CHARACTERIZATION OF DNA BINDING PROPERTIES OF TWO NUCLEAR PROTEINS FROM BRAIN

A Thesis submitted for **degree of**

Doctor of Philosophy

by

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to the

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TABLE OF CONTENTS

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July 1993

This is to certify that I, A. Raghunathan, have carried out the research work embodied in the present thesis under the guidance of Dr. Mohan C. Vemuri, for the full period prescribed under Ph.D. ordinances of the University.

I declare to the best of my knowledge that no part of this thesis was earlier submitted for the award of research degree of any University.

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ACKNOWLEDGEMENTS

I have no words to thank, admire and appreciate the person behind this thesis, Dr. Mohan C. Vemuri, my research supervisor, who introduced me into this field of Molecular Biology. His invaluable suggestions and incessant encouragement helped me to love the subject and understand it better. My heartfelt thanks to him for his valuable advices and guidance.

My sincere thanks to Prof. N.C.Subrahmanyam, Dean, School of Life Sciences, and the former Deans, Prof. P.R.K. Reddy and Prof. K. Subba Rao for extending all necessary facilities for my research work.

I thank Dr. Ch.R.K. Murthy for his suggestions and critical reading of the manuscript and Dr. P.Reddanna for his help and cooperation. My thanks to Dr. G. Ranganayakulu for his help in Genius binding assays and Dr. Gopalakrishna for his help in CD studies. My thanks are due to Dr. Anand Kumar for his valuable discussions regarding CD studies.

My sincere thanks to my colleague, Mr. N. Naga Raju for his constant support and cooperation during my research. My other labmates Dr. Prakash Babu, Mr. K.S.G. Haviryaji, Mr. K. Mahadev also deserve an appreciation for their cooperation.

My thanks for all my other colleagues and friends among whom Dr. Palaniappan, Dr. Balagopalakrishna, Mr. Ravi, Ms. Madhavi, Ms. Gayathri, Mr. Sreenivas, Mr. Bhasker, Mr. Vinay Kumar, Mr. Sailesh, Mr. Satya Prasanna and Mr. Mathews deserve a special mention.

Partial financial assistance from CSIR and ICMR is gratefully acknowledged.

I wish to express my deepest gratitude to my mother, brothers and sister who always wished me the best in life, and gave me all support throughout my academic career.

Last but not the least I would like to thank my friend and beloved wife, Poornima for giving a regular inflow of zeal and enthusiasm at times when I needed them most.

A. RAGHUNATHAN

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
cm	Centimeter
2DE	Two dimensional electrophoresis
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetate
hr	Hour
IEF	Isoelectric focussing
kDa	Kilo daltons
M	Molar
μCi	Micro curie
μL	Micro liter
μM	Micro molar
mA	Milli amperes
mM	Milli molar
min	Minutes
mL	Milli liter
Mol. Wt.	Molecular weight
$MgCl_2$	Magnesium chloride
N	Normality
NaOH	Sodium hydroxide
NP-40	Nonidet P-40
PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric point
PMSF	Phenyl methyl sulphonyl fluoride
RNA	Ribonucleic acid

SDS	..	Sodium dodecyl sulphate
Sec	..	Seconds
Tris	..	Tris (hydroxy methyl) amino methane
TEMED	..	N,N,N,N' tetramethyl ethylene -diamine
W/V	..	weight/volume

INTRODUCTION

Gene expression in eukaryotes follow the central dogma of molecular biology, DNA makes RNA makes protein. The expression of genes from DNA to RNA and the controlling steps constitutes transcriptional regulation followed by the steps of RNA to protein comprising translational control. In a particular animal, any tissue contains cells of identical embryological origin and the genetic material, DNA, remains virtually unchanged in all the tissues. In spite of not having a qualitative change in the DNA, the type of tissue organization and functional specialities are attained due to tissue specific gene expression. It is majorly because, some regulatory sequences specify the cells in which a given gene is expressed. Some of the sequences identified are 1) promoters, which specify the starting point and direction of transcription, 2) enhancers, which facilitate the transcription of the adjoining DNA, independent of whether it is upstream or downstream, and 3) termination signals (for review, Tobin and Khrestchatisky, 1989).

Binding to a specific DNA target site (be it a promoter or enhancer) is the basic capacity exploited by regulatory proteins or factors which regulate functions such as transcription, replication and recombination (Mathews, 1992) .

In eukaryotes, usually between 60-120 nucleotides upstream from the start site of any gene, promoter sequences like TATA, CCAAT and GGGCG sequences are encountered. Mutations in these sequences decreased transcription (Mathews, 1992). It is likely that in mammals there are hundreds of different proteins that interact with these promoter- proximal sequences. Such proteins

binding these sequences, initiating transcription are also termed DNA binding regulatory proteins (Muller et al., 1988).

Another group of gene activators, the enhancers, can enhance transcription when placed either close to (`100 bases) or at a distance (upto 5 kb or more) from the RNA start site of a target gene (Darnell et.al, 1986). They can be either upstream or downstream from the gene, but are usually oriented upstream. An enhancer sequence, 200 bp long of SV40, when linked to a gene, could enhance transcription from this gene more than one hundred fold. This activation was found to function also over large distances of more than 3000 bp downstream, and even from a position behind the gene (Banerji et al., 1983). The property of remote control of eukaryotic gene expression by enhancers may also be involved in pathological processes, particularly in certain types of cancer (Muller et al, 1981). During viral infection, an enhancer- containing segment of the virus can be inserted in the vicinity of a cellular gene of the host animal or humans that controls cell proliferation (a protooncogene). Owing to this insertion, the gene now referred to as an oncogene is deregulated, leading to an uncontrolled cell growth and malignant transformation (Muller et al., 1981). DNA-binding proteins (DBPs) are a group of proteins which have an affinity to bind to a sequence of nucleotides in a specific and non- specific manner. Apart from histones, several non-histone chromosomal proteins are present in the nucleus which bind to DNA at an appropriate stage of development or depending on the developmental requirements. While many DNA binding proteins have been studied in prokaryotes (Moreau, 1987; Prasad & Chiu, 1987; Chase & Williams, 1986; Levens

& Howley, 1985; LaBonne & Dumas, 1985; Takeda et al., 1983), very few DBPs are characterized in eukaryotes. In eukaryotes the DBPs are less abundant, thus identification, purification and characterization becomes a very cumbersome process. Progress has been made in this field over the past few years largely by x-ray difraction analysis, 2D-NMR spectroscopy, site directed mutagenesis, CD spectral analysis etc., after purification by conventional chromatography, retardation assays, radiolabelled techniques or sequence specific binding techniques.

DBPs are identified and studied in many eukaryotes which act as regulatory elements (Beekman et al., 1991; Mayer et al., 1991), transcription factors (Pabo & Sauer, 1992; Segil et al., 1991; Polyanovsky & Stepchenko, 1990), hormone receptors (Schwabe & Rhodes, 1991; Thampan, 1987), enhancers (Reddy & Shen, 1991) transcriptional regulators (Giese et al., 1991; Schreiber et.al, 1990) and also oncogene products (Blackwell et al., 1990; Bonde & Privalsky, 1990; Patel et al., 1990). They also have been implicated to play a role in the maintenance of chromatin structure or DNA repair (Hasegawa et al., 1991), differentiation and transformation (Whitelaw, 1989) . The genes for several transcription factors starting from yeast to mammals have been cloned. These include members of steroid receptor superfamily with steroid, thyroid (Gehring, 1987) and retinoic acid receptors (Petkovich, 1987; Giguere, 1987) and also factors AP-1 (Bohmann et al., 1987) and SP1 (Kadonaga et al., 1987). Some of the factors which are well identified and characterized have been shown in a separate table (Table 1).

Most of the eukaryotic protein-coding genes require

TABLE 1

Some eukaryotic site-specific DNA-binding proteins:

Class/gene pdt	Organism	Comment
HELIX-TURN-HELIX		
MAT al	Yeast	Activates α-specific genes
MAT α2	Yeast	Inactivates a-specific genes
MAT al	Yeast	Combines with a2 to repress haploid specific genes
Antennapedia	Drosophila	Homeotic gene
Ultrabithorax	Drosophila	Homeotic gene
Engrailed	Drosophila	Segment-polarity gene
Fushi tarazu	Drosophila	Pair-rule gene
Paired	Drosophila	Pair-rule gene
HOX*	Mouse	Potential development regulator: a group of different proteins
Unc86#	Nematode	Determines cell lineage in worms
Oct1#	Human	Generally distributed activator
Oct2#	Human	Lymphoid-specific activator
Pit#	Mouse	Pituitary-specific activator
ZINC FINGER		
GAL4	Yeast	Galactose-dependent activator
HAP1	Yeast	Inducible activator of cyt C
SW15	Yeast	Activates HO transcription in mother cells
Krüppel	Drosophila	Gap genes
Hunchback	Drosophila	Gap genes
Steroid receptors	Vertebrates	Positive and negative acting
SP1	Vertebrates	Widespread activator

. . . contd •

HOMO- AND HETERODIMERS OF AMPHIPATHIC HELIX

LEUCINE-ZIPPER

GCN4	Yeast	Activates genes for enzymes to make amino acids
C/EBP	Mammals	Activates genes in liver and other cells; limited cell distribution
c-Fos/c-Jun	Mammals	Growth regulation
JunB	Mouse	Widespread in cells; growth regulators
JunD	Mammals	Oncogene product
FosB	Mammals	Oncogene product
Fra-1	Mammals	

HELIX-LOOP-HELIX

Daughterless	Drosophila	Developmental role in nervous system
Achaete-scute (T3)	Drosophila	Developmental role in nervous system
MyoD1	Mammals	Muscle differentiation
E12 and E47	Mammals	Immunoglobulin activation
Oct-2	Mammals	Oncogene product
c-Myc	Mammals	Oncogene product
N-Myc	Mammals	Oncogene product
L-Myc	Mammals	Oncogene product

Protein contains homeobox sequence.

Protein contains a homeobox sequence and a second domain called POU that is highly conserved.

Sources: P.F.Johnson and S.L.McKnight, 1989, Ann.Rev.Biochem., 58:799; and C.Murre et al., 1989, Cell, 56:777.

activators. The proteins necessary for beginning transcription of protein-coding genes are the TATA (transcription factor IID, or $TF_{II}D$), RNA polymerase II, and accessory proteins ($TF_{II}B$ and $TF_{II}E$ & TF_{I} F). The initiation of transcription of eukaryotic genes by RNA polymerase II, even when the TATA box and TATA factors are present, is not a frequent event. The process is accelerated by activators, which are required for normal transcription of most eukaryotic genes. In yeast and other simple organisms, a single upstream activating sequence (UAS) can be sufficient: the UAS is usually located near a gene (upstream) and often a single protein factor binds to a 15-20 bp segment in the UAS. In contrast, all mammalian genes contain multiple 15-20 bp protein-binding sites each of which is necessary for optimal transcription. Many of the binding sites in DNA have dyad symmetry, suggesting that binding proteins may act as dimers.

STRUCTURAL DESIGNS OF EUKARYOTIC DNA-BINDING PROTEINS

General structural designs appear to be present in the DNA-binding proteins whose amino acid sequences have been determined. These designs are termed the Helix-turn-helix, Zinc-finger and Amphipathic helix motifs.

1. **Helix-turn-helix proteins and Homeoboxes:** These HTH proteins were first identified in bacteria as regulatory proteins whose three dimensional structure was determined, and all bind to DNA in a similar manner. They bind as dimers and have three α-helical regions separated by short turns in their structures. This arrangement was termed *helix- turn- helix* motif (Fig. 1). It has been proved that amino acid substitutions in the a helices nearest

Taken From: A. Laughon, Biochemistry 30:11357-11367, 1991

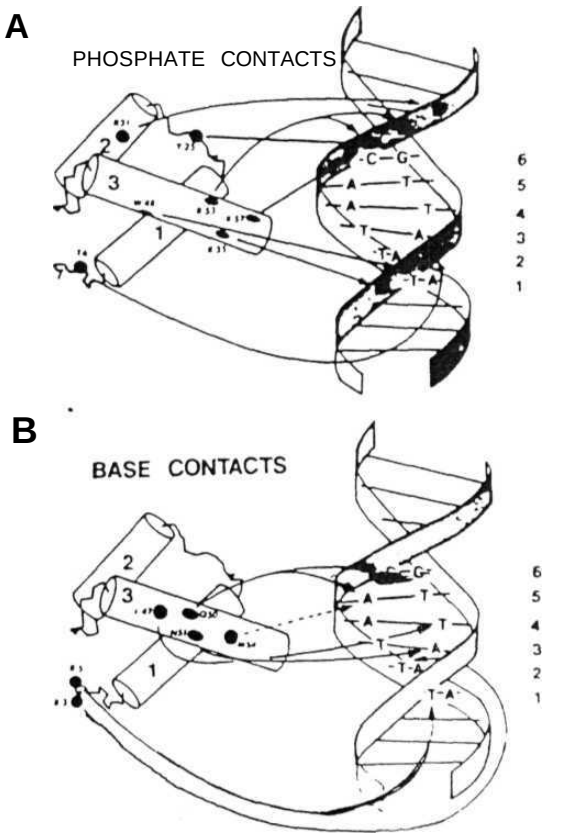


FIGURE 1: Homeodomain-DNA contacts (A) Phosphate contacts Lines with arrows connect amino acids with the phosphates (shown as solid circles) that they contact in the *en* complex (B) Base contacts Lines with arrows connect amino acids with the specific bases they contact in the *en* or *Antp* structures The drawings of homeodomain and DNA arc adapted from Kissinpcr et al (1990) for the *en* homeodomain-DNA complex, except that Met 54, corresponding to *Antp*, is shown instead of Ala 54 of *er*. The three n helices arc numbered

to the carboxyl end of each subunit of the dimer disturb the binding in the major groove of the DNA. These findings together with X-ray studies showed clearly that a protein helix in each of the dimers occupies the major groove in two successive turns of the DNA helix. When the nucleotide sequences of many homeotic genes were studied it was seen that a region encoding about 60 amino acids was remarkably conserved in all these genes. This region, called the homeobox, is conserved well enough to be easily recognized in genes from Drosophila to mammals. A number of other genes encoding vertebrate DNA-binding proteins contain regions homologous to the homeoboxes originally found in Drosophila homeo-box genes.

Three dimensional structure studies of two homeodomain-DNA complexes (Otting et al., 1990; Kissinger et al., 1990) confirm previous predictions that homeodomains utilize a helix-turn-helix (HTH) fold to contact DNA in the major groove (Laughon & Scott, 1984; Shepherd et al., 1984) but genetic studies (Hanes & Brent, 1989; Treisman et al., 1989) revealed that homeodomain HTH-DNA contacts are different from the prokaryotic HTH paradigm. In addition, homeodomains utilize an N- terminal arm to contact DNA in the minor groove (Laughon, 1991). This minor-groove contact is sequence-specific and contributes substantially to the high affinity of homeodomains for DNA (Affolter et al., 1990; Percival-Smith et al., 1990; Florence et al., 1991).

Some mammalian transcription factors also have the HTH motif, which is contained in a large conserved region consisiting of a 60 amino acid homeodomain, a spacer and another 76-78 amino acid domain. The 76-78 amino acid domain region is unique to the

pituitary-specific factor (Pit-1) and the two octamer-binding proteins (Oct-1 and Oct-2) and is referred to as the POU specific domain (Schreiber, et al., 1990). POU domain containing proteins, which are distantly related to homeobox proteins have been implicated in transcription and replication in the central nervous system (Le moine & Young, 1992).

Myb-related proteins from plant to humans are characterized by a DNA-binding domain which contains 2-3 imperfect repeats of approximately 50 amino acids each. It is proposed that each Myb repeat consists of 3 alpha helices packed over a hydrophobic core which is built around the 3 highly conserved tryptophan residues. The C-terminal forms part of the helix-turn-helix motif and can be positioned into the major groove of B-form DNA, allowing prediction of residues critical for the specificity of interaction (Frampton et al., 1992).

2. Zinc-finger proteins: The first eukaryotic positive-acting regulatory protein to be well characterized was transcription factor IIIA ($TF_{III}A$), which is required for RNA polymerase III transcription of the 5S-rRNA genes. This protein has nine repeated domains that contain cysteines and histidines spaced at regular intervals. The purified protein has zinc associated with it which is required for activity. Furthermore, their structure contains DNA-binding motifs with a coordinated binding of Zn atoms through properly spaced cysteines and/or histidines to impart tetrahedral symmetry to the coordinate complex. Residues between the coordinate amino acid loop out in a finger like projection (Fig.2). In the zinc-finger folded structure of TF A, the repeated domains form loops in such a way that a zinc ion is bound

Taken from: Kaptein, R., Current Opin Str. Biol 2:109-115, 1991

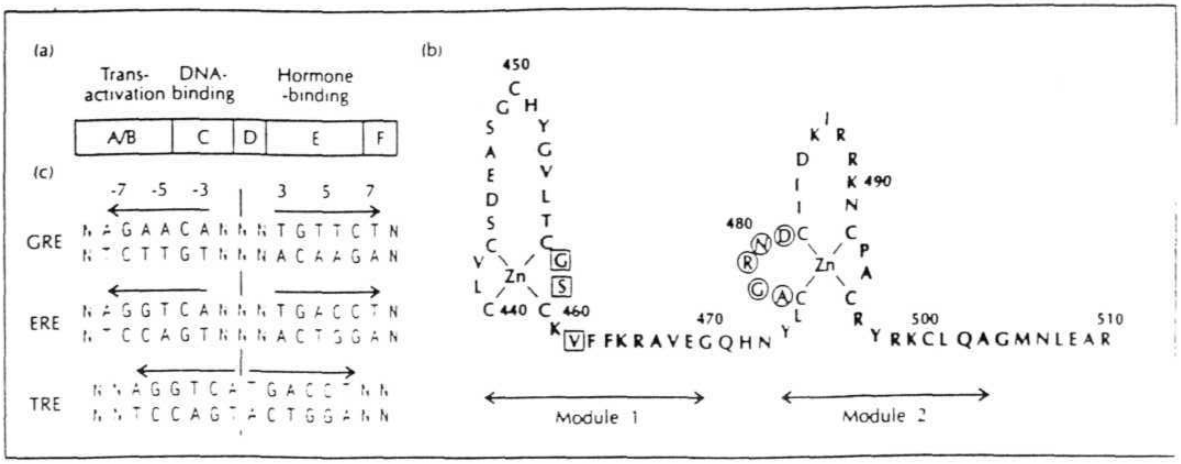


Fig. 2 (a) Structural organization of the nuclear receptor proteins in separate functional domains. (b) Amino acid sequence of the glucocorticoid receptor (GR. C-domain (numbering scheme of the rat GR is used). The α-helical regions are shaded; boxed residues in module 1 discriminate between the glucocorticoid and oestrogen responsive elements; residues involved in dimerization of the two protein when it binds to DNA (D-loop) are circled. (c) Palindromic hormone response elements for glucocorticoid (GRE), oestrogen (ERE) and thyroid (TRE) hormones. The GRE shown is the symmetrized version of the stronger binding half-site hexamer TGTTCT, as occurs in the consensus sequence.

between a pair of cysteines and a pair of histidines. A phenylalanine or tyrosine residue and a leucine residue occur at a nearly constant position in the loops, which are required for binding to DNA. A yeast protein that regulates a cytochrome c gene, several early Drosophila developmental regulators and a whole class of proteins that bind steroid hormones in vertebrates belong to this category. The zinc-finger motif is a common structure in eukaryotes from steroid receptors to factors associated with sex determination (Evans & Hollenberg, 1988).

