 100,000*g* showed high specific activity of the enzymes, indicating the purity of plasma membrane (data not shown).

SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (10) with modifications as suggested by Thomas and Kornberg (20). The purified plasma membrane pellets were solubilized in SDS sample buffer [2% SDS, 10 mM Tris-HCl (pH 6.8), 5% 2-mercaptoethanol, and 8% sucrose] and heated at 100°C for 5 min. Samples were electrophoresed on 18% acrylamide gels following the modifications suggested by Thomas and Kornberg (20). The modifications include (a) an increase in the concentration of Tris to 0.75 M in the resolving gel; (b) lowering the ratio of acrylamide to *N,N*-methylene bisacrylamide to 40 : 0.40; and (c) changes in the electrode buffer composition (0.05 M Tris, 0.38 M glycine, and 0.1% SDS). These modifications allow a better resolution of proteins ranging from 90 to 9 kD. The gels were stained with Coomassie blue R-250. The SDS-PAGE gels were scanned for protein analysis by automated laser scanning unit, and results were analyzed using the software program of Biomed Instruments (1988).

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was performed essentially as described by O'Farrel (13). Purified plasma membrane pellets were suspended in 10 μl of 10 mM Tris-HCl, pH 7.8, and solubilized in 40 μl of 9.5 M urea with 2% ampholines (1.6% of pH 5-7 and 0.4% of pH 3.5-9.5 ampholines) and 5% 2-mercaptoethanol by swirling at room temperature. The IEF tube gel (110 X 2.5 mm) was prefocused before the addition of sample at 200 V for 15 min, 300 V for 30 min, and 400 V for 30 min. After prefocusing, the samples were loaded at the cathodic end and the gels were run for 8000 V h (600 V for 12 h and 800 V for the last 1 h). The isoelectrofocused gels were removed and equilibrated for 15 min in sample buffer. They were transferred onto SDS polyacrylamide slab gel with a stacking gel of 4.75% polyacrylamide/bisacrylamide and a resolving gel of 11.75% polyacrylamide/bisacrylamide. After completion of the electrophoresis in the second dimension, the gels were fixed and stained.

Silver Staining

Proteins in the gels were visualized by a silver staining procedure (1). Briefly, the protocol consists of the following steps. The gels were fixed in 50% methanol with 7.5% acetic acid and 0.5 ml of 37% formaldehyde for 1 h and washed in 50% ethanol three times, each 20 min. The gels were treated with sodium thiosulfate (0.2 g/liter) for 1 min, rinsed with water, and impregnated with silver nitrate (2 g/liter containing 0.75 ml of 37% formaldehyde) for 10 min. The gels were rinsed in water twice for 20 s, and

protein spots were developed in sodium carbonate (60 g/liter) and 0.5 ml of 37% formaldehyde/liter. Soon after the protein spots developed, the gels were washed twice in water and stored in 50% ethanol. Polypeptides were identified by their relative molecular mass with respect to the standards (Sigma). The isoelectric point (PI) was determined from the pH gradient measured in the IEF gel extrusion into deionized water. Assessment of alterations in control and ethanol-treated plasma membrane protein patterns was by direct visual scanning.

Protein Estimation

The protein levels in the cerebral cortex of control and ethanol-treated rats was estimated by the method of Lowry et al. (11).

Results

In the present study ethanol was administered to rats as sole drinking fluid in the form of 5% ethanolic tap water and the experiments were carried out over a period of ~70 days. The experimental group of rats did not show any overt behavioral or pathological symptoms except that their sleeping time was increased and the animals were flaccid. The righting reflex indicated they were intoxicated. The alcohol dose consumed by animals did not cause any mortality, and throughout the experimental period the animals were healthy but inebriant.

A 5% decrease (Table 1) in body weight was observed in the experimental group of rats when compared to water- or sucrose-fed rats (Figure 1). The decrease in body weight observed in the ethanol-treated group of rats agrees with the reports of Matsubara et al. (12). There was no significant variation in the food consumption in the experimental group of animals when compared with the controls (Figure 2). These results were in agreement with the values reported by Ward (21). The average consumption of ethanol was 7.5 g/kg body weight/day. From the fluid consumption pattern (Figure 3), as well as from ethanol levels in blood, blood alcohol concentration (BAC) was found to be 0.15%, suggesting that the animals were under the intoxication range. The fluid intake pattern suggested that the absolute amount of ethanol consumed was relatively consistent.

Total Protein Levels

Following ~70 days of treatment, analysis for protein variation showed no significant differences between control and sucrose-fed rats. Therefore comparison was made between control (tap water-fed rats) and ethanol liquid-fed rats only.

Table 1

Changes in Body and Brain Weight (g) of Rats during Chronic Ethanol Treatment

Parameter	Control	Sucrose-fed	Ethanol-fed
Body weight (initial)	221 ± 27	222 ± 18	226 ± 25
Body weight (final)	328 ± 65	329 ± 62	**311** ± 55
Brain weight	2.04 ± 0.11	2.00 ± 0.19	1.98 ± **0.21**

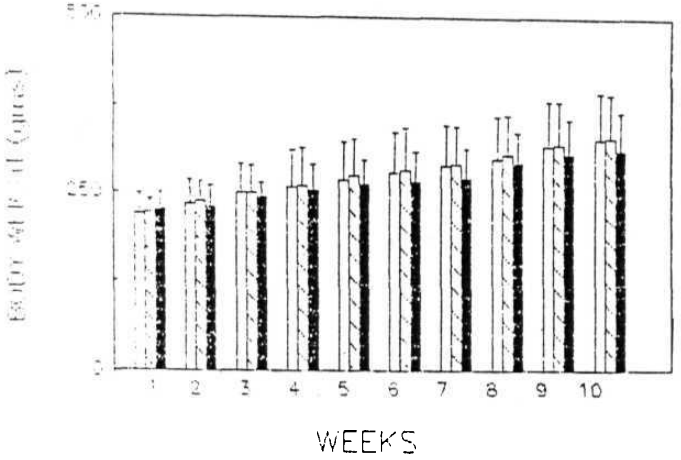

Figure 1. Changes in average body weights in control (•), sucrose-fed (D), and chronic ethanol-fed (•) rats. Ethanol at 5% (v/v) was given to rats from 80 days of age as sole drinking fluid. Each bar is represented as mean ±SD of 10 samples.