3. **Amphipathic** helical proteins: Leucine-Zipper and **Helix-loop-Helix** proteins: The first and most extensively studied protein of this large group is C/EBP (enhancer binding protein initially thought to bind to both enhancer core and CCAAT boxes). It is present in high levels in hepatocytes, fat cells, intestinal epithelium and brain, and functions in liver-specific gene expression. A helical domain with leucines projecting uniformly on one side is a common feature of several regulatory proteins. Landschulz et al proposed this DNA-binding motif for C/EBP, a dimeric protein. This model describes the protein dimerization domain as a region with a repeat of five leucines (L1 to L5) forming leucine zipper and an adjacent DNA-binding domain containing clusters of basic amino acids. The leucine zipper is highly conserved in several other transcription factors and has been identified in many non-nuclear proteins (Busch & Corsi, 1990). The helical region is amphipathic: i.e., one side has charged amino acids such as arginine, glutamine and aspartic acid with hydrophilicity. The array of leucines on the other side is hydrophobic. It was originally proposed that the surfaces of

protein coils bearing the leucine might interdigitate to "zip up". Two highly conserved alanines (Ala-238 and Ala-239 in Yeast GCN4) and an invariant aspargine in the basic region have been proposed to play an important role in DNA sequence recognition by bZIP (leucin- zipper proteins with basic regions) proteins (Pu and Struhl, 1991). Jun and Fos, oncoproteins, are leucine-zipper containing transcriptional factors and function as transcriptional regulators in the form of homo- or heterodimeric complexes that bind to DNA. While Fos functions specifically as heterodimeric complex, Jun functions both as homo- and heterodimeric complex (Okuno et al., 1991) .

A similar but distinguishable design has been recognized in a group of proteins termed helix-loop-helix proteins. The first such proteins studied are termed E12 and E47 - bind to two regions in the enhancer of genes encoding the kappa chains of immunoglobulins. A stretch of 100-200 amino acids in the carboxyl-terminal end of both E12 and E47 contain two regions that can be folded into amphipathic α helices and another nearby region rich in basic amino acids. A new class of DBPs with a HLH structure has been recently described (Visvader & Begley, 1991). Many of these transcriptional regulators are known to play a central role in cell specification and differentiation processes. SCL, LYL-1, c-myc and E2A are implicated in the development of human lymphoid malignancies as a result of aberrant expression following chromosomal translocation events (Visvadev and Begley, 1991) .

4. **HMG group** of **proteins:** (Fig.3) These proteins have a very acidic C-terminus. Of the total amino acids, 64% are acidic

Taken From: Ner S.S., Current Biol 2:208-2 10, 1992

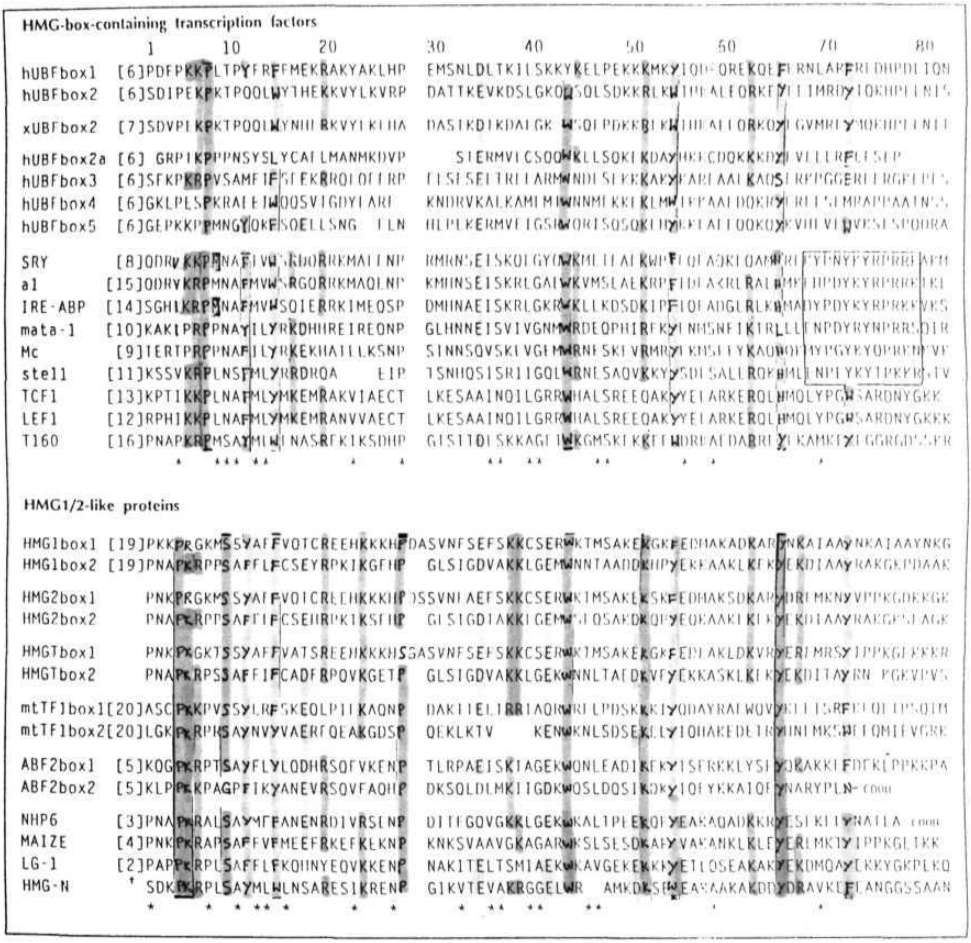


Fig. 3. Alignment of HMG-boxes separated into (top) the sequences from HMG-box-containing transcription (actors and (bottom) the HMG1/2-like proteins. Residues that are invariant, or nearly so, within a column are coloured red; ' denotes a position where residues are conserved; green, position of aromatic residues; blue, positively charged residues; yellow, negatively charged residues. Residues highly conserved among the regulators of sex-determination, which recognize a similar DNA sequence, are boxed. Amino acids known to alter DNA-binding in IRE-ABP and SRY are coloured pink. †, *Drosophilamelanogaste*HMC1-lil<e protein (SSN and AA Travers unpublished

including poly glutamic/aspartic acid residues. The remaining residues in the acidic C-terminus tail are exclusively serine (25%), aspargine (4%) and glycine (7%). The acidic C-terminal tail is necessary for efficient DNA binding. HMG1 and HMG2 bind to AT-rich sequences *in vitro* and prefer single strand to double strand DNA (Wright and Dixon, 1988). Upstream binding factor (UBF) in contrast to the AT-rich sequences bound by HMG1 & 2, binds specifically to sequences that are generally, (but not exclusively) GC-rich. The interaction of UBF with DNA is sequence specific, while that of HMG1 & 2 is not. It is possible that the HMG domain of UBF has evolved from a primordial non-specific DNA-binding structure. The sequence specificity of UBF is significantly influenced by protein-protein interaction with SL1. Another difference between HMG1 & 2 and UBF is that the latter contains 3 HMG boxes. It is speculated that each box is capable of binding to DNA on its own with weak affinity, but that together they allow high affinity binding to adjacent sites on the template. Alternatively, one HMG box could be mostly responsible for non-specific DNA-binding where no others would direct sequence specific contacts. hUBF has a fourth (of the six) HMG box (amino acids 287-372) which is more diverged from HMG1 than the other HMG boxes (Ner, 1992).

'Cold shock domain' (CSD) a putative DNA-binding motif is found in prokaryote cold shock proteins and some eukaryotic DNA-binding proteins. It consists of multiple repeats, primarily of (3 sheet in contrast to many eukaryotic DBPs. The core of one CSD repeat is encoded in a single exon. The open reading frame transcribed from the *unr* gene (upstream of N-ras) in mammals

consists of these CSD motif (Doniger et al., 1992).

SEQUENCE SPECIFICITY AND TISSUE SPECIFICITY OF DBPS:

Seguence specific DNA-binding proteins are the mediators of transcription, initiation of DNA replication and site-specific recombination. Studies in phage and bacterial genetics allowed identification and characterization of a number of prokaryotic sequence-specific DNA- binding proteins. However, very few such proteins have been identified in eukaryotes, due to the low abundance of many sequence-specific DNA- binding proteins. Some sequence-specific DNA binding proteins are directly involved in the regulation of mRNA transcription initiation in higher organisms (reviews: Dynan & Tjian, 1985, Serfling, et al., 1985; Kadonaga et al., 1986). More than 100 such sequence specific DNA binding proteins - mainly from yeasts, Drosophila, rodents and humans - have been so far isolated and their genes cloned and sequenced. The deduced amino acid sequences suggest that a limited number of families of site-specific DNA-binding proteins exist, although the total number of such proteins encoded by vertebrate genomes probably is in few hundreds. A few DNA-binding proteins have been shown directly to stimulate transcription by RNA polymerase II in vitro. How increased transcription is mediated by various types of transcription factors still remains an important research topic.

Sequence-specific DNA binding proteins are purified by chromatography through affinity resins containing the proper DNA sequences attached to an immobile support. A number of DNA binding proteins including various RNA and DNA polymerases,

hormone receptors and repressors, have been purified by non specific DNA-cellulose and DNA-agarose affinity chromatography (Mayer et.al, 1991; Thampan, 1987; Rajkumar & Shanmugam, 1986; Shelton et.al. 1990), while purifications of sequence-specific DBPs has also been done in few cases (Levens & Howley, 1985; Blanks and Laughlin, 1988; Magor & Wright, 1992; Sorger et al., 1989; Weideli & Gehring, 1980). Complementary chemically synthesized oligodeoxynucleotides possessing a recognition site for a sequence specific DBP are annealed and ligated to give oligomers. This DNA is then covalently bound to Sepharose CL-2B with cyanogen bromide to form an affinity resin which is used as a matrix in affinity chromatography to purify the sequence-specific DBP (Kadonaga & Tjian, 1986).

Sequence specific DNA-binding proteins act as positive or negative regulators in transcription (Traber et al., 1992; Zhou et al., 1992). Element A, element B, element C and element S bind specifically to sequences CCAAAAGTGG, TTATTTTTA, TATTTATT and TATTACCTTTAT respectively which are proximal promoters in Cardiac myosin light chain-2 (MLC2). Element B & C (on proximal promoter -53 to +1) are required for muscle-specific transcription. While A acts as a positive regulator, element S has a negative effect on transcription. This was revealed by site specific mutations of the promoter following transient transfections of cardiac muscle cells in culture (Zhou et al. , 1992). Studies by Zhou et al., (1992) also showed that the contribution of the functionally opposed cis-elements depends upon an interplay between the positively and negatively acting DNA-binding proteins via protein-protein interactions to mediate either activation or suppression

of gene expression.

Remarkable specificity is seen in the function of some DNA-binding proteins. The intestine specific transcription of the SI gene (sucrose isomaltase) is positively regulated by DNA-binding proteins SIF1, SIF2 and SIF3 (Traber et al., 1992). Similarly two DNA-binding proteins (13 & 30 kDa) participate in formation of an active transcription initiation complex with the testis H1t promoter in rat (Grimes et al., 1992). There are reports on DBPs from different tissues and species, but there has been almost no studies on DBPs from brain which is very essential in terms of understanding the mechanisms involved in brain specific gene expression.

It is known that a substantial portion of genetic information is required for development and function of any tissue and this is particularly evident in brain from measurements of sequence complexity of mRNA (Bantle & Hahn, 1976; Hahn et al., 1986; Kaplan, 1986). Several genes expressed in brain are also expressed in many other organs; but quantitative difference in the expression of these genes is evident (Milner & Sutcliffe, 1983). There are about 30,000 putative brain specific mRNAs which presumably encode for proteins that are of specific adaptive value in the development and function of brain (Milner and Sutcliffe, 1983). Studies reveal that the (poly A) mRNA in brain is much more complex than that found in other tissues and that it contains an equally complex and different set of sequences transcribed into a polysome-associated RNA that lacks long (>10) terminal sequences of polyadenine (poly A~ RNA) i.e. it contains an approximately equal quantities of (poly A^+) and (poly A~) RNA. In such a case,

the brain must synthesize a very large number of proteins, perhaps as many as 10^5, and many of these proteins may be specific to the brain (Milner & Sutcliffe, 1983). But it is noted that the number of protein species detectable by high resolution two-dimensional electrophoresis in neural cells is much less than predicted, from the complexity of mRNA population (Schubert et al., 1986). The exact number of specific proteins is not yet established. Hybridization experiments with poly (A) mRNA from other organs reveal that more than half are specific to brain while high resolution hybridization assays show the presence of some brain restricted mRNAs (Hahn et al., 1986). It is presumed that these proteins might function in specific developmental and physiological processes that are unique to this organ. It is suggested that during evolution there has been a selection for a substantial number of genes that are specifically expressed in brain (Hahn & Owens, 1988).

Brain undergoes enormous changes during postnatal development. The processes of a neuron (i.e. axon and dendrites) grow depending on the extent and nature of the connectivity (Hahn & Owens, 1988). About 5000-10,000 new mRNAs appear during the course of post natal development of rodent brain (Sutcliffe et al., 1986), while the number of proteins studied and characterized, according to the present literature, is an underestimate considering the various functions of the brain. An investigation in this field might possibly give some clues regarding brain specific expression and contribute to the understanding of functional complexity of brain.

SCOPE

SCOPE OF THE STUDY

Analysis of nuclear protein interactions with genome may to some extent help in explaining cell type specific gene expression, which is usually brought about by DNA binding regulatory proteins. DNA binding proteins constitute both histones and non-histones. While histones are basic in nature and are established to have a role in the conformation of nucleosomes, non-histone nuclear proteins are the best candidates to probe into in such a study. In order to search for such regulatory proteins of brain, in this study DNA binding non-histone proteins were analyzed from rat brain. Working with a preparation of non-histone nuclear proteins, rather than whole cell protein extracts, should increase the chances of isolating DNA binding proteins invovled in nuclear events.

Brain specific gene expression and gene regulation in different subsets of cells need a crucial involvement of non-histone nuclear (NHC) proteins. It has also been shown that NHC proteins in brain particularly in neurons exceed their ratio to either histones or DNA relative to other tissues (Wu et al., 1973). It is known that about 5000-10,000 new mRNAs appear during the course of post natal development of rodent brain, inclusive of brain specific mRNAs, while the number of proteins studied and characterized according to the present literature is an underestimate considering the various functions of the brain. A considerable number of these proteins could be DNA binding regulatory proteins. A better understanding of the fuctional

complexity of brain could be accessed by having a close insight into the properties and functions of these DNA binding proteins. With this background, an attempt is made in this study to characterize DNA binding proteins possibly specific to brain.

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

two nuclear non-histone proteins from brain, isolated and intensively characterized for their DNA binding property and function.

MATERIALS & METHODS

Animals

Wistar strain albino rats of both sexes and different ages were used for the experiments.

Chemicals

Acrylamide, bis-acrylamide, calf thymus DNA, dithiothreitol, PMSF, trypsin, urea, molecular weight markers (BSA, α-lactalbumin, ovalbumin, trypsin inhibitor, carbonic anhydrase, β-galactosidase, trypsinogen) were purchased from Sigma Co. Ltd, St. Louis, USA. Ampholines were purchased from LKB-Pharmacia, [^{3}H-methyl]thymidine from Bhabha Atomic Research Centre, Bombay, India.

Other chemicals were of analytical grade and purchased locally from Indian manufacturers.

Isolation of nuclei and preparation of DNA-free nuclear protein extracts from rat brain

Rats were killed by decapitation, brains were rapidly removed and placed in ice. Meninges and blood vessels were removed by rolling the tissue on wet Whatman filter paper. The tissue was chopped and homogenized to give a 20% (w/v) homogenate in ice-cold TEMN buffer (10 mM Tris-HCl pH 7.4, 1mM EDTA, 6mM β-mercaptoethanol and 50 mM NaCl) containing lmM PMSF in a Dounce homogenizer using pestle 'B'. Homogenate was diluted with equal volume of TEMN buffer and filtered through one layer of muslin cloth and centrifuged at 750 x g for 10 min at 4°C. The pellet was resuspended in TEMN buffer and nuclear extracts were prepared using Dounce homogenizer with pestle 'A' followed by ammonium sulphate precipitation. The protein precipitating at 60% ammonium sulphate saturation was collected by centrifuging at 10,000 x g at 4°C for 10 min. The 60% ammonium sulphate pellet was resuspended in TEMN buffer and dialysed overnight against 4 changes of TEMN buffer. DNA in the extract was precipitated with 10% polyethylene

glycol. The DNA- free nuclear protein extract was dialysed against TEMN buffer and used for chromatography.

Protein estimation

Protein content was determined by the method of Lowry et al., (1951) using bovine serum albumin as standard.

Single and double strand DNA-cellulose chromatography

DNA-cellulose chromatography was performed as described by Alberts and Herrick (1978). Nuclear protein (70 mg) was loaded onto a single strand (ss) DNA-cellulose column (10 cms X 1.5 cms), preequilibriated with TEMN buffer. Proteins bound to ssDNA-cellulose were eluted step-wise with TEMN buffer containing 0.1, 0.2, 0.5, 1.0 and 2.0 M NaCl. Absorbance of the fractions was measured at 280 nm and analyzed by electrophoresis. Double strand DNA-cellulose chromatography was also performed in similar manner.

SDS-Poly acrylamide electrophoresis (SDS-PAGE)

SDS-PAGE was performed essentially as done by Laemmli (1970) with modifications as suggested by Thomas and Kornberg (1975). These include (1) concentration of Tris in the resolving gel was increased to 0.75 M from 0.375 M; (2) the ratio of acrylamide to N,N-methylene bis- acrylamide was changed to 40:1 and (3) the electrode buffer contained 0.05 M Tris, 0.19 M glycine and 0.1% SDS. The proteins were separated on a 12% acrylamide gel. The slab gel was 1mm thick and 15 cms long, run for 150 V for approximately 5 hrs or till the dye front reached the bottom of the gel.

Two-dimensional gel electrophoresis (2D)

Two dimensional electrophoresis was done as described by O'Farrell (1975). Protein sample was solubilized in 30µl of 9.5M

urea, 2% ampholines (1.6% - 5 to 7 pH & 0.4% - 3 to 10 pH) and loaded on to isoelectrofocussing gel. Isoelectrofocussing (IEF) was done in glass tubes (12 cms X 0.3 cm) in a disc gel electrophoresis unit. Prefocussing was done at 200V for 15 min, 300V for 30 min and 400V for 30 min. Then the samples were loaded and electrophoressed for 8000V hrs (600 V for 12 hrs and 800 V for 1 hr) with 0.01 M phosphoric acid and 0.03 M sodium hydroxide as anode and cathode buffers respectively. The gels were extruded out of the tube and equilibriated in 'equilibriation buffer' (0.0625 M Tris-HCl, pH 6.8, 10% (w/v) glycerol, 0.005 M DTT and 2.3% SDS) for 1 hr.

These gels were subjected to second dimensional run. The IEF gels were placed on the stacking gel (4.75% polyacrylamide/bis-acrylamide in 0.125 M Tris HC1 pH 6.8 and 0.1% SDS) while polymerizing. The resolving gel was 11.75% polyacrylamide/bis-acrylamide in 0.55 M Tris HC1 pH 8.8 and 0.12% SDS. Hot agarose was used to seal the IEF gel with the stacking gel of second dimension. The gel was run at 15 mA/gel till the dye entered the resolving gel followed by 20 mA till the end of the run. The running buffer contained 0.025 M Tris base, 0.192 M glycine and 0.1% SDS. Standard molecular weight markers were co-electrophoressed to determine molecular weight of the proteins in the samples.

Silver staining

Silver staining of proteins in gels was done as described by Blum et al., (1987). Briefly, the gels were fixed in a fixative containing 50% methanol, 7.5% acetic acid and 0.5 ml of 37% formaldehyde/liter, for 1 hr and washed in 50% ethanol thrice for 20 min to prevent deformation of gels. Gels were then treated with

sodium thiosulphate (0.2 g/liter) exactly for 1 min and rinsed thrice with water to remove excess of thiosulphate. The gels were then impregnated with freshly prepared silver nitrate (2 g silver nitrate and 0.5 ml of 37% formaldehyde per liter) for 20 min, washed in distilled water thrice for 20 secs and protein spots were developed in sodium carbonate (60 g sodium carbonate and 0.5 ml of 37% formaldehyde/liter). As soon as protein spots appeared, gels were removed from the developer and washed with water followed by a wash in 50% methanol for 20 min. Gels were immediately dried or/and preserved in 50% methanol for a short period of time.

Determination of Isoelectric point of the protein

One of the IEF gels during isoelectrofocussing was run without loading any sample. This gel was extruded at the end of the IEF and cut into pieces of 1 cm each and immersed into an Eppendorf tube containing 1 mL of double distilled water. The tube was vortexed intermittently and the pH of the water was measured after 24 hrs. Data was plotted on a graph, pH versus length of gel in cms and the value was extrapolated to determine the pI of the purified protein.

Determination of molecular weight of the purified protein

Standard molecular weight markers from Sigma Chemical Co. Ltd., were run simultaneously on an 12% SDS-PAGE and stained by silver staining. The distance moved (rf) was plotted against **the** molecular weight **of** the marker on a semi log graph and the value was extrapolated to determine the molecular weight of the purified protein.

DNA binding protein dot blot assays

Dot blot studies were done according to the Genius system to confirm the DNA binding nature of the proteins using non-radioactively labelled DNA. Briefly, 8.5 µgs DNA was digested with excess amount of EcoRI (20 units) and 5 units of Pst overnight at 37°C. The DNA was extracted successively with Phenol:chloroform and chloroform, precipitated with ethanol, washed with 70% ethanol and vaccum dried. The DNA was dissolved in sterile water. An aliquot of DNA was incubated in a total of 20 µl containing 9.5 µl Pd(N) Primers (10 mg/mL, Pharmacia), 2.0

µl Nucleotide mix (Boehringer), 2.0 *ul* of Vogel 10X, 1.0 *ul* of Klenow Pol I enzyme (6 units) (BRL) and 5.5 µl of sterile water for 2 hrs at 12°C and 1 hr at room temperature. The reaction was stopped by adding 2 *ul* 200 mM EDTA pH 8.0, followed by 3 µl 4 M LiCl (-20°C). 3 volumes of absolute ethanol and 10 µl carrier DNA (10mg/mL, Amersham) was added, vortexed and briefly centrifuged in a microfuge. The mixture was incubated at -70°C for 30 min centrifuged at 15000 x g for 15 min at 4°C. The pellet was washed with 70% ethanol and centrifuged at 10000 x g for 10 min. The DNA was vaccum dried for 15 min and dissolved in 50 µl sterile water.

Blots were done using Gene Screen plus membranes. The membrane was soaked in 40 mM Tris-HCl pH 7.5 for 30 min and air dried with the 'A' side (convex surface) facing upwards. The purified protein (2.5 µgs) was spotted on the membrane at different dilutions (12.5%, 25%, 50% and 100% w/v in 40 mM Tris-HCl, pH 7.5) and air dried for 20 min. The membrane was then inserted into a polythene bag and incubated in hybrimix (50% formamide, 1% SDS, 1 M NaCl, 10% dextran sulfate in sterile water) at 42°C for 1 hr. Simultaneously 5 µL labelled DNA (kept earlier

at 95°C for 10 min) was quickly transferred into a ethanol/dry ice/NaCl bath. The polythene bag was cut open and 5 μL of the labelled DNA was added, sealed and incubated for 18 hrs at 42°C. The membrane was then removed and washed in 125 mM SSC (saline-sodium citrate), 125 mM SDS for 10 min with 2 changes, 25 mM SSC/SDS for 30 min with 2 changes and washed once with 100 mM Tris-HCl pH 7.5. The filters were air dried and the DNA-protein complex retained on them was detected with NBT.

Incorporation of [^{3}H-methyl] thymidine into E. coli DNA

Incorporation was done essentially as described by Mahler (1967). The E. coli culture medium contained 1 gm ammonium chloride, 0.5 gm NaCl, 2 gm magnesium sulphate, 3 gm monobasic potassium phosphate and 6 gm dibasic sodium phosphate in 1 liter distilled water. 1 mM calcium chloride was added and the medium was made 1% with glucose. The overnight primary culture was transferred into a secondary culture flask and [H-methyl]thymidine was added to the medium after 2 hrs. Cells were given atleast one doubling time after addition and harvested after 30 min. Labelled DNA was later isolated as described by Marmur (1961).

Isolation of DNA

Labelled DNA was isolated from E. coli according to the method of Marmur (1961). The cells were harvested, washed once with saline-EDTA (0.15M NaCl, 0.1M EDTA, pH 8.0) and suspended in a total volume of 25 mL of saline-EDTA. Cells were lysed by addition of 2 mL of 25% SDS and by incubating the mixture at 60°C in a water bath for 10 min. After cooling the samples to room temperature, sodium perchlorate (5 M) was added (final concentration of 1 M) to the viscous, lysed suspension. To this

an equal volume of chloroform-isoamyl alcohol (24:1) was added and mixed for 30 min. The resulting emulsion was separated into 3 layers by centrifugation at 10,000 rpm for 5 min. The upper aqueous phase was carefully transferred to a tube and the DNA was precipitated by adding 2 volumes of ethanol and spooled onto a glass rod. The precipitate was dissolved in saline-citrate (0.15M NaCl, 0.015 M trisodium citrate) and mixed with an equal volume of chloroform-isoamyl alcohol for 15 min, centrifuged and the supernatant was saved. This was repeatedly deproteinized with chloroform-isoamyl alcohol until very little protein was seen at the interface. The DNA in the final supernatant was precipitated with ethyl alcohol. The precipitate was dispersed in saline-citrate and treated with ribonuclease (50μg/mL) for 30 min at 37°C. The digest was again subjected to a series of deproteinizations until little or no denatured protein was visible at the interface after centrifugation. The DNA in the final supernatant was again precipitated with ethyl alcohol and the DNA was dissolved in 9 mL of saline- citrate followed by the addition of 1 mL of acetate-EDTA (3.0 M sodium acetate, 0.001 M EDTA pH 7.0). To this 0.54 vols of isopropyl alcohol was added drop-wise with rapid stirring. Precipitated DNA was washed free of acetate and salt by gently stirring the precipitate adhered to the glass rod in progressively increasing concentrations of aqueous alcohol. DNA was dissolved in required solvent. Labelled DNA was stored at 4°C with a few drops of chloroform when not in use. The specific activity of the DNA was 800 CPM per μg.

Binding of proteins to native [^{3}H] DNA

Binding of proteins to [^{3}H]DNA was carried out as described by Labonne et al., (1983) with few modifications as suggested by

Reddy and Suryanarayana (1988). Binding mixture (100 µl) contained 20 mM Tris- HC1 pH 7.6, 150 mM KCl, 3 µg of native [^{3}H]DNA, 20 µg ovalbumin and different concentrations of proteins at a DNA/protein ratio of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0. After incubation at 30°C for 10 min, the assay mixture was diluted with 1 mL buffer (same composition as assay mixture) and filtered through nitrocellulose membranes (Schleicher and Schuell, 45µ). Filters were washed with additional 5 mL of assay buffer, dried and radioactivity retained on the membranes was determined in Beckmann scintillation counter. Prior to use, membranes were boiled for 10 min in 20 mM Tris-HCl pH 7.6 and 6 mM β-mercapto-ethanol).

Gel retardation assay

The gel retardation assays or Mobility shift assays were performed essentially as described by Garner and Revzin (1981). DNA-protein complexes were formed in 10 mM Tris-HCl pH 7.6, 0.1 mM Na_2-EDTA, 22 mM NaCl in a total volume of 30 µl, at 37°C for 10 min. 2 µl 50% (v/v) glycerol containing 0.04% Bromophenol blue was added, and the samples were loaded on a 4% acrylamide gel. The running buffer contained 0.0375 M Tris, 0.019 M glycine and 0.1% SDS. The gel was run at 100 V/cm till the dye reached the end. The DNA in the gel was visualized by fluorescence after immersing the gel in a solution containing ethidium bromide (0.5 µg/mL of distilled water).

Absorption spectrum

Absorption spectrum of the protein (10 µg) was studied using a Shimadzu UV/VIS spectrophotometer between 200-400 nm.

Intrinsic fluorescence spectra

Emission spectra (excitation at 268 nm) and excitation spectra (emission at 340 nm) of the 56 kDa protein was recorded at 25°C using F-3010 Hitachi Fluorescence spectrophotometer. 6µg/mL of protein was used in a buffer 1mM Tris-HCl pH 7.4 and 50 mM NaCl.

Circular Dichroism studies

Circular Dichroism of the 56 kDa protein was recorded using Jasco-20 Scanning spectrometer. The instrument was calibrated using an aqueous solution of d-10 camphor sulfonic acid. The spectropolarimeter was continuously purged with dry 99.8% N_2 before and during the experiment. The protein was taken in 1mM Tris-HCl pH 7.4, 20 mM NaCl in a cell of 0.1 cm path length. All spectral data were plotted as mean residue ellipticity (θ) using the formula:

$$\text{Molar ellipticity } (\theta)_{MRW} = \frac{\theta \text{ obs (in degrees X 100 X MRW)}}{C \times l}$$

where 8 obs = ellipticity observed in degrees

MRW = mean residue molecular weight

C = Concentration of protein sample in gm/mL

1 = path length in cm.,

e is expressed as deg Cm^2decimole

Amino acid analysis

50 μg of protein was hydrolysed by standard acid hydrolysis conditions using 6N HC1 at 110°C for 22 hrs in evacuated tube and hydrolysates were analyzed in a Pharmacia LKB alpha plus automatic amino acid analyzer. Protection was done for cysteine, methionine and tyrosine using proper protecting reagents.

Fourth derivative spectra

Fourth derivative spectra of the purified protein (in 1mM Tris- HC1 pH 7.4, 20 mM NaCl) were recorded in a Shimadzu UV/Vis spectrophotometer at 25°C.

T_m studies

Melting curves of calf thymus DNA in the presence and absence of 56 kDa DBP was done as described by Reddy and Suryanarayana (1988). Thermal denaturation profiles of DNA in the absence and presence of DBPs were obtained by heating the DNA in 3 mL of lmM Tris-HCl pH 7.2 and 25 mM NaCl. The rate of heating was 2°C per minute. Increase in absorbance at 260 nm was measured in a programmed Hitachi double beam UV/Vis spectrophotometer attached with a thermoprogrammer. The melting curves were simultaneously recorded. The protein was added to the DNA before heating, gently mixed and incubated for 5 min at the starting temperature. Separately, buffer blank and protein solutions were also heated to observe any temperature-dependent variation in absorbance, which was found to be negligible.

DNA-Polymerase (α and β) assay

The DBP was incubated in an assay mixture (40 mM Tris-HCl, pH 7.5, 8mM MgCl , 1mM β-mercaptoethanol, 4mM ATP, 100mM dATP, 100mM dGTP, 100mM dCTP, 25mm dTTP, 1mCi [^{3}H]-TTP for α-polymerase assay (Syvaoja et al., 1990) and 50 mM Tris-HCl, pH 7.5, 10mM

$MgCl_2$, 500mM KC1, 0.5mM DTT, 2mM ATP, 100mM dATP, 100mM dGTP, 100mM dCTP, 25mm dTTP, 1mCi [H]-TTP for β-condition (assay for polymerase-β) . Both were incubated for 20 min at 37 c (Accomando et al., 1989). 200 mg of DNA and BSA were added as carriers. Reaction was stopped by adding 1 ml of cold 10% TCA followed by 10mM sodium pyrophosphate and kept on ice for 10 min. It was centrifuged at 4000 rpm for 5 min, precipitate was washed twice with 5% TCA and twice with 95% ethanol. Final pellet was dissolved in 200 mL of 0.1N NaOH. An aliquot was taken into Brays mixture (4 gm PPO, 200 mg POPOP, 60 gm naphthalene in 100 mL methanol and 20 mL ethylene glycol, made upto 1 litre with 1,4 Dioxan) and the radioactivity was determined. Specific activity was expressed as picomoles of [H]-TTP incorporated into the acid insoluble portion/mg protein/hr.

Peptide mapping of DBPs

Peptide mapping of DBPs was done after partial proteolysis of the proteins using Staphylococcus aureus enzyme. The DBPs were separated on SDS-PAGE (1 mm thickness) and the bands were cut and inserted into the wells of another polyacrylamide gel (1.5 mm thickness). Corresponding bands of cow brain protein were also loaded in a separate well. 5 µl of the enzyme was loaded over the gel piece in the well. The separating gel was 14% and 7.5 cms long, while the stacking gel was 3% and 5 cms long. After the protein entered the stacking gel the power was put off for 30 min for *in situ* proteolysis. The gel was run at a constant voltage of 120 V till the dye front reaches the bottom of the gel.

Production of polyclonal antibodies to DBPs

Polyclonal antibodies were raised against purified protein in albino rabbits. The protein (100 µg in 1 mL of TEMN) was

emulsified with equal volume of Freund's complete adjuvant and injected subcutaneously into rabbit at multiple sites. After 15 days, booster dose seach of 50 μg protein in Freund's incomplete adjuvant were given subcutaneously on the 2nd, 3rd and 4th weeks. Rabbits were bled in the 5th week through the pinna vein. Blood was allowed to clot and serum was collected after centrifugation.

Western Blotting

Western blotting was performed according to Towbin et al., (1979) with minor modifications. The proteins were electrophoressed in 12% gels and transferred onto a nitrocellulose membranes (NC) of 0.45 μ pore size in a buffer containing 0.025 M Tris-HCl pH 8.3, 0.192 M glycine and 20% (v/v) methanol. The transfer was done at 0.8 mA/cm^2 using a LKB-2117 Multiphor II electrophoresis unit (Nova Blot) for 2 hrs. After complete transfer, the NC membranes were washed with a buffer containing 0.01 M Tris-HCl pH 7.5, 0.15 M NaCl and 0.1 % (v/v) Tween-20 for 10 min and blocked in 5% skimmed milk in the same buffer for 1 hr. The NC membrane was then incubated with 5% primary antibody (polyclonal antibody raised against the purified protein) in blocking solution for 2 hrs with gentle shaking and washed for 1 hr with 6 changes of buffer. Simultaneously, a similar blot was processed with preimmune sera. The blots were later incubated with secondary antibody (anti-rabbit IgG- peroxidase conjugate; diluted 1:2000) for 2 hrs followed by washing for 60 min with 6 changes. Blots were visualized with 0.01 M Tris-HCl, 0.15 M NaCl, 0.03 % hydrogen peroxide and 0.275 μg 4-chloro-1-naphthol. The reaction was terminated by washing the blot with water.

Age--specificity of the purified DBP

Total nuclear proteins were extracted from the brains of 1, 10, 30, 60 and 90 day old rats. The proteins were separated by SDS-PAGE and transferred on to a nitrocellulose sheet. Western blots were done using polyclonal antisera to DBPs as antibody probes to study the age- specific pattern of the DBP.

Regional specificity of the purified DBP

Total nuclear proteins extracted from different regions of the brain (cerebral cortex, cerebellum, mid brain and brain stem) from 1 month old rats were resolved on a SDS-PAGE. The proteins were transferred onto a NC membrane and the presence of the DBP was checked by western analysis to study the regional specificity of the DBP in brain.

Tissue specificity of the purified DBP

Total nuclear proteins were extracted from heart, liver, kidney, lung and muscle of rat apart from brain. The proteins were subjected to SDS-PAGE followed by western blotting to test the tissue specificity of the DBP.

Immunocytofluorescence studies

A cell suspension was prepared from cerebral cortex by dissociating the tissue with collagenase (50 µl of 1% collagenase per 2 mL DME medium) and trypsin (50 µl of 0.25% trypsin per 2 mL DME medium) at room temperature. The trypsin and collagenase were inactivated after 10 min by the addition of an equal volume of fetal calf serum (FCS). Cell suspension was passed through needles of different gauges (18, 20, 22, and 26) slowly. The separated cells were taken in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS and were allowed to settle overnight on cover slips at 37°c in a CO incubator , in the presence of 5% CO .

Care was taken to prevent drying of cover slips. The cover slips containing the settled cells were processed the for immunofluorescence.

The cells were fixed in chilled methanol at -20°C for 5 min. The cells were washed in PBS (phosphate buffered saline - 8.0 gm NaCl, 0.2 gm KC1, 1.5 gm dibasic sodium phosphate, 0.2 gm mono-basic potassium phosphate, 0.2 gm sodium azide in 1 Liter distilled water) with 0.05% (v/v) Tween-20 for 30 min (3 changes 10 min each). Care was taken not to drop any solution directly on the cells. Blocking of non-specific binding of the primary and secondary antibodies was done by applying 3% (w/v) BSA in PBS for 30 min at room temperature. Cells were treated with 100 µl of primary antibody diluted (5% v/v) in PBS overnight at 4°C or for 2 hrs at room temperature. Care was taken to prevent drying of cover slips. The cells were washed in PBS-Tween 20 for 30 min (3 changes of 10 min each). Further steps were carried out in dark. 100 µl of secondary antibody conjugated to fluorescein iso thiocyanate (FITC) diluted in PBS was applied and incubated for 1 hr at room temperature. The cells were then washed in PBS-Tween 20 for 30 min (3 changes of 10 min each). The cover slips were mounted onto glass slides with glycerol and sealed with a glass adhesive. The slides were observed under phase contrast microscope equipped with fluorescence attachment or stored at 4°C in the dark when not in use.

Cancer cell lines - cell culture

The following cancer cell lines were obtained from NFATCC, Pune, INDIA, and grown in 25 cm^2 T-flasks as described separately. All the cell lines were maintained in logarthmic growth in CO incubator.

C6 glioma cells

Rat C6 glioma cells (39th 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 and 95% humidity) in 82.5% nutrient mixture F-10 (HAM) medium supplemented with 15% horse serum, 2.5% fetal bovine serum and 0.015 μg/mL phenol red, 0.01 M sodium pyruvate, 0.002 M L-glutamine, 1% (v/v) MEM vitamins, 1% (v/v) non-essential amino acids, 100 U/mL pencillin, 0.1 μg/mL streptomycin and 0.03 μg/mL gentamycin. This cell line was coded as ATCC CCL 107 in the ATCC catalogue.

Neuro-2A

Neuronal cell types and small round stem cells containing neuroblastoma was established from a spontaneous tumor of strain A albino mouse. The 172nd passage of this neuroblastoma line was cultured in MEM with non-essential amino acids and BSS (90%), fetal bovine serum (10%) and 0.015 μg/mL phenol red, 0.01 M sodium pyruvate, 0.002 M L- glutamine, vitamins, 100 U/mL pencillin, 0.1 μg/mL streptomycin and 0.03 μg/mL gentamycin under standard tissue culture conditions. These cells are coded ATCC CCL 131.