The protein content of cerebral cortex decreased significantly due to chronic ethanol intake (Table 2). A similar decrease in total protein content was reported in humans over a 1-year alcohol treatment (9). In cultured glial cells, the protein content was reported to have been reduced by 50% during a *2%* ethanol treatment of cells beginning on day 6 and harvested on day 10 (3). It was suggested that an insufficient supply of essential amino acids could be responsible for low protein levels, but this possibility was ruled out as we found that the food consumption in the experimental group of animals is not

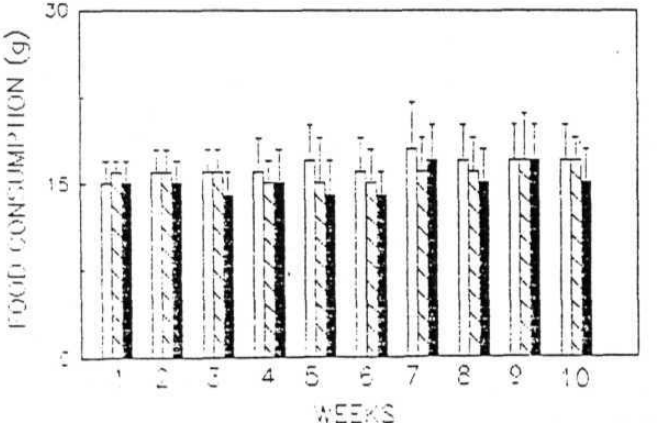

Figure 2. Changes in average food intake pattern in control (D), sucrose-fed E), and chronic ethanol-fed (•) rats. Ethanol at 5% (v/v) was given to rats from 80 days of age as sole drinking fluid. Each bar is represented as mean ±SD of 10 samples.

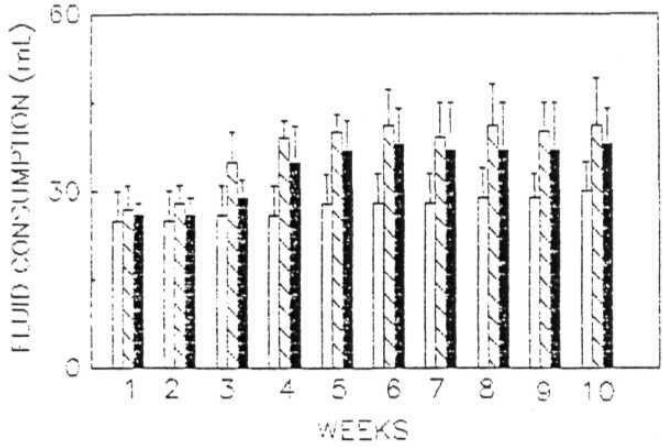

Figure 3. Changes in average fluid intake pattern in control (□), sucrose-fed (▨), and chronic ethanol-fed (•) rats. Ethanol at 5% (v/v) was given to rats from 80 days of age as sole drinking fluid. Each bar is represented as mean ±SD of 10 samples.

altered significantly (Figures 2, and 3), thereby ensuring a similar supply of essential amino acids.

SDS-PAGE Profiles of Plasma Membrane Proteins

The protein compositions of the plasma membrane fractions from control and ethanol-treated rat brains were analyzed by SDS-PAGE analysis. Significant differences were observed in the membrane protein profiles of cerebral cortex of control and ethanol-treated rats (Figure 4). The SDS-PAGE scans were developed using the same volume (Figure 4A and B) and the same amount of protein (Figure 4A and C). These scans revealed either a decrease in the content or a total loss of proteins in the molecular weight range from 70 to 20 kD. The changes in protein pattern could be noticed in which either the same volume and the same amount of protein was loaded on to the gels;

Table 2
Changes in Protein Levels in the Cerebral Cortex of Rat during Chronic Ethanol Treatment

Treatment	Protein[a] (mg/g weight)
Control (no treatment)	9.77 ± 0.7
Control (sucrose-fed)	9.75 ± 0.6
Ethanol-treated	7.48 ± 0.5
Percent change	−23.43
t-Test	$p < .01$

[a]Values are mean and SEM of 10 samples.