Hep G2

Human hepatoma cell line coded ATCC HB 8065. 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 μg/mL phenol red, 0.01 M sodium pyruvate, 0.002 M L-glutamine, 1% (v/v) MEM vitamins, 100 U/mL pencillin, 0.1 μg/mL streptomycin and 0.03 μg/mL gentamycin under standard tissue culture conditions.

K-562

This cell line is a human erythroleukemia line. This cell line is widely used as a sensitive *in vitro* target for the natural killer assay. These cells were grown in RPMI medium 1640, fetal bovine serum (10%) in antibiotic-free condition.

KG-1

KG-1 is a acute myelogenous leukemia line grown in Iscove's modified Dulbeccos's medium (80%), fetal bovine serum (20%) in an antibiotic-free condition. The cells were maintained in standard tissue culture conditions. This is coded ATCC CCL 246 by the ATCC.

As soon as the cells reached confluency, they were passaged. The medium was changed every 3 days. The monolayer cells were detached by trypsinization (0.1% trypsin for 5 min). Following detachment, the trypsin was inactivated by adding 10 mL medium containing serum. Cells were harvested by centrifugation at 1000 x g for 10 min followed by washing with medium. The cell pellet was suspended in a small 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 min. The cell pellet was washed thrice with TEMN buffer and suspended in TEMN buffer containing 0.1% digitonin. The cells were homogenized manually in a Dounce homogenizer using pestle 'B' with 20 up and down strokes. The nuclei were collected by centrifugation at 3000 x g for 10 min. 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 fro 45 min

in SW 60 rotor in a Beckman Ultracentrifuge to get a pure nuclear pellet.

Screening for 56 ssDBP in cancer cell **lines**

Nuclear proteins (100 μg) from the cancer cell lines were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. They were immunoblotted as described earlier **and** stained with 4-chloro- naphthol.

RESULTS

Isolation of nuclei and preparation of DNA-free Nuclear proteins from brain

The integrity of the nuclei from brain was monitored by phase contrast microscopy and DAPI fluorescence. Nuclear proteins were extracted into TEMN buffer and the DNA was removed by PEG precipitation followed by dialysis against TEMN buffer before DNA-cellulose chromatography.

Isolation of double strand **DNA** binding protein

The protein profiles at various stages of DNA cellulose chromatography are shown in Fig. 4. Several minor proteins were eluted in 0.1 and 0.2M NaCl fractions during double strand DNA-cellulose chromatography but they were ignored as proteins bound to DNA cellulose below 150 mM ionic strength were not of significantly binding type (Campbell et al., 1986). The 2.0 M NaCl fractions contained almost only one protein on SDS-polyacrylamide gels. The 2.0M NaCl eluate from the DNA cellulose column was highly enriched with this protein and was denoted as 67 dsDBP. Isolation of the 67 dsDBP was carried out only by double strand DNA cellulose chromatography.

The 2.0 M eluate fraction containing 67 dsDBP was further subjected to single strand DNA-cellulose chromatography (Fig. 5). The electrophoretic pattern showed the presence of 67 dsDBP only in the flow through; but not in eluate fractions of different ionic strengths indicating preferential affinity of the protein to double strand DNA but not to single strand DNA.

Molecular mass of dsDBP

The dsDBP was stable in TEMN buffer containing 2.0 M NaCl. Salt was removed by dialysis against TEMN buffer and the subunit molecular mass, as determined by SDS-PAGE, was 67 kDa.

Two-dimensional gel analysis of the 67 dsDBP

To determine the purity and pI of the 67 dsDBP, the DNA-affinity column fraction was subjected to 2DE analysis (Fig. 6). The 67 kDa dsDBP was localized at an isoelectric point range of 5.1-5.4 suggesting an acidic pI and its specific feature of having a broad pI rather than a narrow spot-type pI, which could possibly be due to post- translational moidification.

DNA binding assays

The extent of binding of 67 dsDBP to DNA was measured by the standard filter binding technique by mixing the dsDBP with E coli [H]-DNA. After incubation, radioactivity in the DNA-protein complexes on nitrocellulose filters was determined. Binding of protein to DNA was measured in terms of radioactive DNA-protein complex retained on filters. The data indicated an increased binding with an initial increase in the concentration of protein suggesting that the protein is binding to double strand DNA. Maximum binding was observed at a protein to DNA ratio (w/w) of 1.0, followed by a plateau till a ratio of 3.0 (Fig.7).

Partial peptide mapping of 67 dsDBP

To analyse for the presence of 67 dsDBP in other mammals, its presence in the cow brain was examined. The cow brain nuclear extracts were processed in the same manner through DNA cellulose affinity chromatography and the 2.0 M NaCl eluate was separated on SDS-PAGE and the 67 dsDBP from rat and cow brain was subjected to partial proteolysis as described in Methods. The partial peptide maps generated are shown in Fig. 8. The peptide pattern indicated a clear homology of the 67 dsdBP suggesting its existence in rat as well as cow.

Cellular localization of 67 dsDBP

Since the protein has been eluted at 2.0M NaCl concentration, it is logical that it binds tightly to the DNA. Therefore, the DNA in the nuclei was precipitated using PEG and the tight binding proteins which get co-aggregated with the DNA were examined for the presence of 67 dsDBP. The proteins in the precipitate were extracted into TEMN buffer and analysed by 2DE. This process was done with neuronal and glial nuclei. The results show that 67 dsDBP was present only in neurons but not in glial nuclei suggesting that the protein possibly could be specific to neuronal cell population in brain (Fig. 9,10).

Basing on the molecular weight and isoelectric point and peptide mapping pattern generated by partial proteolysis, a search was made in the literature (Eva Luderus et al., 1992). This search revealed that possibly the 67 dsDBP might be nuclear Lamin B . But several of the earlier studies have shown that lamin B is not a DNA binding protein (Nakayasu & Berezney, 1991). However, recently it is reported that lamin B binds to a high AT rich specific sequence called matrix associated region (Eva Luderus et al., 1992).

Isolation of 56 kDa ssDNA binding protein

In order to identify the brain proteins capable of binding to single strand DNA, ssDNA-cellulose affinity chromatography was employed. The DNA free nuclear proteins were absorbed on to ssDNA- cellulose column and the proteins were eluted in a stepwise gradient with increasing ionic strength buffer containing NaCl (Fig. 11). one milliliter fractions were collected and analysed for proteins. Several minor proteins were eluted with 0.1 M NaCl, but they were once again ignored as proteins bound to DNA cellu-

lose below 150 mM salt concentration were not of significantly binding type. The O.D 280 peak fractions of 0.2M NaCl contained a prominent single protein band with a molecular mass of 56,000 (Fig. 12).

On a preliminary search on protein data base net work, employing the molecular mass and isoelectric point (see below), no such protein with DNA binding activity was found from existing protein data base. However, literature search revealed the presence of a 56 kDa protein in the subplate of cerebral cortex involved in developmental regulation (Naegele et al., 1992). However, on further examination, it was found to be different but for the similarity in having identical molecular mass. Since no other protein was identified, it is presumed that 56 ssDBP from brain is a novel protein and hence further work was carried out with this protein which will be refered hereafter as 56 ssDBP.

Two-dimensional gel analysis of the 56 ssDBP

To determine the purity, molecular weight and pI of the ssDBP, the 0.2M fraction from DNA-affinity column was subjected to 2DE analysis. The 56 kDa protein band was resolved at a pI of 5.1-5.2 and showed no molecular heterogenity in SDS-PAGE gels (Fig. 13).

UV absorption spectrum of 56 ssDBP

The spectral characteristic of the 56 ssDNA binding protein was studied by determining the absorption spectra of the protein and also by a change in the absorption spectrum in the presence of DNA (Fig. 14). The protein was found to have a maximum absorption at 268 nm and the absorbance showed no shift in the presence of DNA.

Intrinsic fluorescence spectra

The fluorescence emission spectrum of the 56 ssDBP was recorded in a Hitachi fluorescence spectrophotometer (Fig. 15). The protein had a maximum emission at 340 nm which suggests the presence of tryptophan in the protein, but in a buried condition. Proteins with tryptophan exposed in the conformational structure show a maximum emission at around 358 nm. The excitation spectra for 56 ssDBP showed a broad maximum excitation at 270 nm. This fluorescence was increased by binding to ssDNA. Titration of a fixed amount of protein with increasing amounts of ssDNA revealed an initial steep increase in fluorescence followed by a shallower increase at high concentrations of ssDNA.

Ultra-violet fourth derivative spectra

Contribution of aromatic amino acids to UV absorption in proteins can be resolved with the help of fourth derivative spectrophotometry (Padros et al., 1984, Reddy & Suryanarayana, 1988) The fourth derivative spectrum of 56 ssDBP showed a peak at 300 nm. The fourth derivative peaks of tryptophan are chiefly in the minimum of largest wave length (280-300 nm). The peak observed in the present study at 300 nm suggest that tryptophan is in a buried conformation (Fig. 16). The data are in agreement with the fluorescence studies.

Circular dichroism studies of 56 ssDBP

The CD spectrum of 56 ssDBP was recorded as described in materials and methods. The CD spectrum studies on the interaction of the 56 ssDBP with DNA suggest that the protein has no influence on the conformation of ct DNA as no change is observed in the wavelength from 240-300 nm (Fig. 17) ruling out the possible involvement of the ssDBP in structural regulation of DNA/

chromatin. However, stoichiometric and titration studies are required further to confirm this result.

Amino acid analysis of 56 kDa

The amino acid composition of the 56 kDa protein has been determined as shown in the table (Table II) . The protein is relatively rich in glycine, serine and glutamic acid. The mole % of the acidic amino acid is abut 22.41% while the basic amino acids is of 7.28%. Cysteine, methionine, proline and tyrosine were completely absent, while arginine and histidine were in trace amounts. Fluorescence emission spectra indicated the presence of tryptophan, but however, in the amino acid analysis, it could not be identified as acid hydrolysis of the protein results in the breakage of the indole ring of tryptophan.

DNA binding protein dot blot assay

After having identified that 56 ssDBP is a DNA binding protein, it was attempted to identify the extent of binding of 56 ssDBP with ssDNA from rat brain. This was accomplished by a dot blot technique involving adsorbtion of the protein on a Gene-screen-plus membrane and the latter was probed with non-radioactively labelled ssDNA. After incubation as described in methods, DNA-protein complexes were retained on Genescreen-plus and the DNA was stained with Nitro blue tetrazolium (NBT) to visualize DNA-protein complexes. This method allows the detection of 0.1 pg of homologous DNA. Single copy genes can also be detected in 1 ng of mammalian DNA. The data obtained in the present study showed (Fig. 18) an increased affinity of the ssDBP to the DNA with increasing concentrations of protein. The DNA binding protein dot blot assay indicated that 56 ssDBP preferentially binds to ssDNA from rat brain. Thus, this technique

provides a rapid and efficient method to detect DNA-protein complexes.

Gel retardation assay

The gel retardation experiments carried out in this study were based on the method of Garner and Revzin (1981). This is a simple and rapid gel electrophoresis method for quantitative study of DNA-protein interactions. In principle, in a mixture of DNA-protein solution on a gel, the unbound DNA separates from bound DNA-protein complex and hence the DNA-protein complex has a diminished electrophoretic mobility and the retarded DNA in the DNa-protein complex can be visualized as a fluorescent complex after ethidium bromide treatment.

In the present study, a fixed amount of pBR322 DNA (4.2 kb fragment) was allowed to bind with different amounts of 56 ssDBP and the mixture of DNA-protein complex was subjected to PAGE. The data suggested a clear binding of 56 ssDBP to pBR322 DNA. Increasing in protein concentration (1, 2, 4, 6, 8 and 10 μg) attenuated the mobility of DNA and the relationship was almost linear upto a protein concentration of 10 *uq* (Fig. 19). This data complements the results obtained in DNA binding protein dot blot assays confirming the ssDNA binding nature of 56 ssDBP.

Thermal denaturation studies

The effect of 56 ssDBP on the melting curve of DNA was studied under various ionic conditions and by varying protein to DNA ratio. Majority of these studies were carried out in 10 mM Tris-HCl (pH 7.5) containing 25 mM NaCl. This buffer was used as phosphate or cacodylate were reported to interact directly with arginine residues through ionic and hydrogen binding and reduce protein-DNA interaction. Under the present experimental condi-

tions the T_m of calf thymus DNA was found to be 79°C (Fig. 20). Addition of 56 ssDBP had no significant effect on the melting of DNA. DNA/protein ratio of 0.5, 1.0, 1.5 and 2.0 also showed similar melting features suggesting that the 56 ssDBP has no effect on the melting property of DNA.

DNA-polymerase (a **and** β) assay of 56 ssDBP

The enzyme DNA polymerase (a and β) activities were assayed in the presence and absence of 56 ssDBP to see 1. whether the 56 ssDBDP has any polymerase activity by itself and 2. if not, does it involve in DNA replication and repair by altering DNA polymerase (a, β) activities. The results suggest that the protein has no DNA polymerase activity and further had no influence on the enzyme activity (Table III) .

Polyclonal antibodies of **DBPs**

Several efforts to raise polyclonal antibodies against the 67 dsDBP in rabbits were not successful suggesting the poor antigenicity of the dsDBP. However, polyclonal antibodies were succesfully raised against the 56 ssDBP. The antibodies for 56 ssDBP were used for further studies on 56 ssDBP.

Tissue specific distribution of 56 ssDBP

In order to identify the possible distribution of 56 ssDBP, the nuclear protein extracts from different tissues (heart, liver, kidney, lung, muscle and brain) of rat were immunoblotted and probed with polyclonal sera as described in methods. The western blots showed the presence of this protein only in neural tissue but not in other tissues suggesting that ssDBP is specific to central nervous system (Fig. 21).

Regional distribution of 56 ssDBP in brain

Since the ssDBP was specific to brain, the distribution of ssDBP in brain regions, brain stem, mid brain, cerebellum and cerebral cortex was examined employing Western blots. All the four regions of brain showed the presence of 56 ssDBDP. However, brain stem had a relatively higher amount of ssDBP (approximately 3-fold) than mid brain followed by cerebellum and cerebral cortex (Fig. 22). The fact that it is a ssDNA binding protein and its selective expression in brain and relatively in high levels in brain stem suggests that the protein although might involve in brain specific gene expression, difference in regional distribution of this protein indicated that it may have some crucial role in brain stem function.

Developmental distribution of 56 ssDBP

Changes of ssDBP during cerebral development was examined by preparing nuclear extracts from the brains of rats of different ages from 1 day to 90 days old and the nuclear proteins were separated on SDS-PAGE, and Western blotted. The analysis (Fig. 23) showed that the protein starts appearing from around 30 days of age, and its concentration increased with age upto 90 days. In the early days of development i.e., before 30 days, the protein might either be absent or might be below the level of detection by the present method. However, the fact that the protein appeared after a specific stage of development suggested the possibility of involvement in regulating brain specific functions.

Immunofluorescence studies to localize 56 ssDBP

Polyclonal antibodies against the 56 ssDBP were used to probe for the presence of ssDBP in different cell types of brain. Indirect immunofluorescence was performed to examine the cellular

localization of the protein. Immobilized brain cells on cover slips were probed with antibody against the 56 ssDBP and stained with secondary antibody conjugated to FITC as explained in materials and methods (Fig. 24.). Fluorescence was localized within cell nuclei but not in the cytoplasm suggesting 1) the absence of the 56 ssDBP in the cytoplasm and 2) localization of the protein in the nuclei.

Screening for 56 **ssDBP in cancer** cell **lines**

An attempt was made to screen for the presence of the 56 ssDBP in some cancer cell lines of neural as well as non-neural cell origin. Immunoblot studies with nuclear proteins showed the presence of 56 ssDBP only in Neuro-2A cells and C6 glioma (Fig. 25). This suggested that the protein was specific to central nervous system, as in other tumor cells such as leukemia cells (KG-1 and K562) and hepatoma cells (Hep G2), the protein was absent. This data also suggested that this protein might not be related to cell or DNA replication events since it is found in normal dividing cells as well as in neoplastic cells.

FIGURES & TABLES

TABLE II

Report of amino acid analysis of 56 ssDBP

Amino acid	composition mo 17.
Aspartic acid	6.715
Threonine	4.103
Serine	17.025
Glutamic acid	15.687
Glycine	27.654
Alanine	9.757
Valine	3.492
Isoleucine	1.406
Leucine	3.292
Phenylalanine	2.634
Histidine	0.951
Lysine	6.754
Arginine	0.527
Total	100.00

TABLE III

a and β DNA Polymerase activity of 56ssDBP

Sample	assay condition (dpm)	
	a polymerase	β polymerase
Blank	251.04	205.65
5μg protein	247.45	140.73
10μg protein	186.55	97.40

Since the blank values for both α and β DNA polymerase activity of 56ssDBP is higher than the test values (i.e. 5 and 10 μg protein), the 56ssDBP fails to act like a and β DNA polymerase.

Effect of 56ss DBP on a and β DNA polymerase

Sample	Specific activity in (pmoles/mg protein)	
	a polymerase	β polymerase
Control	18.28	16.26
5μg protein	19.29	18.85
10μg protein	16.73	16.65

All values are average of quadruplicate experiments. SEM is < 5%

Fig. 4 dsDNA-cellulose column chromatography of nuclear proteins of rat brain. Bound proteins were eluted with 0.1M (fraction nos. 1-6), 0.2M (7-11), 0.35M (12-16), 0.4M (17-21), 0.6M (22-26). 1M (27-32) and 2M NaCl (33-39). Absorbance at 260 nm was measured and plotted.

Fig.

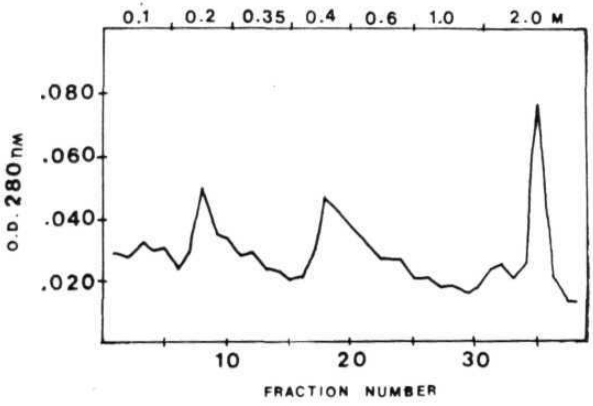

0.1
0.2
0.35
0.4
0.6
1.0
2.0 M
.080
.060
.040
.020
O.D. 280 nM
10
20
30
FRACTION NUMBER

Fig.4a. Profile of peak fractions of 2M eluates analysed by SDS-PAGE (25 μg of protein was loaded on a 5% stacking gel and 15% resolving gel). The proteins were silver stained and scanned by BIOMED computerised auto scanner.

Fig. 4a.