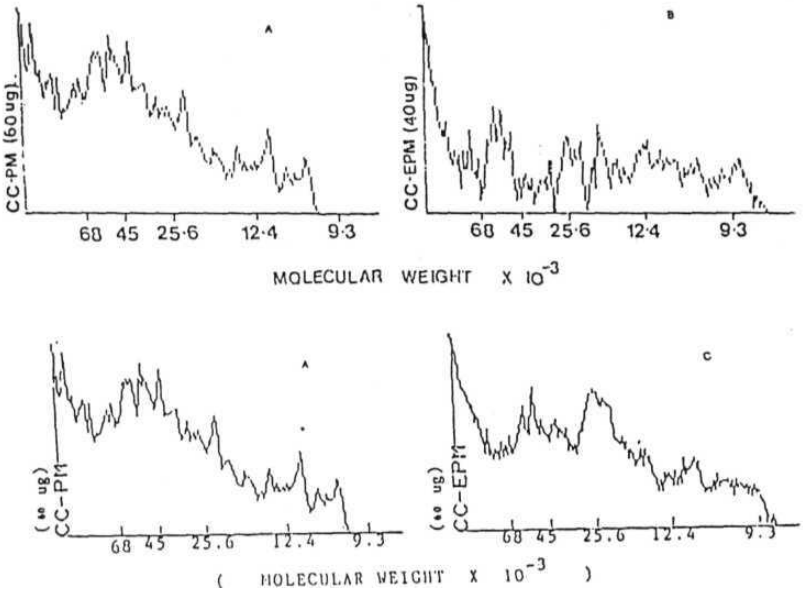

Figure 4. Automatd laser scan SDS-PAGE profiles of plasma membrane proteins from cerebral cortex of control and ethanol treated rat brains. Membrane fractions are obtained from cerebral cortex of control rat (CC-PM) and from cerebral cortex of ethanol-treated rat (CC-EPM). Molecular weight is shown on *x* axis. Purified membrane fractions are analyzed by SDS-PAGE (see Materials and Methods) (A, B) on a constant-volume basis and (A, C) on the basis of same amount of protein. The amount of protein loaded into gels is shown in parentheses.

however, the changes were more conspicuous and can be easily recognized in gels where protein is loaded on a same-volume basis.

Two-Dimensional Gel Electrophoretic Profiles of Plasma Membrane Proteins

A marked decrease in plasma membrane-associated proteins in rat brain by ethanol as noticed in SDS-PAGE gels prompted more detailed analysis to identify the category of protein species affected or unaltered. As the resolution of proteins in SDS-PAGE is limited, the membrane proteins were separated on high-resolution, two-dimensional electrophoresis followed by ultrasensitive silver staining. The pH gradient in representative IEF gels was measured by a surface pH electrode, and it showed a linear gradient from pH 3 8 to 8.1. The presence of proteins did not affect the pH gradient in the IEF gel. Using two-dimensional electrophoretograms a catalog of the proteins sensitive and insensitive to ethanol is generated (Figure 5 (A and B) and Table 3) Proteins insensitive to ethanol action have been designated with numbers and termed ethanol-,n ens tive proteins (EISP). Those proteins that were decreased or completely missing in membrane fraction obtained from ethanol-treated rats but were present in the membranes o contro rats were termed ethanol-sensitive proteins (ESP) and designated with letters of the alphabet. Ethanol in vivo appears to be very effective as a membrane-disordering agent,

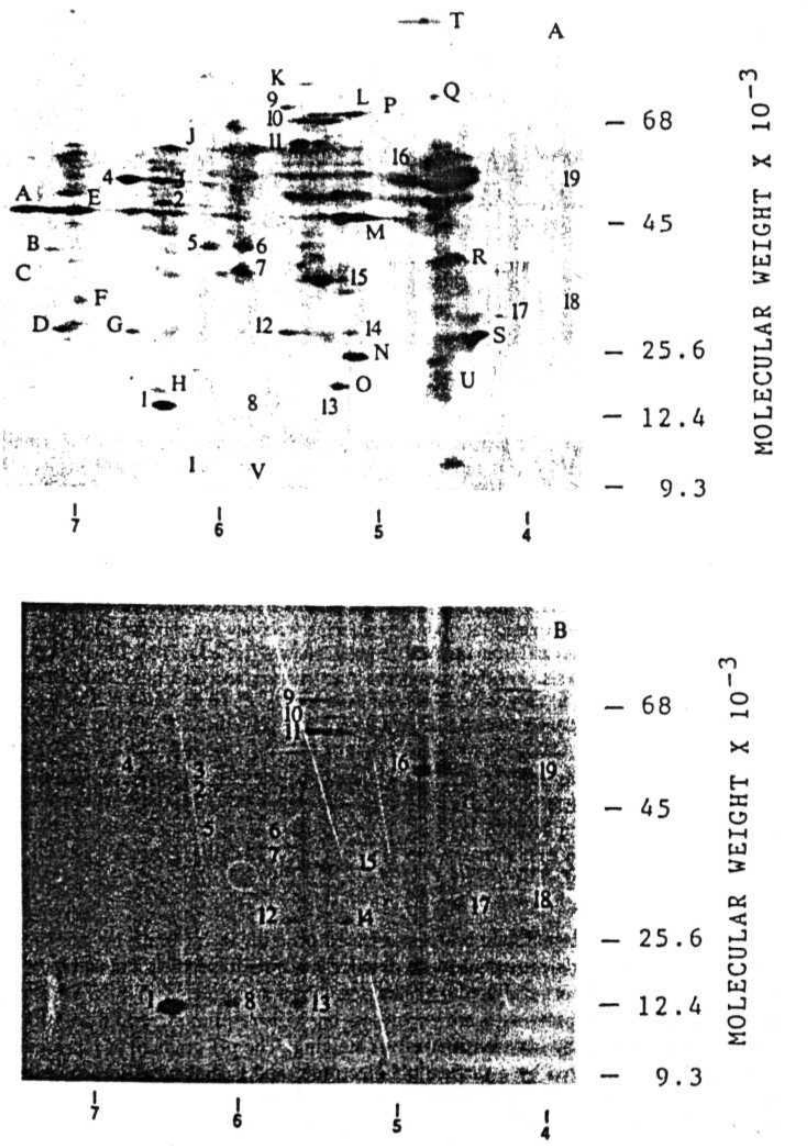

Figure 5. Two-dimensional gels of plasma membrane proteins from (A) control (60 μg protein) and (B) ethanolic rat cerebral cortex (40 μg of protein). Proteins were separated over a pH gradient of 3.8 to 8.1. Molecular weight is shown on the right side. Proteins designated by numbers in the figure indicate ethanol-insensitive (and less sensitive) proteins (EISP), while proteins designated with letters of the alphabet refer to ethanol-sensitive proteins (ESP).