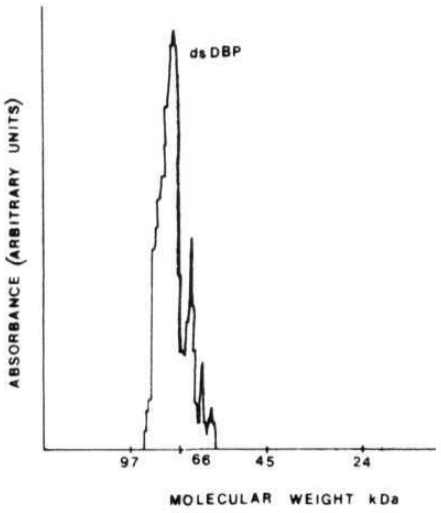

ds DBP
ABSORBANCE (ARBITRARY UNITS)
97
66
45
24
MOLECULAR WEIGHT kDa

Fig. 5 SDS-PAGE of flow through and eluates of **dsDBP** on **ssDNA-cellulose** column. Lane 1, 2 and 3 - alternate fractions of **flow** through, lane 4, 5 **and** 6 - 0.5M, 1M and 2M **NaCl** eluates.

Fig. 5

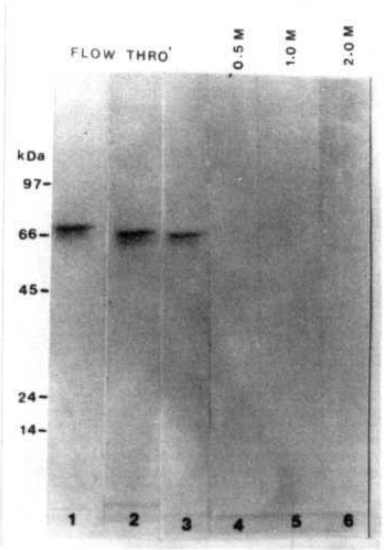


Fig.6 2-DE profiles of total nuclear (500 μg, panel a) and dsDNA binding protein (25 μg, panel b). Arrow indicates the protein dsDBP. IEF was done in tubes (3 mm ID) using 2% ampholines (1.6% 5-7 pH and 0.4% 3-10 pH) and second dimension in a 10% polyacrylamide resolving gel with a 4.75% stacking gel. The IEF gels were equilibrated in a buffer containing 10% glycerol, 5mM DDT, 2.3% SDS and 0.0625 M Tris-HCl pH 6.8 for 1 hr before loading on to second dimension.

Fig. 6

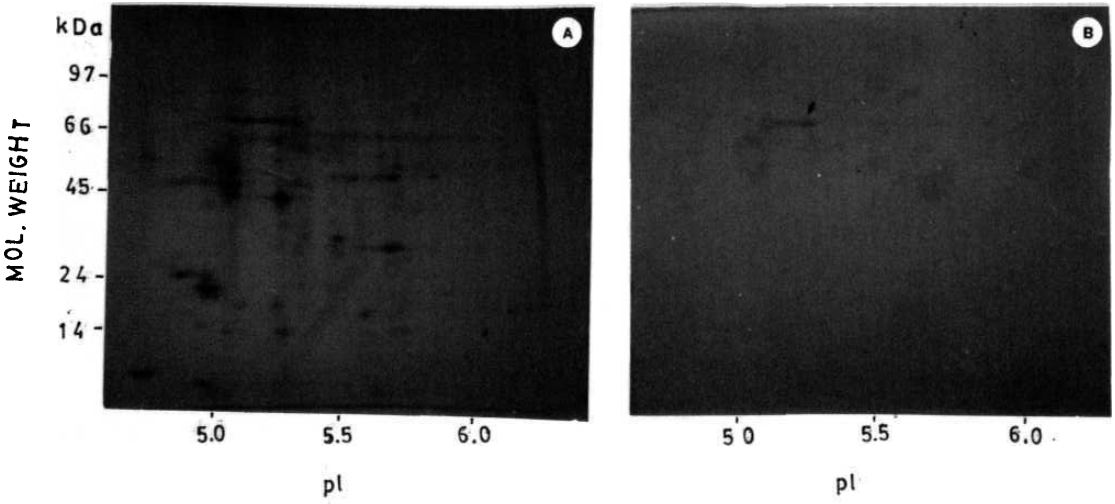


Fig.7 Nitrocelluolse filter binding assay of dsDBP to [^{3}H] E. coli DNA. Assay was done as mentioned in methods and radioactivity measured in a liquid scintillation counter.

Fig.7

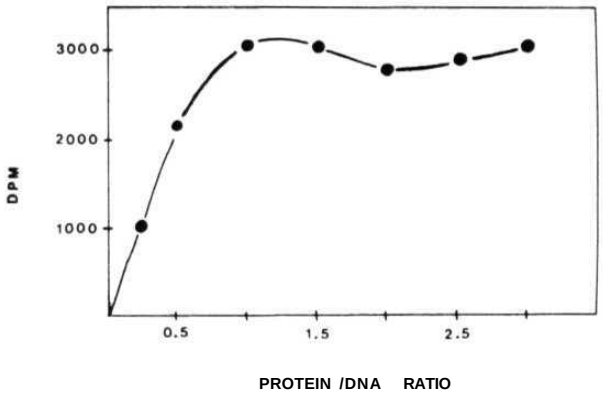


Fig.8. Partial proteolysis of dsDBP from rat and cow brain. 67 kDa protein was digested partially (as in methods) and subjected to electrophoresis and stained with silver.

Fig. 8

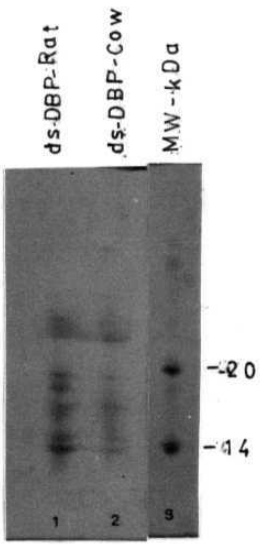


Fig. 9 2-DE profiles (IEF-SDS-PAGE) of PEG pelletable proteins from a) Neuronal and b) Glial nuclei. Arrow indicates the neuron specific DBP.

Fig. 9

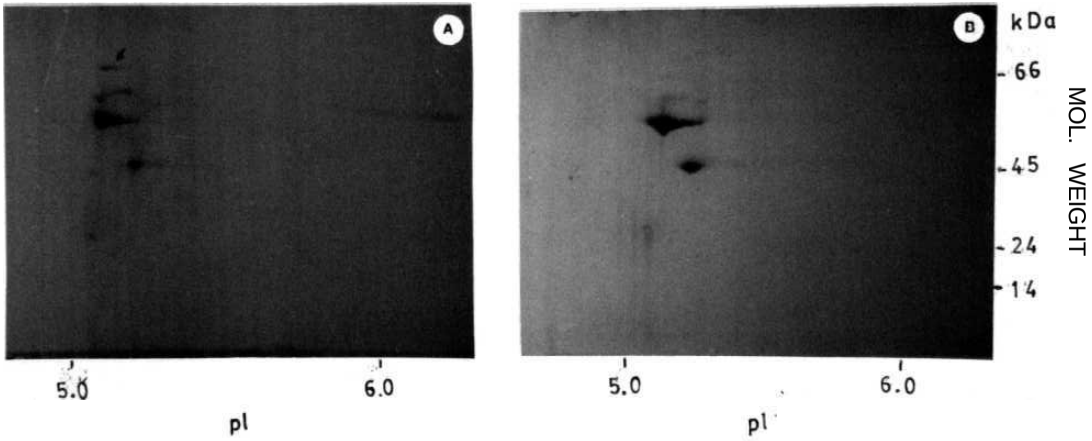


Fig.10 SDS-PAGE profiles of 2M NaCl eluates of dsDNA cellulose chromatography to show age dependent expression of dsDBP. Lane 1 - Total nuclear proteins (100 μg), lane 2, 3, 4 and 5- 2M NaCl eluates from 2, 5, 10 and 30 days old rat brain respectively. The gels were stained with silver.

Fig. 10

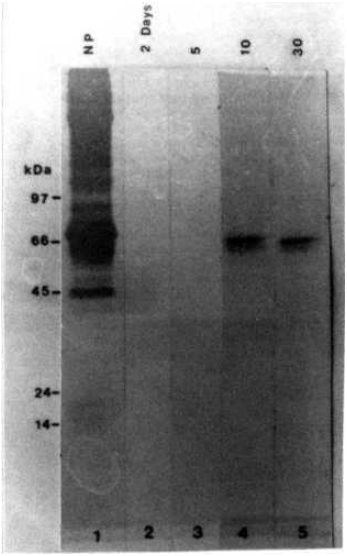


Fig.11 Elutlon profile of ssDNA-cellulose chromatography of nuclear proteins from rat brain. Bound proteins were eluted using increasing salt concentrations. Absorbance at 280 nm was measured. O.D of Fractions 1-6 (0.1 M), 7-13 (0.2 M), 14-20 (0.35 M), 21-28 (0.5 M), 29-37 (1.0 M) and 38-46 (2.0 M) was plotted against fraction number.

Fig - 11

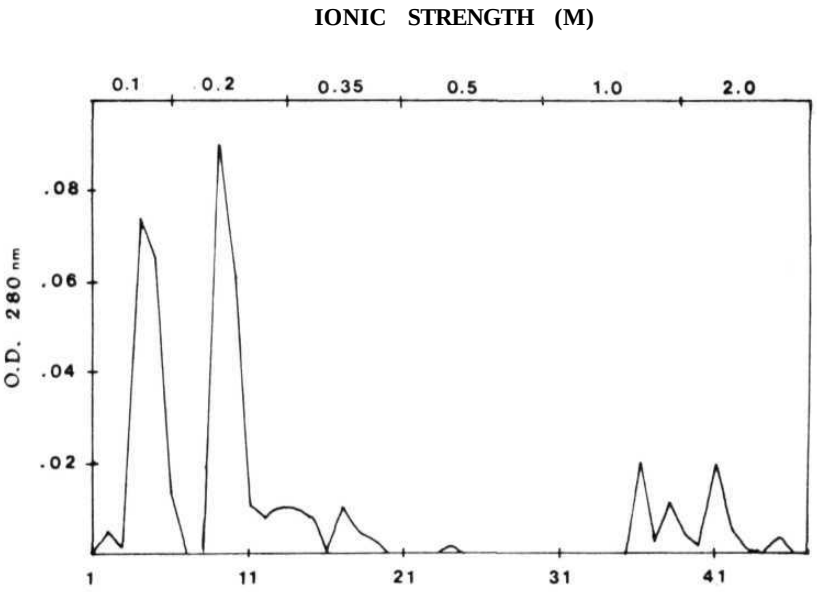

IONIC STRENGTH (M)
0.1
0.2
0.35
0.5
1.0
2.0
O.D. 280 nm
.08
.06
.04
.02
1
11
21
31
41
FRACTION NO.

Fig.12 SDS-PAGE of total nuclear proteins and 56 ssDBP. Lane 1 - ssDBP eluted at 0.2 M NaCl, lane 2 - total nuclear proteins, lane 3 - molecular weight markers.

Fig. 12

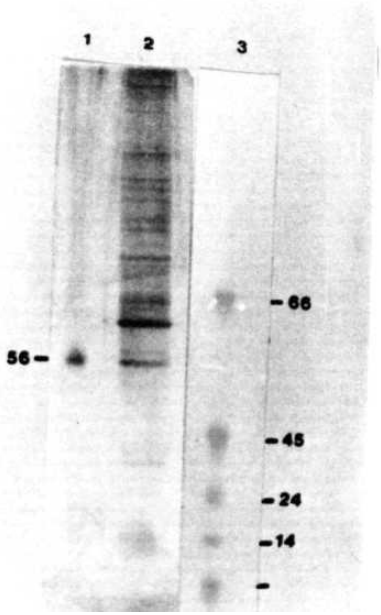


Fig. 12a. Determination of molecular mass of the ssDBP on SDS-PAGE. Molecular weight standards were co-electrophoresed along with the ssDBP and relative mobility (rF) **was** plotted against the mobility on the semi log scale. • indicates the position of **the** ssDBP **in** the gel. 1) BSA, 2) Ovalbumin, 3) Glyceraldehyde 3-phosphate dehydrogenase 4) carbonic anhydrase, 5) trypsinogen, 6) trypsin inhibitor and 7) α-lactalbumin.

Fig. 12a.

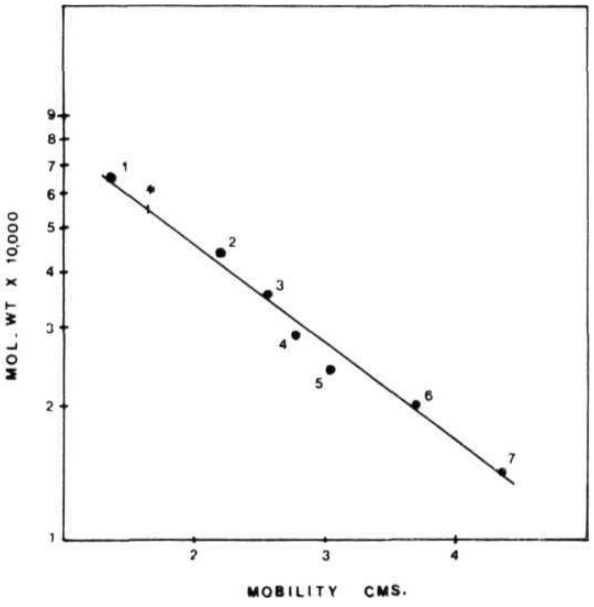


Fig.13 Two-dimensional electrophoresis of 56 ssDBP protein. IEF was done in tubes (3 mm ID) using 2% ampholine (1.6% 5-7 pH and 0.4% 3-10 pH) and second dimension in a 10% polyacrylamide resolving gel with a 4.75% stacking gel. The IEF gels were equilibrated in a buffer containing 10% glycerol, 5mM DDT, 2.3% SDS and 0.0625 M Tris-HCl pH 6.8 for 1 hr before loading on to second dimension.

A) Total nuclear proteins B) 56 ssDBP

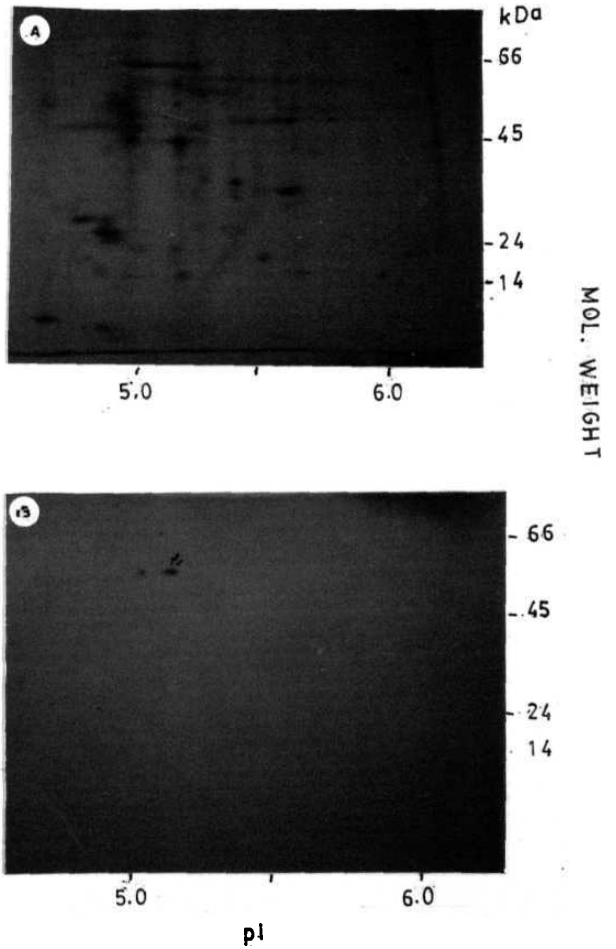

A
kDa
66
45
24
14
5.0
6.0
MOL. WEIGHT
B
66
45
24
14
5.0
6.0
pI

Fig.14 Absorption spectra of 56 ssDBP protein. The protein was taken in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl and spectrum was taken between 200-400 nm in Shimadzu UV/VIS spectrophotometer.

Fig 14

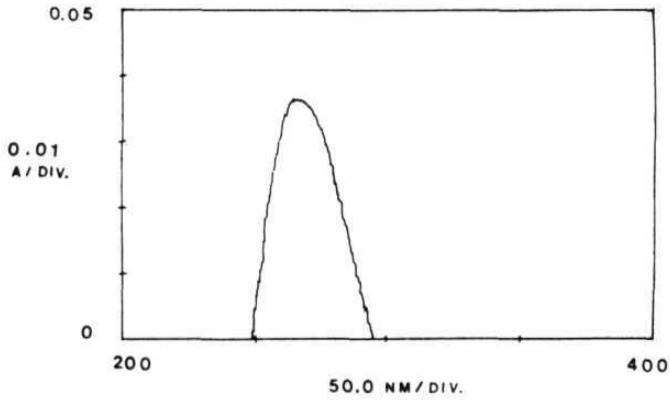


Fig.15. Intrinsic fluorescence spectrum of 56 ssDBP. 30 μg protein was taken in 1 mM Tris-HCl pH 7.4, 50 mM NaCl. Spectrum was recorded in the absence of and increased amounts of calf thymus ssDNA.

Panel A) Fluorescence spectrum of a) 56 ssDBP alone, 30 μg, b - g) 30 μg of 56 ssDBP plus 0.3, 0.6, 0.9, 1.5, 3.0, 6.0 μg of ssDNA.

Panel B) Fluorescence spectrum of a) only buffer (1 mM Tris-HCl, pH 7.4, 50 mM NaCl) and b) 6 μg ssDNA recorded as control for the experiment.

Fig. 15A

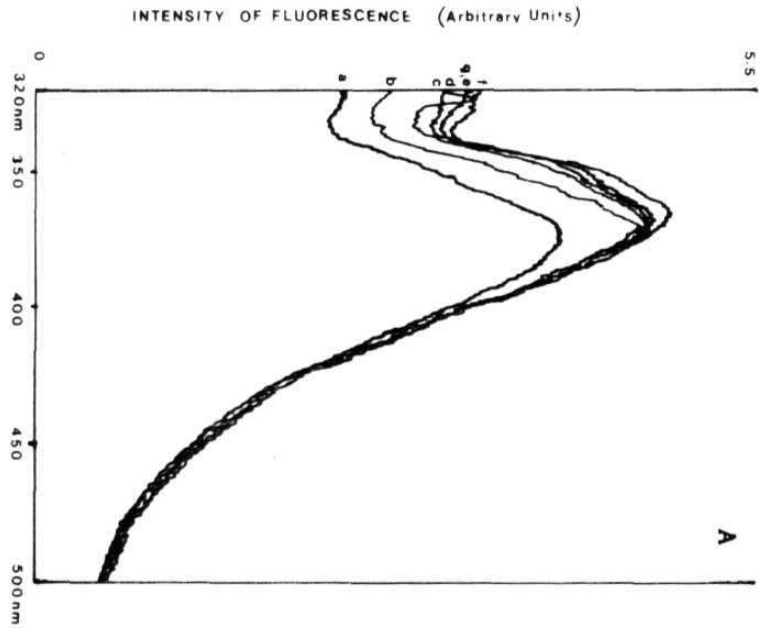

INTENSITY OF FLUORESCENCE (Arbitrary Units)
0
5.5
320nm
350
400
450
500nm
a
b
c
d
e
f
A

Fig. 15 B

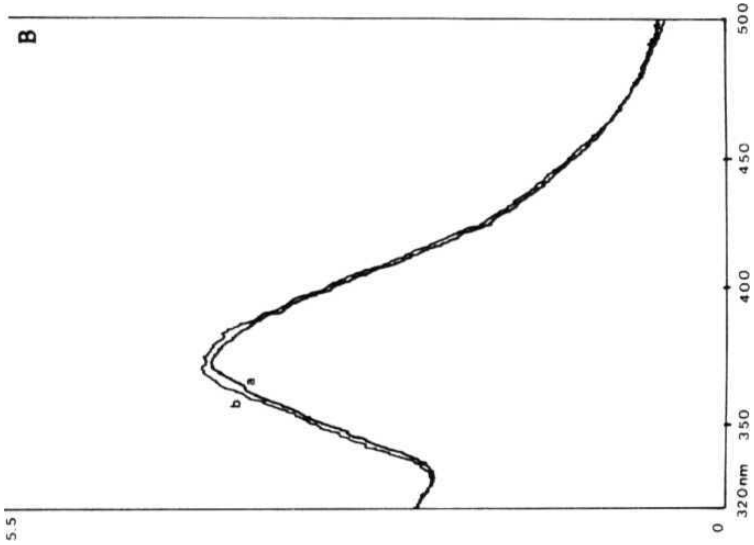


Fig.16 Fourth derivative spectra of 56 ssDBP protein. The protein was taken in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl and the derivative was recorded using a Shimadzu UV/VIS spectrophotometer.

Fig. *16*

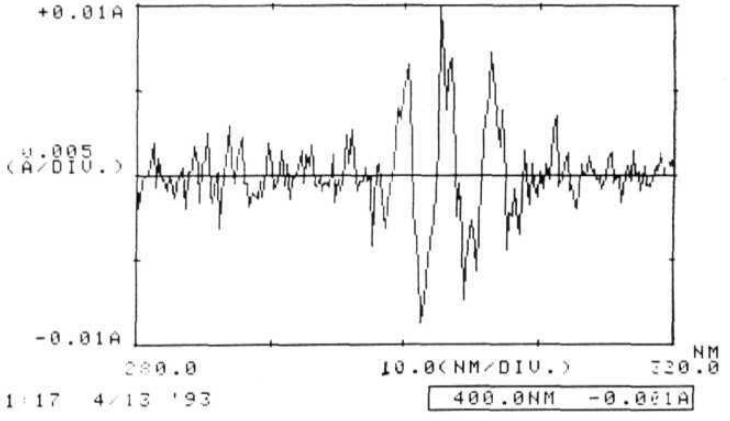

+0.01A
0.005
(A/DIV.)
-0.01A
380.0
10.0(NM/DIV.)
420.0
NM
1:17 4/13 '93
400.0NM -0.001A

Fig.17 Circular Dichroism studies of calf thymus DNA in the presence (——) and absence (——)of 56 ssDBP protein. The spectra were recorded in 1 mM Tris-HCl, pH 7.4 and 20 mM NaCl using a 0.1 cm path length cuvette.

Fig. 17.

CD spectra of ctDNA in the presence and absence of 56 ssDBP

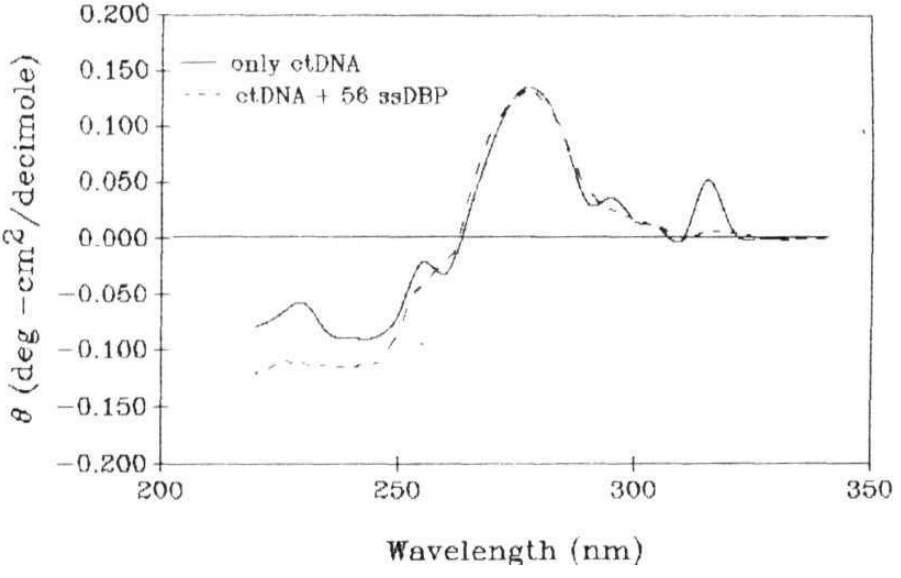
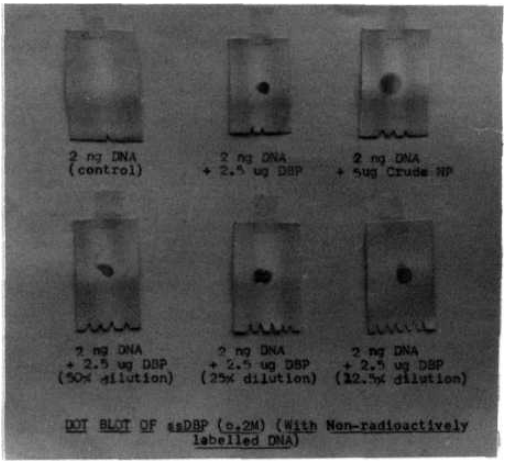


Fig. 18 Dot blot analysis of ssDBP using non-radioactively labelled DNA. 2.5 μg 56 ssDBP protein at different dilutions (100%, 50%, 25% and 12.5%) and crude nuclear proteins (NP) were blotted onto a Gene Screen plus membrane and later hybridized with 2 ng of non-radioactively labelled DNA and the complex was stained with NBT (as described in methods).

Fig 18



DOT BLOT OF ssDBP (0.2M) (With Non-radioactively labelled DNA)

Fig. 19 Gel retardation assay. pBR322 DNA (2μg) was incubated with 1, 2, 4, 6, 8 and 10 μg of 56 ssDBP (ssDBP) in a binding mixture (details in methods) and electrophoresed in a 5'/. polyacrylamide gel. Only pBR322 DNA was co-electrophoresed in the first lane.

Fig. 19

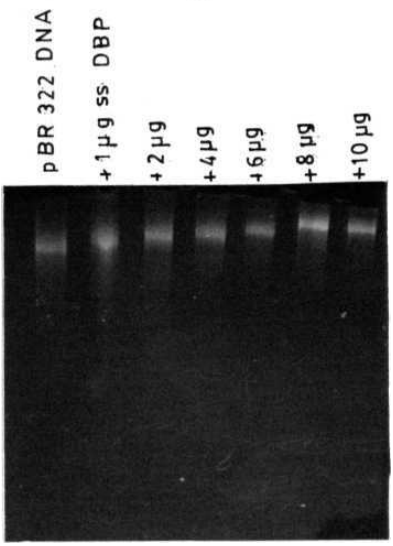


Fig. 20 Effect of 56 ssDBP protein on the melting of calf thymus DNA. Melting of DNA was performed in buffers containing 1 mM Tris-HCl, pH 7.4, 1 mM EDTA and 25 mM NaCi in the absence (●—●) of 56 ssDBP protein, protein/DNA ratio of 0.5 (■—■), 1.0 (Δ—Δ), 1.5 (▲—▲) and 2.0 (□—□).

Fig. 20

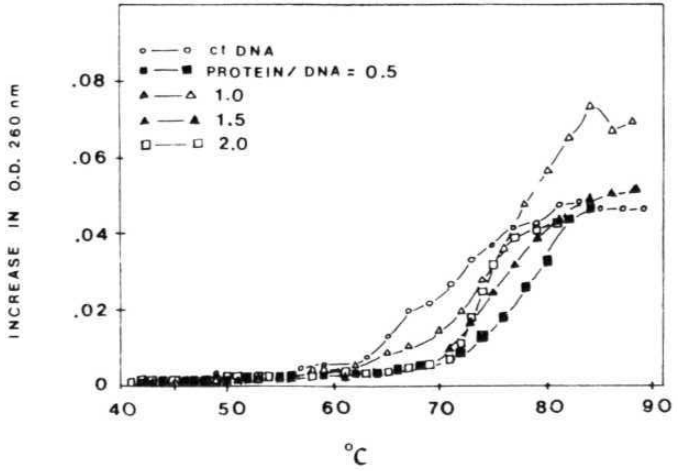

ct DNA
PROTEIN/ DNA = 0.5
1.0
1.5
2.0
INCREASE IN O.D. 260 nm
.08
.06
.04
.02
0
40
50
60
70
80
90
°C

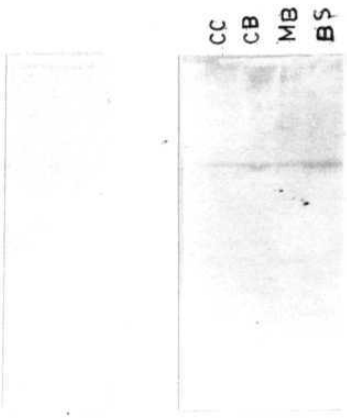
Fig. 21. Western blot to study the tissue specific expression of the 56 ssDBP. Nuclear proteins were extracted from Cerebral cortex (CC), Cerebellum (CB), Mid brain (MB), Brain stem (BS), H (Heart), Li (Liver), Ki (Kidney), Lu (lung) and Mu (Muscle) of 60 day old rat brain. The proteins were separated on a 12% SDS-PAGE and transferred onto a Nitro-cellulose membrane, treated with primary antibody and stained (as in methods).

Fig 21

Fig. 22 Western blot to study the regional distribution of 56′ssDBP. Nuclear proteins were extracted from Cerebral cortex (CC), Cerebellum (CB), Mid brain (MB) and Brain stem (BS) from brain. Proteins were separated on a 12% SDS-PAGE and transferred onto a nitrocellulose membrane and processed as in Methods. A) Two lanes were treated with preimmune sera to see non-specific cross reactivity B) Treated with antisera to 56 ssDBP

Fig. 23 Western blot analysis to study the developmental distribution of 56 ssDBP. Nuclear proteins were extracted from 1, 10. 30, 60 and 90 days old rat brain and subjected to Western blotting (as in Methods).

Fig. 22.



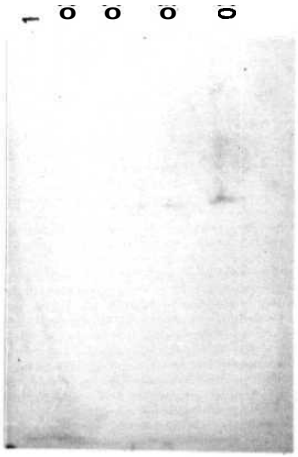


Fig. 23

Fig. 24 Cellular localization of 56 ssDBP by immunocytofluorescence. The cells were fixed on glass slides and treated with polyclonal antibodies raised against 56 ssDBP. The protein was localized using FITC-conjugated anti rabbit IgG and observed under a fluorescence microscope. A) under normal phase contrast microscope B) under fluorescence

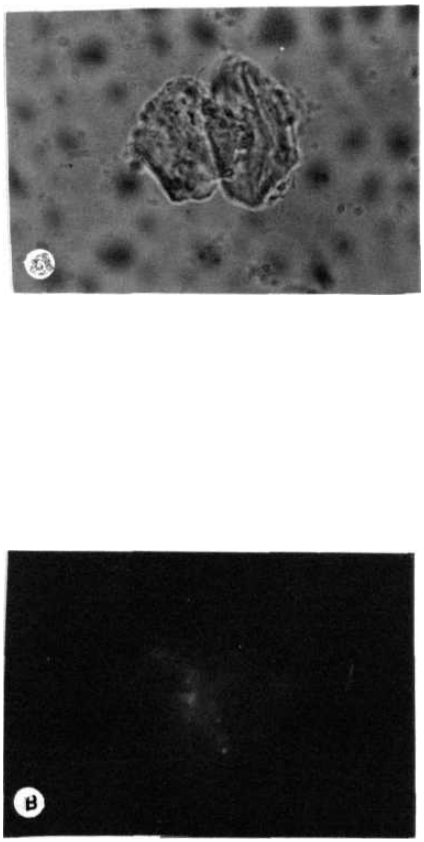


Fig. 24

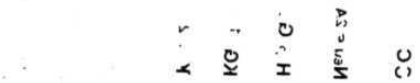


Fig. 25 Screening for 56 ssDBP in cancer cell lines. Nuclear proteins were extracted from C6 glioma, Neuro-2A, Hep G2, KG-1 and K562 cell lines and separated on a 12% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and treated as in Methods.

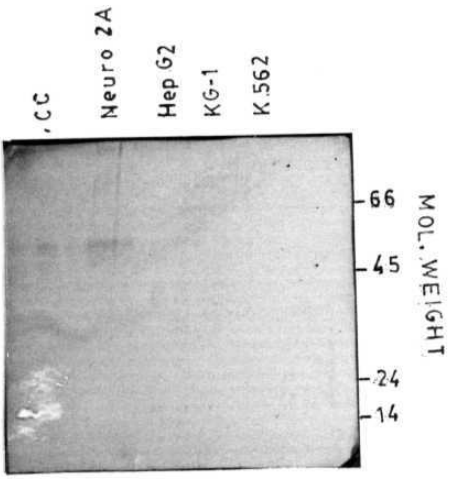


Fig 25

DISCUSSION

Brain is a complex organ with cellular, regional and functional heterogenity (Lajtha, 1982; Korr, 1986; Tobin & Khrestchatisky, 1989). The cellular diversity in the central nervous system is further remarkable for the variety in cell types, which differ distinctly by size, shape, pattern or connections and by the neurochemical composition. Inspite of these variations, the brain is known for its excellent performance by way of its enormous storage, retrieval and meticulously coordinated information processing. The diverse functions and complexity of brain is partly attributed to the flexibility of gene regulation in brain cells, which is speculated to be effectively carried out by DNA-protein interactions by regulatory proteins.

Cell type specific gene expression involves an interplay of DNA- protein interactions (Heizman et al., 1982; Sutcliffe et al., 1984). Of late a number of transcription factors and regulatory proteins exhibiting cell and sequence specific binding and expression have been studied (Lillycrop & Latchman, 1992; Green and Begley, 1992; An et al., 1992; Lu et al., 1992; de-Vetten et al., 1992; Elder et al., 1992). The mechanisms by which DNA binding proteins regulate eukaryotic gene expression vary to a large extent. For example, lytic control element- binding protein, (LCP-1) differentially regulates the JC virus early transcription unit prior to DNA replication in the infected host (Tada & Khalili, 1992); the Nuclear factor-1 (NF1) motif contained in the JC virus shows glial specific expression in differentiated human embryoinc carcinoma cells in vivo (Kumar et al., 1993).

Further, cellular regulation achieved by DNA-protein interactions involve subtle complexities in the sense that a particular DNA sequence may be the target of two or three different proteins (Pfeifer et al., 1987a); or a given DNA binding protein can recognize several distinct but related binding sites (Kadonaga et al., 1986). In rare cases, it is also possible that a transcriptional factor can bind to two apparently unrelated promoter sequences (Pfeifer et al., 1987b). Therefore it is very essential to extensively characterize any newly detected DBP.

The goal of the present study is to identify, isolate and characterize DNA binding proteins from rat brain, which could act as regulatory proteins. Several studies have reported DBPs from other tissues, but very few reports are available about DBPs in brain. These include a 35 kDa protein in 'mitotically arrested neurons' (Kuenzle, 1984); 24 and 30 kDa proteins from rat brain having preferential affinity for single strand DNA (Falaschi et al., 1984); *pax3,* a novel murine DBP expressed during early neurogenesis (Goulding et al., 1991); Zif 268 and Krox 20 transcriptional regulatory factors that contain zinc finger DNA-binding domain, which are rapidly regulated in rat brain by neuronal stimulation (Bhat et al., 1992). Astrocytes and glioblastoma cells express novel octamer DBPs, designated as N-oct proteins which are distinct from the ubiquitous oct-1 and B cell type oct-2 protein and are considered as transcriptional activators for genes specifically expressed in cells of nervous system (Schreiber et al., 1990). In the present study isolation and characterization of two DNA binding proteins 1) a double strand DNA binding protein

(67 dsDBDP) and 2) a single strand DNA binding protein (56 ssDBP) has been accomplished.

In the first part of this study a double strand DNA binding protein of molecular weight 67 kDa has been isolated. The molecular size and the pI of the protein do not match with histones (Hlinica, 1972) and therefore has been considered as a non-histone chromosomal protein. The protein was designated as 67 dsDBP, since it showed a marked preference for double strand DNA, as it was not retained by single strand DNA cellulose column.

The fact that this protein is nuclear and could be purified by DNA-cellulose chromatography under conditions of 2.0 M ionic strength suggests that it has a high affinity to double strand DNA. Studies on cell specific presence indicated localization of this protein in the neurons. It was considered at this point whether it is in anyway related to another protein, np526, which was recently reported to be expressed in mammalian neurons (Paden et al., 1992). However, it was found to be different as np526 is a 42 kDa protein and is localized in neurons and astroglial cells, but not in microglia and oligodendroglial cells (Paden et al., 1992). The 67 dsDBP is significantly expressed from 10th day in rat brain (Fig. 10) suggesting that the protein could possibly act as repressor for cellular differentiation, since the neuronal cell division in rat stops approximately around 10-15 days of development. It is possible that this protein could be completely absent till 10th day or present in very low quantities eluding detection by silver staining. Unless specific antibody probes are used, the presence of the protein in very low quantities can not

be established. Nevertheless, the fact that this protein is encountered in neuronal nuclei from day 10 and further all along the development suggests that this proteins might have a less functional significance before day 10 and assume a significant functional role thereafter.

Considering the molecular size of this protein it is anticipated that the protein could be Lamin B1. The pI of the protein also matches the acidic pI of lamin Bl. All the lamins A, B and C are reported to be associated with nuclear matrix (Nakayasu & Berezney, 1991). However, some studies have shown that Lamin B and lamin B do not bind to DNA (Hakes & Berezney, 1991) . Conversely, a recent report showed binding of matrix attachment regions to lamin B on a DNA binding protein blot assay in a two dimensional gel of total matrix proteins (Eva Luderus, et al., 1992) suggesting that lamin B is a DNA binding nuclear matrix protein. It argues for a role of nuclear envelope reassembly in cells lacking lamins A and C. These observations from our study and the similar characterisitic features of lamin B such as molecular size, isoelectric pH (based on two dimensional electrophoretic profiles), association with nuclear matrix (Nagaraju, 1993), high affinity for double strand DNA, DNA binding property in DNA cellulose affinity chromatography, nitrocellulose filter binding assay and poor antigenic nature of the protein, suggest that the protein isolated in the present study might be most likely lamin B . However, peptide mapping studies are required to confirm this possiblility. The precise role of different lamins is not clearly known in chromatin conformation

particularly in different cell types of brain. How do they complement functionally in case of cells where a specific lamin is absent is also not known. However, it may be concluded from the present study that if the 67 dsDBP is same as Lamin B , of the brain cell types, only neurons possess lamin B and has DNA binding property, while it is absent in non-neuronal cell types, such as glial cells. This implicates a lamin family diversity in brain and requires further investigation on different roles of lamin types.

Since the primary goal of this study was to identify DNA binding proteins from brain, single strand DNA binding proteins were analyzed and the attempts yielded a 56 kDa single strand DNA binding protein designated as 56 ssDBP; which was subjected to extensive characterization.