Table 3
Characteristic Features of Ethanol-Sensitive (ESP) (A-V) and Ethanol-Insensitive Proteins (EISP) (1-19) Associated with Plasma Membrane Fraction on Cerebral Cortex of Rat Brain

Ethanol-sensitive Proteins			Ethanol-insensitive Proteins		
Protein	PI	Molecular weight* ($\times 10^{-3}$)	protein	PI	Molecular weight ($\times 10^{-3}$)
A	7.5–7.8	47.00	1	6.6	12.40
B	7.4	40.00	2	6.6	54.00
C	7.5	35.60	3	6.6	58.00
D	7.1–7.4	25, 60[a]	4	6.8	58.00
E	7.1–7.4	45, 48[a]	5	6.3	41.00
F	7.2	30.00	6	6.0	41.00
G	6.7	25.00	7	6.0	38.00
H	6.2–6.6	19, 12.4, 8.5[a]	8	6.1	12.40
I	6.5	9.00	9	5.8	72.00
J	6.4–6.6	60.00	10	5.4–5.7	70.00[a]
K	5.5–5.7	73.00	11	5.5–5.8	68.00[a]
L	5.3–5.4	70.00[a]	12	5.8	29.00
M	5.2–5.5	45.00[a]	13	5.7	12.00
N	5.2–5.4	22.00	14	5.3	25.00
O	5.4	13.00	15	5.4–5.7	37.00[a]
P	5.0	68.00	16	4.5–4.9	55.60[a]
Q	4.8	70.00	17	4.3	32.00
R	4.6–4.9	38.00	18	4.0	33.00
S	4.4–4.6	24.00	19	4.0	58.00
T	4.8–5.0	108.00			
U	4.7	12.00			
V	6.04	9.50			

[a]Group of proteins. *In kilo daltons.

as several proteins could be identified (Table 3) undergoing change quantitatively and/or qualitatively.

Discussion

These results represent an initial attempt to identify the changes in membrane protein composition and thereby explain the mechanism of membrane disorder induced by ethanol. Though proteins were suspected to be involved in the membrane disorder, experimental evidence for protein involvement is lacking. Hence, in the present study, protein defects were analyzed in plasma membrane of rat brain cortex in response to chronic ethanol intake in vivo. In this study, we have shown that membrane proteins observed in one-dimensional SDS-PAGE could be resolved further by using 2-DE into individual polypeptides based on their isoelectric point and relative molecular mass. There have been previous studies of this kind, but all of them have focused on proteins of different

cell types in brain or regional variation of brain proteins in disease states (7, 8). The results of 2-DE showed differences in the quantitative and qualitative profiles of individual plasma membrane proteins of brain from control and ethanol-treated rats. The total number of plasma membrane proteins detectable from control fractions was more than 100. Though it was possible to resolve all these proteins, only the most abundant and easily recognizable proteins were analyzed. Emphasis was laid on the proteins that were missing completely after chronic ethanol intake. However, the number of membrane proteins visualized in two-dimensional gels might still be partial in terms of percent messenger-RNA-specific or destined to membrane proteins in rat brain. Considering the brain-specific translational regulation and efficiency (18), it could be possible that some of the messenger RNAs might be scarcely translated. Further, reduced ribosomal binding to stable mRNA in brain in the presence of ethanol (19) might be responsible for the reduced number of polypeptides noticed in the cerebral cortex from ethanol-treated rats.

The aim of this study was mainly to observe the action of ethanol on the proteins associated with the plasma membrane of brain. It is obvious from this study that ethanol can be very effective in vivo, as several protein species were found to decrease quantitatively or disappear completely. The present report might be the first one to demonstrate changes in the proteins associated with the membrane in brain after chronic ethanol treatment. Though the metabolic fate of these missing proteins is not known at present, we propose that the very loss of proteins from membrane disrupts the membrane. The loss of proteins indicates that these missing proteins embedded in the too-fluid-lipid environment of plasma membrane might be responsible for altering membrane stability and bilayer fluidity during the influence of ethanol. It is not known at present whether such a loss could be due to proteolysis of specific membrane proteins or due to synthesis of respective messenger RNAs, or whether the proteins are lost during their translocation into other subcellular organelles or due to an impairment in signal transduction. Studies are being conducted in this direction to resolve these possibilities.

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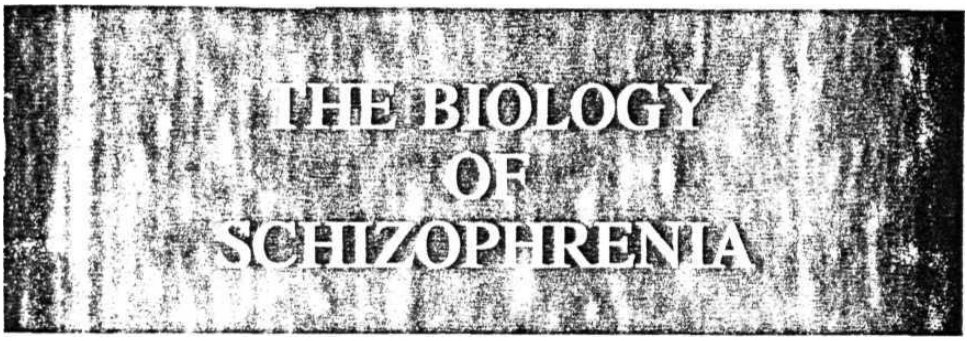
PROGRAM AND ABSTRACTS



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TWO NOVEL SEX-LINKED PROTEINS IN MODERATELY MENTALLY RETARDAD (SCHIZOPHRENIA) PATIENT

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Cerebrospinal fluid (CSF) from six moderately mentally retarded male patients were compared with their age matched normal CSF samples. Two novel proteins with relative molecular mass 16.5 and 16.8 with isoelectric point of 7.5 and 7.2 respectively were identified in the Schizophrenic's (retarded) CSF samples. These two proteins are X-chxomosome linked and are stable for a long period (3 months) at room temperature. These patients had common physical features Like normal birh, minimal neurological deficits, narrow fore-head, large mouth, wide ear lobules and hyper extensible finger joints. Despite unknown origins and identity, the presence of proteins in CSF of a specific group of mentally retarded male patients suggests their possible clinical utility in Schizophrenics.