A single strand DNA binding protein was isolated from DNA cellulose affinity chromatography at a 200 mM ionic strength buffer. It could not be quantitated in ssDBP fractions or prior to DNA cellulose chromatography and therefore must be indirectly calculated from the sample recovered after the steps of elution. On the average of 50 μg of this protein could be recovered from 70 mg of DNA free nuclear protein loaded onto the DNA affinity column. It had a molecular weight of 56,000 as estimated by SDS-polyacrylamide gel electrophoresis. The isolectric pH of 56 ssDBP was 5.1-5.2 as measured in isoelectrofocussing gels. In the two dimensional gels this protein appears as a clear spot.

DNA-ligand interactions can be monitored by ultraviolet and circular dichroism spectral changes. In the present study, the

circular dichroic spectra of the protein in the presence and absence of calf thymus DNA showed no alteration excepting for the usual CD spectral peak of DNA betwee 240-300 nm. Nevertheless, more experiments involving CD titration of DNA-protein complex are necessary to infer the significance of this feature. Similarly, the UV-spectral properties showed no significant change. The results of ultraviolet and circular dichroic spectral analysis imply that the interaction of 56 ssDBP with DNA might not alter the conformation of DNA.

Ability of 56 ssDBP to reduce the melting temperature of double strand DNA by preferentially binding to transient single strand DNA and preventing it from renaturation was assessed by studying the thermal denaturation profiles of ctDNA in the presence and absence of 56 ssDBP under varying protein/DNA ratios. The results suggested that the T_m of ctDNA was not altered by the protein, which indicated that the DNA- ssDBDP interaction does not involve a change in the denaturation profiles of DNA or in the melting kinetics. However, at a high protein concentration slight decrease in T_m was noticed. This could be due to the presence of both double strand and single strand DNA. Usually under these conditions the double strand DNA has higher negative charge density and therefore the peptide binding constant to double strand DNA is higher than that of single strand DNA. This arguement holds right for proteins like histones. But in this study, the 56ssDBP has more affinity for single strand DNA than for double strand DNA. However, under conditions of high protein concentration chances exist for the protein to bind to DNA and

cause a minor alteration in the T as noticed in the present study.

As a next step, the fourth derivative spectral features of the 56ssDBP were determined, which indicate the characteristic spectral peak of tryptophan presence probably in a buried conformation within the protein. This was further supported from the intrinsic fluorescence spectral properties of the protein as described already. The fluorescence properties of tryptophan and tyrosine are often used to study the conformation, environmental properties and the interaction of proteins containing these amino acids (Lackovicz, 1983). The fluorescence titration of ssDBP with single strand DNA, indicated increased fluorescence moderately which suggests that the ssDBP indeed binds to single strand DNA, but with a very moderate affinity, a feature characteristic to regulatory proteins eluted at low ionic strength buffers (such as 200-300 nm) from DNA affinity columns.

The binding nature of 56 ssDBP with DNA was further probed by a DNA binding protein dot blot assay, a sensitive assay to identify the DBPs and the results indicated binding of the protein with DNA in a concentration dependent manner. This pattern was reaffirmed on gel retardation assays also, using a 4.2 kb fragment of pBR322 DNA. The data from these two experiments suggested that the 56 ssDBP binds with single strand DNA. In these two different assays, in one case rat brain single strand DNA was employed, while in gel retardation assays double strand DNA from pBR322 was employed.

The DNA-binding protein dot blot assay provides a means to screen for proteins which bind to DNA and this particular method has an additional feature of not requiring large quantities of purified protein. This assay has a distinct advantage of being a sensitive one when screening to detect small quantities of single strand DNA binding proteins particularly from higher eukaryotes such as mammals. Additional evidence suggesting DNA-binding nature was provided by gel retardation assays based on simple fact that the presence of bound protein retards the migration of nucleic acid through the gel. However, misleading gel shifts could result particularly when screening for single strand DNA binding activities using a crude nuclear extract. But in the present study, purified and fairly characterized protein was employed and the data, in addition to other means of screening, suggested that the protein indeed binds to single strand DNA. Binding of 56 ssDBP to single strand DNA implicated that functionally it might have a regulatory role either in transcription or in DNA replication, and a structural role in DNA organization can possibly be excluded basing on the experiments of CD analysis and thermal denaturation kinetics. Therefore, the interaction of ssDBP with DNA polymerase α and β in brain were assessed. As such, the 56 ssDBP preparation was found to be devoid of a and β polymerase activity. Further, when DNA polymerase α and (3 activities were assayed in the presence and absence of 56 ssDBP, their activities were found to be unaltered in the presence of 56 ssDBP. Thus, this protein may not be involved in DNA replication and repair in brain. The possibility of its involvement in

transcriptional activities remains to be tested.

Immunological studies using the antibodies to 56 ssDBP by Western blots showed interesting observations. In terms of distribution, the protein was found to be tissue specific - highly specific to brain; other tissues do not show even weak response, a feature suggesting a selective enrichment and presence of this protein only in brain. Even within the brain, regional heterogenity was observed in the distribution of this protein being more in brainstem and less in cerebral cortex. As it is, even though the regional distribution of the 56 ssDBP has been examined only in few brain regions, the study indicates a very subtle and clear profile of the protein distribution in a gradual increasing pattern from the deeper most regions of brain to outer most brain regions, such as brain stem, mid brain, cerebellum and cerebral cortex. Further developmentally, the protein starts appearing in the brain around the age of 30 days.

The cell culture experiments showed that of the different cell lines studied; C6, neuro-2A, Hep G2, KG-1 and K562, the protein was present (as assessed by western blots) only in Neuro2A and C6-glioma cells indicating the distribution of the protein in both neurons and astrocytes in a non-selective manner. The biological significance of the occurence of 56 ssDBP in brain is speculative because of the complexity of brain. The protein is less abundant and based on its isolation, it can be categorized into a rare DNA binding regulatory proteins selectively present in brain and therefore might involve in brain specific gene expression.

Nuclear functions in the CNS are of special interest due to exceptional level of specific gene activity, which is further complexed by regional, cellular and functional heterogenity. Therefore, identification of nuclear constituents contributing to neural function is very crucial to understand specific gene expression in brain. This study is one such attempt and has lead to the identification, isolation and characterization of the double strand and single strand DNA binding proteins from brain. The 67 double strand DNA binding protein basing on its features appear to be a lamin of B type while 56 ssDBP is a novel protein specific to brain and enriched in brainstem. Various techniques employed to characterize the protein indicated its binding to single strand DNA preferentially and functional assays examined excluded its possible involvement in DNA replication, DNA repair and structural organization of DNA and thus leaves scope for a role only in transcriptional regulation.

Identification, isolation and characterization of 56 ssDBP selectively from the nuclei of brain regions offers a novel opportunity now for examining brain specific gene expression further. The selectivity of tissue-specific gene expression depends primarily on the transcription factors present in a given cell type and their recognition of specific sequences of promoter and enhancer of particular gene to be transcribed. The modular arrangement of DNA through promoter and enhancer elements and the interplay of specific nuclear factors at these DNA modules results in tissue/cell specific gene regulation. The 56 ssDBP isolated in the present study has to be now tested for such selective binding

to specific sequences of DNA in order to identify its regulatory role with specific promoter or/and enhancers contributing to brain specific gene expression.

CONCLUSION

DNA-binding non-histone proteins were analyzed from rat brain in this study. The results and observations implicate the following conclusions:

1. Two DNA-binding proteins, one 67 dsDBP and another 56 ssDBP, have been isolated and characterized.
2. The 67 dsDBP binds only to double strand DNA and has a wide pI range of 5.1 to 5.4.
3. It is specific to neurons and is not encountered in glial cells. Moreover, it is expressed only around 10 days of postnatal development.
4. Based on the physical characters, the 67 dsDBP shares similarities with lamin B .
5. Results support that lamin B is a DNA binding protein and in our study is selectively located in neurons and not in glial cells.
6. Results implicate a lamin family diversity in brain and requires further investigation on different roles of lamin types.
7. The 56 ssDBP has an affinity to single strand DNA and is eluted at an ionic strength of 200 mM NaCl.
8. 56 ssDBP has a pI of 5.1 to 5.2.
9. It has a maximum absorbance at 268 nm.
10. The protein (56 ssDBP) has a maximum emission (intrinsic fluorescence) at 340 nm suggesting presence of tryptophan in buried condition. Thisissupported by the fourth derivative peak around 310 nm.

11. 56 ssDBP has no significant influence on conformation and melting profiles of calf thymus DNA.

12. DNA binding nature of 56 ssDBP is confirmed by gel retardation and dot blot analysis.

13. The protein has no α or β polymerase activity, neither does it have an influence on the enzyme activities; suggesting no role in DNA replication or repair.

14. The 56 ssDBP is a brain specific protein, expressed around 30 days of postnatal development, with relaative abundance (about 3 fold) in brainstem than in mid brain, followed by cerebellum and cerebral cortex.

15. 56 ssDBP is present only in Neuro-2A and C6 glioma cell lines, but not in the other cell lines screened like Hep G2 (hepatoma), KG-1 and K562 (leukemia) of non-neural origin; suggesting that the 56 ssDBP is seen only in tissues of neural origin.

16. Results exclude its involvement in DNA replication, DNA repair and structural organization of DNA and leaves scope for a role only in transcriptional regulation.

17. 56 ssDBP is a novel protein and belongs to a group of rare DNA binding regulatory proteins specific to brain and might involve a role in brain specific expression.

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PUBLICATIONS

Biochimica et Biophysica Acta, 1167 (1993) 102-1 OK
 0005-2760/93/$06.00

BBALIP 54128

Differential effects of 15-HPETE and 15-HETE on BHK-21 cell proliferation and macromolecular composition

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(Received 10 September 1992)

Key words: 15-Hydroperoxyeicosatetraenoic acid; Prostaglandin D_2; BHK-21 cell

Arachidonate and/or linoleate metabolites have been implicated in modulating cell growth, replication and cell transformations. In studies with BHK-21 cells, we found lipoxygenase and cyclooxygenase inhibitors (NDGA and indomethacin, respectively) to be antiproliferative. Studies on the metabolism of arachidonic acid in BHK-21 cells have demonstrated that prostaglandin D_2 is the major cyclooxygenase product, and 15-hydroxyeicosatetraenoic acid (15-HETE) is the major lipoxygenase product. Addition of D_2 showed a significant decrease in the BHK-21 cell number showing antiproliferative action. Addition of lipoxygenase products, on the other hand, showed differential effects in that 15-HPETE decreased the cell number while 15-HETE increased. NDGA and 15-HPETE decreased DNA, RNA and protein contents, while 15-HETE significantly increased them. 5-HPETE and 5-HETE also showed similar results but were less potent than 15-H(P)ETEs. The differential effects of 15-HPETE and 15-HETE could be due to the generation of free radicals by the hydroperoxide and mitogenic response by hydroxide.

Introduction

The metabolites of the arachidonic acid cascade are known to influence tumor growth. This was evident primarily through the studies using different inhibitors of cyclooxygenase and lipoxygenase pathways, the two major routes of the arachidonic acid cascade. The results of in vivo and in vitro studies are partly contradictory, with respect to cyclooxygenase inhibitors which decrease tumor growth in vivo [1,2] but enhance tumor cell proliferation in vitro [3]. However, lipoxygenase inhibitors are shown to inhibit tumor growth both in vitro and in vivo [4–6]. Besides, the *cis*-unsaturated fatty acids are shown to exhibit tumoricidal action by a free radical dependent process [7].

Prostaglandins, the metabolites of the arachidonate cyclooxygenase pathway are known to evoke species-specific and tissue-specific physiological responses [8]. Further, prostaglandins are also implicated in tumor cell growth. Prostaglandin D_2 inhibits the growth of cultured mastocytoma cells [9], and inhibits the synthesis of DNA, RNA and protein in L1210 cells [10]. However, information on lipoxygenase-mediated metabolites of arachidonic acid is very limited in tumor cells. Specific metabolites of arachidonic and linoleic acids via the lipoxygenase pathway have been shown to be exercising a regulatory function in epidermal growth factor signal transduction in fibroblasts [11]. Arachidonate 5-lipoxygenase an enzyme catalyzing the formation of 5-hydroxyeicosatetraenoic acid (5-HETE) was identified and purified in rat basophilic leukemia (RBL-1) cells [12]. The production of leukotrienes, which are formed by way of arachidonate 5-lipoxygenase was reported to increase in malignant cells like rat basophilic leukemia cells [13], But there is no substantial information on the identification of other lipoxygenase metabolites and their influence on tumor cell growth and development.

In the present study we have used suspension type BHK-21 cell lines. The cell growth and proliferation was monitored in the presence and absence of the inhibitors of cyclooxygenase and lipoxygenase pathways. Further, the metabolism of arachidonic acid via the cyclooxygenase and lipoxygenase pathways was monitored and the lipoxygenase products identified were screened for their influence on cell growth, DNA, RNA and protein composition. Since hydroperoxides (HPETEs) and hydroxides (HETEs) exhibit differential effects on free radical generation and lipid peroxidation, both compounds were employed in the present study.

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Materials and Methods

Indomethacin, NDGA (nordihydroguaiaretic acid), arachidonic acid (99% pure), calcium ionophore A_{23187}, prostaglandin standards (PGE_2, PGD_2, $PGF_{2\alpha}$) were obtained from Sigma, St. Louis, USA. [^{3}H]Arachidonic acid was obtained from BARC, India (532 mCi/mmol). 15-HPETE and 15-HETE were synthesized and purified on HPLC in our laboratory by using soybean lipoxygenase.

Cell culture

BHK-21 cells (suspension type, passage 14) were procured from NFATCC Pune, India. They were maintained in Glassgow's modified Eagle's medium with L-glutamine, tryptose (Hi media, India) and 8% FCS (Gibco) under 37°C in a 5% CO_2 95% humid air chamber. Cells were passaged every alternate day. At specific intervals of time as designed in the experiment the cells were collected by centrifugation at 400 rpm and the cell pellet was washed twice with fresh medium and were used for further experiments. Cell viability was examined by the trypan blue dye exclusion method.

Identification of proslaglandins and HETEs

$5 \cdot 10^6$ cells were suspended in 50 mM potassium phosphate buffer pH 7.4 and were sonicated at 20 Hz four times with 15-s pulses. The sonicate was centrifuged at $10000 \times g$ for 10 min. The supernatant was again centrifuged at $105\,000 \times g$ for 1 h. All these centrifugations were carried out at 4°C. To the supernatant 2 mM $CaCl_2$, 2 mM ATP and 10 μCi of 25 μM arachidonic acid were added and incubated at room temperature for 2 min with continuous shaking. The reaction was terminated by acidifying the reaction mixture with 1 M HC1. The products were extracted into hexane/ether (1:1, v/v) and evaporated to dryness on a vacuum rotary evaporator. The dried products were dissolved in 1 ml of methanol and stored at - 20°C for further studies. The process was repeated for the extraction of cyclooxygenase products without the addition of $CaCl_2$ and ATP.

Prostaglandins were identified by separation on TLC where 10 μl of standard PGs (1 μg each of PGD_2, PGE_2, $PGF_{2\alpha}$), endogenous products and incubated products were spotted on whatman silica gel G TLC plates and were resolved with a mobile phase of chloroform/methanol/acetic acid (100:5:1, v/v) at 4°C. Plates were sprayed with 50% sulfuric acid and were heated at 120°C for 3 minutes and the spots were visualized by fluorescence.

An aliquot of the products was reduced with sodium borohydride and evaporated under nitrogen and redissolved in n-hexane/2-propanol/acetic acid (1000:13:1, v/v). The reduced products were separated on HPLC using straight phase column (Shimpack CLC-SIL 0.5 x 25 cm) with the same solvent system at a flow rate of 1 ml/min. The eluant was continuously monitored at 235 nm and the individual peaks were collected separately. The peaks were identified based on their retention times, co-chromatography with appropriate standards, radioactivity and GC-MS analysis.

GC-MS analysis

GC-MS analysis of 15-HETE was carried out at Pennsylvania State University, University Park, PA with the facilities of Dr. C. Channa Reddy. Hewlett Packard 5890 series II gas chromatograph coupled to a Hewlett Packard 5971 mass spectrometer was used. The separation conditions were 15 m fused silica column, 0.20 mm internal diameter with 0.20 μm film thickness, temperature program 3 min/70°C, then 10°C/min to 240°C. The gas carrier was helium, 2 ml/min. The samples (15-HETE) were methylated and silylated using BSTFA of Supleco, Bellefonte, PA.

Treatment with selected inhibitors and products of lipoxygenase and cyclooxygenase pathways

BHK-21 cells (10^5 cells/ml of medium or 2 10^4 cells/well) were incubated at a final concentration with each of the following: (i) Ethanol, 0.05%; (ii) NDGA, 2.5 μM; (iii) 15-HETE, 1 μM; (iv) 15-HPETE, 1 μM; (v) indomethacin, 10μM; (vi) PGD_2, 5 μM and (vii) control (without any addition) for 24 h at 37°C and 5% CO_2. Viable cells were counted.

Time course incorporation of 15-HPETE and 15-HETE

$2 \cdot 10^6$ cells in 20 ml of medium were incubated with 15-HPETE or 15-HETE (each at 1 μM final concentration). At specific intervals of time (0, 8, 16 and 24 h) 5 ml of the incubation mixture was taken out, the cells were sonicated and the contents were extracted as above. The extract was separated on straight phase HPLC with a solvent of hexane/2-propanol/acetic acid (1000:15:1). Individual peaks were collected and 15-HPETE and 15-HETE were identified based on their retention times and cochromatography with standards.

Estimation of DNA, RNA and Protein

Estimation of DNA and RNA was done by the method of Schmidt [14] while the protein was estimated according to Lowry et al. [15].

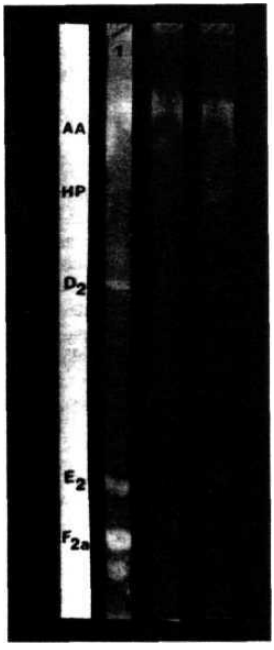
Estimation of cellular peroxidase activity

Selenium-dependent glutathione peroxidase (EC 1.11.1.9, Se-GSH Px) activity levels in the cells were estimated [16] after incubation with 15-HPETE and 15-HETE. Cells were pelleted and suspended in 50 mM Tris-HCl pH 7.6 and were assayed for enzyme activity.

ılts

ıtification of prostaglandins and HETEs

rostaglandins, endogenously generated and those ned by incubating with arachidonic acid were sepa- d on silica gel G TLC plates. The prostaglandins e visualized and identified after spraying with 50% uric acid and heating at 120°C and by comparing ı standard prostaglandins (Fig. 1). The major staglandin formed endogenously in the cell line was ıtified as PGD,. Apart from PGD, no other staglandin was detected in the endogenous sample. s that were incubated with arachidonic acid showed pot with an R_f value similar to that of PGD,. The e was identified as PGD_2 on cochromatogaphy ı standard PGD_2. The samples after reduction with um borohydride were separated on straight phase .C. The individual peaks were collected and cked for radioactivity and for the typical conjugated spectrum. The peak with the retention time of 6 min alone showed radioactivity with the typical ne spectrum (Fig. 2) and was further confirmed on cochromatography with standard 15-HETE and GC-MS analysis. GC-MS analysis of 15-HETE-Me after silylation gave the typical fragments expected for standard 15-HETE (Fig. 3) like 173, 225, 316, 335, 391 and 406 (M^+). None of the major peaks earlier than 14.76 min showed any radioactivity and diene spectrum. The results clearly indicate that the major products of arachidonic acid in the BHK-21 cells are PGD, and 15-HETE.