CYTOBIOS (IN PRESS)

Recent Advances in Nuclear Matrix Function

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Abstract

The nuclear matrix in eukaryotes is a non-histone proteinaceous nucleoskeleton structure having attachment sites for DNA loops during DNA replication. The nuclear matrix has been implicated in transcription, regulation of gene expression, primary transcription processing and provides a mooring for certain hormone receptors. This review presents recent advances concerning the involvement of the nuclear matrix in DNA replication, relaxation of the superhelical strain in DNA, processing of hnRNA and snRNP, and RNA transport. Also, the nuclear matrix protein has linkages to intermediate filaments of the cytoskeleton.

Introduction

Classical ultrastructural studies on the cell nucleus have demonstrated an association of euchromatin and heterochromatin with an extensive non-chromatinous filamentous and granular network in the interior of the nucleus (see Agutter, 1991; Hoffman, 1993). It was shown 50 years ago that a fraction of nuclear proteins resists extraction even with buffers of very high ionic strength (Mayer and Gulick, 1942). This subfraction was found to be made of nucleoprotein fibrillary network. The term 'nuclear matrix' was assigned to this structure by Berezney and Coffey (1974). DNA associated with the nuclear matrix contained A-T rich sequences and therefore was designated as matrix associated regions (MAR) or scaffold associated regions (SAR). This scaffold forms the base chromatin loops during DNA replication in organisms ranging from yeast to man. SARs were shown to be usually located at the borders of different genes, close to 5' or 3'-end cis-regulatory sequences or DNAse I hypersensitive sites suggesting that SARs may participate in transcriptional regulation through regulatory domains (Avramova and Paneva, 1992). The nuclear matrix is now considered to be a valid structure with a few presently defined functions and probably several yet to be identified functions. Some of the nuclear matrix functions supported by experimental proof are:

(a) organization of chromatin in the interphase nucleus of eukaryotes into loops of 30-100 kbp, and each of these loops represents either a replicational or transcriptional single unit.

(b) the localization of replicational machinary (replisomes) in the nuclear matrix (Tubo *et al.*, 1985) is considered to be the site of DNA replication.

(c) the nuclear matrix is enriched with actively transcribed genes (Zehnbauer and Vogelstein, 1985) and is associated with the processing of RNA.

(d) the nuclear matrix acts as a site of interaction for viral proteins (Covey *et al.*, 1984) and viral DNA (Rennie *el al*, 1983).

(e) the nuclear matrix contains binding sites for some hormones, carcinogens (Gupta *et al.*, 1985), tumor promoters (Eisenman *et al.*, 1985), drugs and other substances (Kaufmann *et al.*, 1986; Barrack and Coffey, 1982).

The aims of this article are to show the methodological options available for nuclear matrix preparation and to review the recent information concerning the function of the nuclear matrix.

Methodological options for nuclear matrix preparation

The *in situ* nuclear matrix was first isolated and characterized by Berezney and Coffey (1974) from liver. Subsequently many others (Faken and Hancock, 1974; Cook and Brazell, 1975; Paulson and Laemmli, 1977; Gasser and Laemmli, 1986b) isolated and characterized nuclear matrices from a variety of tissues and organisms. Several of these isolation methods involved treatment of isolated nuclei with nucleases in a non-ionic detergent and a high concentration salt buffer. This sequential extraction of nuclei finally yielded a nuclear matrix fraction usually containing the granular and fibrous internal matrix which forms a web throughout the interior of the nucleus, the residual elements of the nuclear envelope (also termed the pore complex lamina), and residual nucleoli. A major modification of the foregoing method was suggested by Mirkovitch *et al.* (1984)

who used lithium 3,5-diiodosalicylate (LIS) as a chaotrophic agent and a detergent instead of a buffer with high ionic strength. The resultant preparation, termed the "nuclear scaffold" has been widely used to study the DNA sequences which are specifically associated with the matrix. The nuclear scaffold also has been described as the nuclear skeleton, nuclear ghost or nuclear cage. Nuclear matrices are predominantly proteinaceous. Depending on the type of isolation methods employed, several studies have shown minor variations in the biochemical composition of the nuclear matrix, due for example to contamination with RNA. Indeed, if ribonuclease was omitted, the isolated nuclear matrix contained RNA as the second most abundant component. Matrix preparations examined by electron microscopy showed several common structural entities such as the fibrillary and granular internal nuclear matrix, pore complex lamina, and residual nucleoli.

Some studies have suggested that structural integrity of the nuclear matrix involves metallo-protein interactions during matrix isolation, based on the inclusion of Ca^{++}, pr Cu^{++} (Lebkowski and Laemmli, 1982) or Mg^{++} (Bouvier *et al.*, 1985a). Matrix preparations using Mg^{++} were found to be enriched with residual RNP complexes which formed a salt-resistant intra-nuclear network. Digestion with RNase in the presence of low ionic strength EDTA altered the morphology of the network, suggesting a biochemical role for Mg^{++} (Bouvier *et al.*, 1985b). The internal fibrous network of the nuclear matrix is more labile than that of the nuclear lamina. Therefore, most of the nuclear matrix protocols involve a stabilization step to avoid dissociation of the fibrous network. Various methods entail stabilization by fixation with acroline (de Graaf *et al.*, 1991), brief incubation at 37 or 42*C, treatment with Cu^{++} (Razin *et al.*, 1985) or oxidation with sodium tetrathionate (NaTT) (Kaufmann and Shaper, 1984). The mechanisms involved in stabilization are not known except for the method of oxidation with NaTT. NaTT oxidizes sulfhydryl groups to disulfide bridges, which results in the stabilization of the nuclear matrix (Kaufmann *et al.*, 1986). Based on these data, it was

subsequently shown that a reduction of disulfides is important in the disassembly of the nucleus at prophase (Sturrman *et al.,* 1992).

The nuclear matrix and DNA replication

Wanka *et al.* (1982) suggested that the nuclear matrix might be involved in unwinding the DNA double helix in a specific manner so that the daughter DNA molecules can be separated easily from the parent template during DNA replication. But their study could not conclusively demonstrate the matrix binding region on the DNA molecule or specific attachment sites for DNA on the nuclear matrix. Tubo and Berezney (1985) provided evidence, using density shift experiments, for the replisome loop model in eukaryotic DNA replication. On the basis of the relation between loop sites and replication sites, the bases of the DNA loop behave as replication origins. Studies of Dijkwel *et al.* (1986) showed the position of replication origin relative to the nuclear matrix by autoradiographic analysis of nuclear matrix halo structures. Using synchronized BHK cells, a label at the beginning of S-phasc remained matrix associated and later on migrated into the DNA halo, suggesting that replication origins remain matrix bound after the initiation of DNA synthesis. Tubo *et al.* (1985) provided enzymatic evidence that approximately 10% of the total cellular DNA primase activity was associated with the isolated nuclear matrix, suggesting that the nuclear matrix is an important entity in the eukaryotic replication of DNA in the cell nucleus. Further studies by Dave *et al.* (1989) showed terminal deoxyribonucleotidyl transferase(s), a class of DNA polymerase, is involved in the postembryonic DNA synthesis of immunoglobulin gene recombination events in thymus cell nuclei and is associated with the nuclear matrix for its expression. This enzyme catalyses the addition of deoxyribonucleotides to the 3'-(OH) terminus of DNA without template direction. All these reported findings conclusively demonstrate the involvement of the nuclear matrix in DNA replication and DNA synthesis.

Enzymes of DNA and RNA metabolism and the nuclear matrix

Several enzymes involved in DNA and RNA metabolism have been shown to be associated with the nuclear matrix. These include DNA a and [3 polymerases (Foster and Collins, 1985), topoisomerases I and II (Berrios *et al.,* 1985) RNA polymerase II (Lewis *et al.,* 1984), poly (A) polymerase (Schroder *et al.,* 1984), DNA methylase (Burdon *et al.,* 1985) and DNA primase (Tubo and Berezney, 1987a). Though the function of these enzymes is known, it is not understood how and why these enzymes are associated with the nuclear matrix.

Topological states of DNA and the nuclear matrix

The nuclear matrix also has been implicated in the conversion of different topological states of DNA. DNA topoisomerase II, mediates the interconversion of DNA through transient double strand breaks and rejoining. Topoisomerase II can relax positive and negative supercoiled DNA (Wang, 1985). DNA topoisomerase II has been shown to bind in a cooperative manner to SAR. This suggests a role for the nuclear matrix in confirming specific topology to DNA through DNA topoisomerase II. Studies by Tsutsui *et al.* (1988) suggested that the nuclear scaffold exhibits at least two classes of DNA binding sites of which one is specific to supercoiled DNA and does not bind relaxed or linear forms, while the other lacks this specificity. Cockerill and Garrard (1986a) suggested a direct anchorage of topoisomerase II to chromosomal loop domains. They mapped the binding sites in the mouse immunoglobulin-k gene and found common sequences in the binding site and in the corresponding region of other genes (Cockerill and Garrdard, 1986b; Udvardy *et al.,* 1985). The DNA binding site on the nuclear scaffold exhibited a recognition mechanism which was not based on nucleotide sequence but rather was conformation directed, in the sense that tortional stress generated in the looped domain served as a recognition signal. Similarly, in a recent study (Hinzpeter and

Depperet, 1987), it was shown that concatemerized oligonucleotides posses an unwinding nucleation site with strong affinity for the nuclear scaffold, and with augmented SV40 promoter activity. Mutated condatemerized oligonucleotides resisted unwinding, showed weak affinity and a lack of enhancement of promoter activity, suggesting that relaxation of the superhelical structure of DNA by topoisomerase II is important for SAR functions (Bode *et al.*, 1992).

Nuclear matrix and gene expression

Buttyan and Olsson (1986) using androgen dependent genes, demonstrated that actively transcribed genes are protected from nuclease digestion by their association with the nuclear matrix. They further used these nuclear matrix protection assays to analyse the tissue specific expression in a highly related gene family and to predict transcriptional activity of this gene family in a specific tissue. In support of the foregoing, Keppel (1986) provided evidence that transcriptional human ribosomal RNA (rRNA) genes are attached to the matrix and that the tandom repeats of ribosomal DNA (rDNA) are not randomly associated with the matrix but are probably attached at transcriptional complexes. Studies on the visualization of mRNAs for fibronectin and neurotensin using fluoresence hybridization with cDNA and intron specific probes indicate that there is a highly ordered structural organization in association with the nuclear matrix (Xing *et al.*, Carter *et al.*, 1993). Also, it has been demonstrated that a receptor binding factor (RBF-1) for the avian oviduct progesterone receptor (PR) has high affinity binding sites on avian genomic DNA, which are localized in the nuclear matrix. The direct action of progesterone results in the rapid expression of the nuclear matrix protooncogenes *c-myc* and *c-jun* (Schuchard *et al.*, 1991).

Nuclear matrix association with heterogeneous nuclear RNA (hnRNA)

Herman *et al.,* (1978) in a two-step extraction of chromatin were able to remove 99% of the chromatin. The remainder of the RNA is associated with the nuclear matrix. This fraction of residual RNA was found to be chiefly heterogeneous nuclear RNA (hnRNA). They suggested that the integrity of the nuclear matrix is dependent on this RNA. There are pros and cons for this interpretation. For example, Miller *et al.,* (1978a) showed that RNase treatment of the nuclear matrix does not alter its morphology, while electron microscopic studies by Fey *et al.* (1986a) showed a drastic alteration in the morphology of the nuclear matrix when RNase was included in the preparation of the nuclear matrix. The data reported by Smith *et al.* (1986) agTee with other studies showing that internal matrix structures are distorted when nuclear matrix associated RNA is degraded or metal ions are chelated. Their data further suggested that internal nuclear matrix assemblies arc present *in situ* and their absence in biochemical preparations might be an artifact. Knowledge of the composition and organization of hnRNA in the granular and fibrous internal nuclear matrix structure is required to understand how RNA is associated with the nuclear matrix. Gallinaro *et al.* (1983) showed that pre-mRNA in the nuclear matrix and in the salt resistant complexes derived from hnRNP, share a common constitutive unit, suggesting that hnRNP and mRNA are structurally similar.

Small nuclear ribonuclear particles (snRNP) and the nuclear matrix

Like hnRNA, small nuclear ribonuclear particles (snRNPs) were found associated with nuclear matrices prepared from chicken oviduct (Ciezek *et al.,* 1982). The snRNPs (also called U RNAs) play a role in the processing of pre-mRNA. Of these, U1 and U6 RNAs are major U RNAs and U7 to U10 arc minor U RNAs in eukaryotes. Zieve and Penman (1976) showed that U2, U3, U4, U6 RNAs are associated with the nuclear matrix. Though Ul RNA is also associated with the nuclear matrix, it is lost during chromatin extraction. The function of snRNPs is not known. One hypothesis is that they can provide a backbone structure thereby facilitating packaging (Berezney, 1984), post-

transcriptional modification (Herman *et al.,* 1978) and transport of RNA into the cytoplasm (Gallinaro *et al.,* 1983). A specific interaction between snRNPs and nuclear matrix structures has been indicated by a limited number of studies (Padgett *et al.,* 1986; Bringmann and Luhrmann, 1986). However, these fail to establish the functional significance of such an interaction.

RNA transport and the nuclear matrix

Different RNA species are transported at different rates from the nucleus, and in general, smaller mRNAs are transported more rapidly into the cytoplasm than larger mRNAs. Most of the mRNAs are polyadenylated while some are non-polyadenylated such as histone mRNA. Studies by Mariman *et al.,*(1982) showed that adenovirus PIX mRNA (about 9s polyadenylated and unspliced) reaches the cytoplasm within 4 minutes after the start of synthesis while late adenovirus mRNA of the same size reaches the cytoplasm only after 16 minutes. The latter mRNA was shown to be matrix bound. The rate of transcription of mRNA depends on its rate of maturation. Reasons for the different rates of transport of matrix bound mRNAs remain to be elucidated.

Virus specific proteins and the nuclear matrix

Studies have shown that the nuclear matrix is an important site for viral interaction. Viral DNA and viral specific proteins have been found to be enriched in the nuclear matrix. Studies by Hinzpeter and Deppert (1987) showed that the interaction of viral antigens with chromatin and the nuclear matrix is mediated by protein-protein interactions rather than by protein-DNA interactions.

The viral genome of the bovine papilloma virus type-1 (BPV-1) has a stretch of sequences which are located immediately adjacent to the origin of DNA replication. These sequences (672 bp DNA fragment) have been reported to interact with the nuclear

matrix which is responsible ultimately for the replication of virus in the nuclei of tumor and other transformed cells (Adorn and Richard-Fay, 1991).

Phosphorylation of the nuclear matrix

Post-translational modification of proteins is one of the regulatory mechanisms in cellular responses. Phosphorylation reactions involving protein kinases and phosphatases are often involved. The interplay between these two enzyme activities, results in the phosphorylation and dephosphorylation of specific substrates which ultimately bring about signal transduction events. Ca^{++}/calmodulin dependent protein kinases mediate several neuronal events in the cytosol, at synaptosomes, and in the nucleus. Most nuclear Ca^{++}/calmodulin protein kinases have been shown to be associated with the nuclear matrix. Such an association leads to compartmentalization, which provides a mechanism for the regulation of enzyme access to substrates critically involved in nuclear functions. Halikowska and Leiw (1987) characterized a highly phosphorylated nuclear protein (68 kDa; pI 6.5-8.2) associated with mononucleosomal particles and also with the nuclear matrix. This protein showed a high degree of phosphorylation in regenerating liver after partial hepatectomy. Extensive phosphorylation of this protein and its association with the nuclear matrix suggests that it plays a key role in nuclear organization and function. Further, Zhelev *et al.* (1990) showed a marked increase of 125 kDa (pI 6.5) protein in mitotic mammalian cells. This protein was named "mitotin" and was shown to be associated with the nuclear matrix. Accumulation of mitotin in premitotic and mitotic cells was related to the phosphorylation of this protein and metabolic stability of its phosphorylated forms. These events suggest a role for phosphorylation and for the nuclear matrix in the complex events of mitosis. In another study, differences in nuclear matrix phosphoproteins in wild type and nitrogen mustard-resistant rat mammary carcinoma cell lines have been reported (Moy and Tew, 1986). Though the antigens in the

two tumor cell lines were similar, one of them, due to its hypophosphorylation, failed to bind cyclic-AMP (c-AMP) and thus differed from the other. Phosphorylation of matrix proteins leading to structural alterations in the nuclear matrix suggested that the nuclear matrix can change its configuration depending on the functional requirements of the cell.

Cytoskeletal proteins and the nuclear matrix

During transcription in eukaryotes, RNA polymerase II has to be activated by binding to promoter elements such as the TATA box (Breathnach and Chambon, 1981). Eukaryotic cells contain multiple factors which enable the binding of RNA polymerase II to this promoter element. One of these factors has been purified, characterized and was found to be similar to nuclear actin (Egly *et al.*, 1984). Moreover, antibodies to nuclear actin were injected into oocytes, these stopped transcription by RNA polymerase II, suggesting a possible role for nuclear actin in RNA metabolism. Nakayasu and Ueda (1984) have shown an interaction between pre-mRNA and actin filaments in the nuclear matrix of mouse leukemia L5178Y cells. Other studies also detected actin as a major protein in nuclear matrices (Nakayasu and Ueda, 1986). These observations suggest that actin might be involved in a well defined function in RNA synthesis. However, the foregoing possibility requires further study.

Nuclear matrins are major nuclear matrix proteins

The protein composition of the nuclear matrix recently was analysed in detail (Nakayasu and Berezney, 1991). Approximately 12 major proteins were identified, of which nuclear lamins A, B and C and the nucleolar protein B-23, and residual hnRNPs constitute already identified proteins. The rest of the proteins termed nuclear matrins consist of matrin 3 (125 KDa slightly acidic), matrin 4 (105 KDa, basic), matrin D-G (60-75 KDa, basic) and matrins 12 and 13 (42-48 KDa, acidic). Peptide mapping studies showed no homology of these matrins to nuclear lamins. Matrin-3 had an extensive acidic

domain with a nuclear targeting signal sequence, and is a highly conserved protein (Belgrader *et al.*, 1991). Interestingly, matrins D-G comprise two pairs of related proteins (matrins D/E and F/G). The F/G matrin was found to be a DNA binding protein containing two putative zinc finger motifs (Hakes and Berezney, 1991a). A palindromic seven amino acid sequence (Ser-Ser-Thr-Asn-Thr-Ser-Ser) was discovered within one of the zinc finger DNA binding regions. This sequence appeared to be a potential site for phosphorylation and glycosylation and therefore might be involved in a regulatory role within the DNA binding domain.

In addition to F/G lamins A and C (but not B), matrins D and E were reported to be specific DNA binding proteins having preference for single strand DNA (Hakes and Berezney, 1991b), probably each with a separate sequence specificity. In DNA binding assays a preference was demonstrated for nuclear matrix DNA over total genomic DNA. These studies directly demonstrated that internal nuclear matrix proteins (D, E, F, G and 4), in addition to the lamins A and C, can bind to DNA, suggesting that loop attachment sites are internal as well as peripheral in the nucleus during DNA replication.

In another study, a DNA binding protein (SATB1) (human cDNA clone) from thymus, with selective binding to MARs, was reported (Dickinson *et ai,* 1992). SATB1 showed an unusual binding site recognition in the sense that it binds to a ATC rich sequence, wherein one strand consists of mixed A's, T's and C's excluding G's. In mutants deficient in the ATC rich sequence, there was a significant reduction in binding, even when the direct contact sequence remained intact. This suggested that SATB1 binding to MAR's is through the recognition of ATC sequences by sugar-phosphate in the backbone structure of DNA. Luderus *et al.* (1992), employed a heterogenous binding system using matrix preparations from rat liver and the MAR from the histone cluster of *Drosophila.* MAR binding nuclear proteins were identified. They also measured MAR binding to lamins from calf thymus and *Drosophila.* They found a 67 kDa matrix protein, which was identified as lamin B1 and also another minor protein lamin B2. They

further suggested that lamin B interacts with chromosomes by directly binding to MAR sequences leading to the decondensation of the chromatin, the repolymerization of lamina or both. This type of mechanism probably safeguards chromatin organization and argues for a key role of lamin B in certain cells that lack lamin A and C.

Concluding remarks

A proteinaceous fibrillar and granular nucleoskeleton constitutes the major component of the nuclear matrix. Other morphological features of the nuclear matrix, such as the nuclear pore complex and nuclear lamina (also called fibrous lamina) are now generally accepted and immunocytochemical and immunochemical studies have identified some of their constituent molecules (Ris, 1991; Akey, 1991; Dingwall and Laskey, 1992; Hinshaw *et al.*, 1992). However, models describing the association of the nuclear matrix with nuclear pore complex morphology are still evolving; this aspect has been recently reviewed (Maqat, 1991). Recent advances suggest that the participation of the nuclear matrix in DNA replication and gene expression is through replicational assemblies (replisomes) which associate with the nuclear matrix in clusters forming special sites termed "clustersomes" (Berezney, 1991). How exactly DNA synthesis might be initiated on clustersomes is yet to be determined. Also, the stimulation of gene expression at SARs has to be further resolved, as SARs are usually found at the border between different genes which mark the boundaries of chromatin domains. However, there also have been several reports (Jarman and Higgs, 1988; Farache *et al.*, 1990) localizing SARs within the transcribed sequences. The independent regulation of these domains might contribute significantly to gene expression.

Future studies should further clarify the nature of nuclear matrix protein-DNA interactions, the composition of these proteins, and their eurcaryotic cells. Structural and regulatory roles in the nuclear matrix and its protein composition in cancer cells has not been investigated thoroughly. A recent report indicates that a nuclear matrix protein (Mr

56000, P1 6.58) detectable in prostate cancer cells is not seen in normal prostate or benign prostatic hyperplasia (Partin *et al.*, 1993). As the nuclear matrix is involved in cellular regulation, via nuclear events (Xing *et al.*, 1993), there may well be characteristic nuclear matrix lesion(s) in neoplastic cells.

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