ı Separation of arachidonate cyclooxygenase and lipoxygenase ıbolites on thin-layer chromatography. Lane 1, prostaglandin ıdards; lane 2, endogenous arachidonate metabolites; lane 3, ıbolites after incubation with BHK-21 cells. F_{2a}, E,, D, prostaındins F_{2a}, D, and E,; HP. HPETE; AA. arachidonic acid.

TABLE I

Effects of inhibitors and metabolites of cyclooxygenase and lipoxygenase pathways of arachidonic acid on BHK-21 cell proliferation

The experiments were started with 2 10^4 cells/well (initial cell number). Cells were counted after 24 h treatment with the respective compounds. Each value is the mean of six individual observations. N.S., not significant.

Experimental condition	Cell number	Percent change
Control	7.70 ±1.74	
Ethanol (0.05%)	7.60 ± 1.60	
NDGA (2.5 μM)	5.90 ±0.90 ($P<0.05$)	- 22.30
15-HPETE (1.0 μM)	4.27 ±0.75 ($P < 0.001$)	- 43.80
15-HETE (1.0 μM)	8.57 ±0.62 (N.S.)	12.76
Indomethacin (10 μM)	4.73 ±0.04 ($P < 0.001$)	-37.76
Prostaglandin D_2 (5.0 μM)	4.34 ± 0.53 ($P < 0.001$)	-42.80

Effect of selected inhibitors and products of lipoxygenase and cydooxygenase pathways

After incubation of BHK-21 cells with NDGA (2.5 μM) 15-HPETE (1 μM), 15-HETE (1 μM), indomethacin (10 μM) and PGD, (5 μM) for 24 h, the viability of the cells was found to be about 98% sug-

TABLE II

Concentrations of 15-HPETE and 15-HETE remaining after respective time periods of incubation with BHK-21 cells

Cells were incubated with 1 μM 15-HPETE or 1 μM 15-HETE. At specific lime intervals an aliquot of the incubation mixture was taken for the extraction of products. Products were analyzed on HPLC and quantified by their peak areas. Each value is the mean of three individual observations.

Incubation period (h)	15-HPETE		15-HETE
	15-HPETE remaining (μM)	15-HETE formed (MM)	remaining (μM)
0	1.000		1.00
s	0.982		0 54
ıı.	0.978	-	0.55
24	0.975		0.48

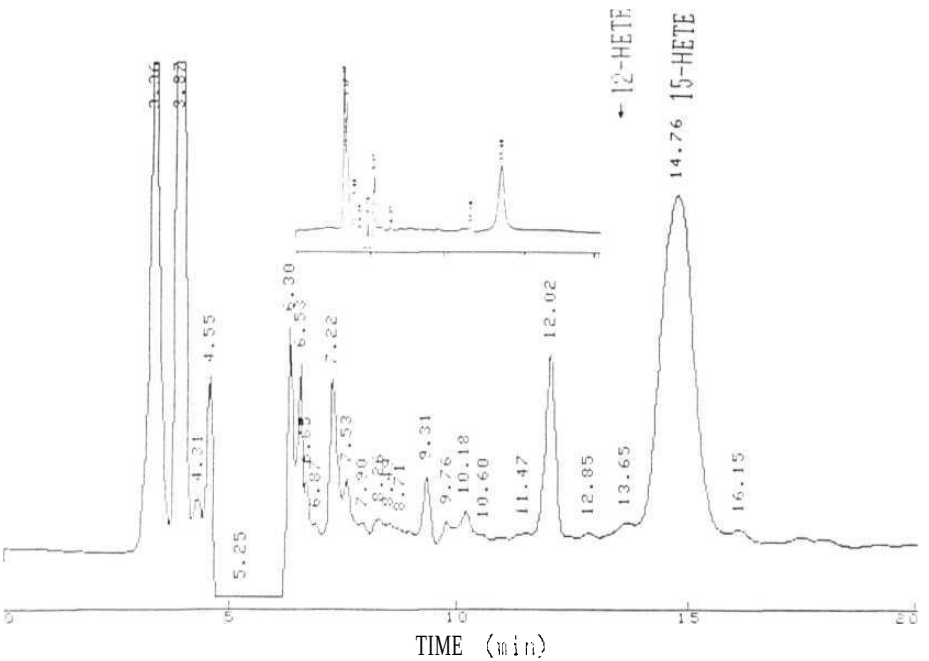


Fig. 2. HPLC separation of metabolites generated by incubating enzyme with [^{3}H]arachidonic acid. Products were generated by incubating the enzyme preparation as described in Materials and Methods. Each peak was collected individually and the radioactivity in them was counted in a liquid scintillation counter. The major peak at 14.76 min was coinjected with standard 15-HETE (inset).

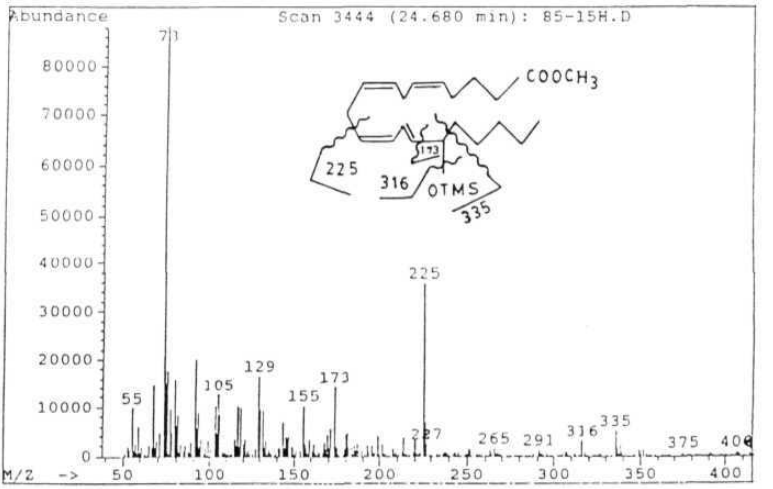


Fig. 3. GC-MS analysis of 15-HETE. 14.76 min peak obtained from HPLC separation was collected. The peak was derived as described in Materials and Methods and analyzed on a Hewlett Packard GC-MS system.

inhibitors and metabolites of cyclooxygenase and lipoxy-hways of arachidonic acid on the macromolecular composi-K-21 cells

a expressed in jug/ml. Each value is the mean of four experiments.

ıtal	DNA	RNA	Protein
	3.350 ±0.980	**2.615 ±0.080**	6.745±0.070
	3.225±0.954	2.750 ± 0.210	6.566±0.088
)	**0.830±0.076**	2.589 + 0.200	5.100 ± 0.140
)	**0.583 ±0.050**	2.090 ±0.090	4.585 ±0.040
)	4.b5 ±0.220	5.495 ± 0.440	11.325 ± 0.049

that the compounds used are not cytotoxic l). NDGA, an inhibitor of the lipoxygenase inhibited (-22%) the cell proliferation mod-15-HPETE, the lipoxygenase product era-showed a higher antiproliferative effect . 15-HETE, a reduced product of 15-HPETE ıther hand showed a seemingly opposite effect :hange with 15-HETE was not stastically signif-is observed from the data that indomethacin D, appeared to be antiproliferative in action at and 5 μM, respectively. The inhibition of cell ıtion was more with PGD_2 (43%) than with hacin (38%).

use levels of 15-HPETE and 15-HETE

evels of 15-HPETE and 15-HETE remaining 8, 16 and 24 h of incubation were determined raction and separation on SP-HPLC. As shown

inhibitor and metabolites of the lipoxygenase pathway of c acid on Se-GSH Px activity of BHK-21 cells

e is the mean of four individual experiments. Enzyme expressed as μmol of NADPH oxidized/min per ml. N.S., .:tnt

ıtal condition	Enzyme activity
	0.216 ± 0.011
	0.175 ± 0.019
	0.170 ± 0.033 (N.S.)
	0.200 ± 0.032 ($P < 0.01$)
	0.184 ± 0.031 (N.S.)

TABLE V

Changes in the cell numbers after incubating BHK-21 cells with 5-HPETE and 5-HETE for 24 h

Experiments were started with $2 \cdot 10^4$ cells/well. N.S., not significant.

Experimental condition	Cell number
Control	7.260 + 0.286
Ethanol (0.05%)	7.269±0.169
5-HPETE (1.0 μM)	6.600 ±0.571 (P<0.01)
5-HETE (1.0 μM)	6.930 ±0.590 (N.S.)

in Table II 15-HPETE content mostly remained unchanged even after 24 h of incubation. In 15-HETE-treated cells a rapid decrease of 15-HETE was observed between 0 h and 8 h. Decrease of 15-HETE continued up until 24 h but at a lower rate as compared with the initial rate of decrease.

Changes in macromolecular composition after treatment

A study of the DNA replication, transcription and translational abilities of the cell will confirm the nature of the compound with reference to its proliferative or antiproliferative properties. Accordingly, the DNA, RNA and protein levels after treatment have been assayed and are shown in Table III. Incubation of BHK-21 cells with NDGA, the lipoxygenase inhibitor, resulted in a drastic decrease in DNA content with no change in RNA content. The protein content also was decreased significantly. 15-HPETE, the lipoxygenase product of arachidonic acid, also decreased DNA, RNA and protein contents significantly, the extent of decrease being maximum in DNA content. 15-HETE, the reduction product of 15-HPETE however, showed the exactly opposite trend in that it increased DNA, RNA and protein contents significantly.

Changes in cellular peroxidase activity levels

Se-GSH Px activity was detected in the $10000 \times g$ supernatant fraction of the BHK-21 cells and the same was determined in control and experimental cells (Table IV). Increased Se-GSH activity was observed in the cells treated with 15-HPETE as well as 15-HETE. But in cells incubated with NDGA, there was no apparent change in the peroxidase activity observed.

Discussion

The association of eicosanoids with multi-stage carcinogenesis arises from a number of experimental observations centering around eicosanoid production in tumors and correlation between inhibitors of eicosanoid biosynthesis and tumor growth. Cell culture systems

have been extensively employed for analyzing the role of eicosanoids in either tumor promotion or inhibition.

Detection of various arachidonate metabolites in tumor cells is one of the crucial steps in understanding the tumor growth. Tumor cells were shown to have an elevated concentration of prostaglandins [17,18]. Of all the prostaglandins that are detected in cancer cells PGD, was shown to be present in the highest concentration [9] and was considered as a potential antineoplastic agent in cultured cells [10]. Inhibitors of prostaglandin synthase or lipoxygenase were reported to inhibit the growth of some cells [5] and there is increasing evidence that metabolites produced by these enzymes affect the growth of cells [19]. We have analyzed the metabolism of arachidonic acid in BHK-21 cells and the data suggest PGD, to be the major cyclooxygenase metabolite of arachidonic acid. No other prostanoids were found in detectable amounts. Incubation with PGD, significantly decreased the BHK-21 cell proliferation supporting the antiproliferative properties of PGD,.

The analysis of the lipoxygenase products of arachidonic acid revealed 15-HETE as the major product. Inhibition of the cyclooxygenase pathway with indomethacin enhanced the production of 15-HETE, suggesting that 15-HETE is formed via the lipoxygenase pathway only. In our study no other lipoxygenase metabolites were detected in substantial amounts.

To obtain the information on the possible role of the lipoxygenase pathway in BHK-21 cell growth, the cells were incubated with NDGA, the selective inhibitor of lipoxygenase pathway. NDGA significantly inhibited the cell proliferation, suggesting the possibility of involvement of lipoxygenase metabolites in the growth of BHK-21 cells. Similar antiproliferative effects of NDGA on a variety of cell lines were reported [5,20.23]. In order to explore the need of arachidonic acid lipoxygenase metabolites to sustain the growth of BHK-21 cells, the cells were incubated with 15-HPETE and its reduction product, 15-HETE. It is interesting to note that 15-HPETE showed antiproliferative effects, while 15-HETE stimulated cell proliferation. These results indicate that the lipoxygenase inhibitors substantially reduce cell proliferation, presumably by inhibiting the production of hydroxy metabolites of arachidonic acid but not the hydroperoxides. Recent studies have indicated that polyunsaturated fatty acids can be employed as anticancer drugs [20]. Such anticancer properties can be explained in terms of the formation of hydroperoxides (which exhibit antiproliferative properties) upon incubation of cells with unsaturated fatty acids. However, the ultimate effects of the lipoxygenase pathway appear to depend on the relative reduction of HPETEs to HETEs by cellular peroxidases. Recent studies by Glassgow et al. [11] have shown that the mitogenic response of the epidermal growth factor can be modulated differentially by linoleic and arachidonic acid metabolites in Syrian hamster embryo fibroblasts.

(Sc-GSH Px) is the major cellular peroxidase involved in the reduction of organic peroxides [24]. In the present study detectable activity of Se-GSH Px was observed in BHK-21 cells and the same was increasd in the presence of 15-HPETE. However, the externally added 15-HPETE levels were not altered substantially in these cells, even after 24 h of incubation, which could be responsible for the observed antiproliferative effecs of 15-HPETE. The low Se-GSH Px observed in these cells may not be efficient in the conversion of 15-HPETE to 15-HETE. Also, the K_m reported for 15-HPETE was 12 μM [25], which is very high especially when the 15-HPETE added in the present study was 1 μM. Further studies on cellular peroxidases, lipoxygenases and peroxide tone could clarify their role in cell proliferation. The rapid decrease in the concentration of 15-HETE upon incubation with BHK-21 cells could be due to its selective incorporation into cellular phospholipids [26].

It is interesting to note that both indomethacin and PGD, suppressed cell proliferation without any cell death, suggesting that the dosage of these compounds was not cytocidal. On the whole these results are in agreement with the earlier findings [5,10]. However, it is not clear how both indomethacin (inhibitor of PGH synthase) and PGD, show antiproliferative properties. It is known that inhibition of the cyclooygenase pathway actually stimulates the lipoxygenase pathway. In the present study the production of 15-HPETE also increased when the cells were incubated with indomethacin, suggesting the possible stimulation of the lipoxygenase pathway (control 100%, indomethacin 112%). This increased amount of 15-HPETE could be responsible for the overall antiproliferatve effects observed in the presence of indomethacin. The effects of the lipoxygenase pathway on BHK-21 cells were also reflected in changes in the macromolecular composition. Both NDGA. the lipoxygenase inhibitor and 15-HPETE, the immediate lipoxygenase metabolite, showed significant reduction of DNA content supporting their antiproliferative roles. 15-HETE, on the contrary, increased the DNA content. In transformed erythroleukemia cells 15-HETE was detected and was shown to increase DNA synthesis in differentiating cells but not in proliferating cells [21]. However, our results indicate that 15-HETE increases DNA synthesis in proliferating cells too. The stimulation of cell proliferation and DNA synthesis by 15-HETE may be brought about by inhibition of diacylglycerol kinase leading to an increase of the cellular diacylglycerol level [22]. Diacylglycerol activates protein kinase C, which stimulates DNA synthesis presumably by phosphorylation of nuclear proteins. Our preliminary stud-

otein phosphorylation indicate that 15-HETE cally involved in phosphorylation of the 54 ein (data not shown). NDGA and 15-HPETE lecreased the RNA and protein levels, while : drastically increased the RNA and protein hese results indicate that 15-HETE is rela- ›re involved in translational activities, while nd 15-HPETE affect the transcriptional activ- BHK-21 cells. These differential effects of and HETEs might be responsible for the g and contradictory reports in the literature ›le of lipoxygenases and eicosanoids in cancer. ıtion of BHK-21 cells with 5-HPETE and 5- so showed similar results to those obseved in :nce of 15-HPETE and 15-HETE, the extent ıch less (Table V). These compounds appear ; potent as they are not the principle metabo- rachidonic acid in BHK-21 cells.

ıclusion, this study demonstrates that arachi- id is metabolized by BHK-21 cells by both :enase and lipoxygenase pathways and the ;es of these pathways are involved in the regu- the growth of these cells. The interesting f the present study is the differential effects of E and 15-HETE on the cell proliferation, tion and translational activities. The peroxi- vity coupled to lipoxygenase seems to be an t determinant of cell proliferaton.

dgements

/ork is supported by grants from the Depart- Biotechnology, New Delhi, India (grant No. 03/25/007/89). M.C.V. is a recepient of entist B award. The authors gratefully ac- ;e Dr. C. Channa Reddy for providing GC-MS and to Mr. Chris for analyzing the samples.

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2-D PAGE '91

Edited by
Michael J. Dunn

Proceedings of the
International Meeting
on Two-Dimensional
Electrophoresis

London, 16th – 18th July, 1991

AGE '91', NHL Press, London, pp. 276-270 CEd. by M.J. Dunn) 1991

MULTIPLE GELS IN 2DE - BETTER RESOLUTION

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1 INTRODUCTION

Identification and characterization of proteins was made 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]. Most 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 aave 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

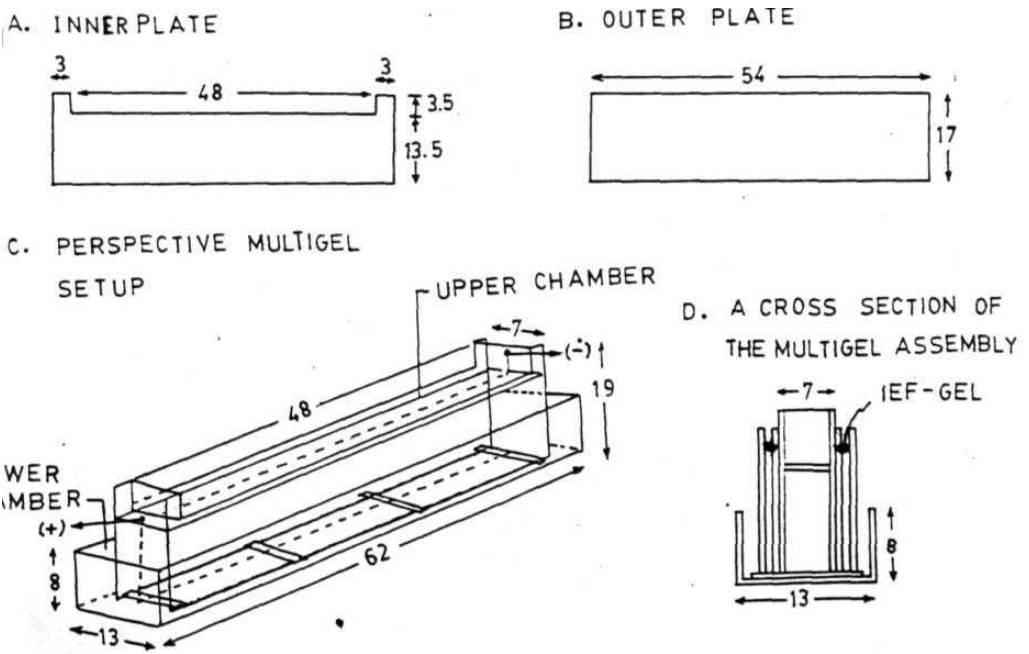

Fig 1: Multigel Unit and its components.

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

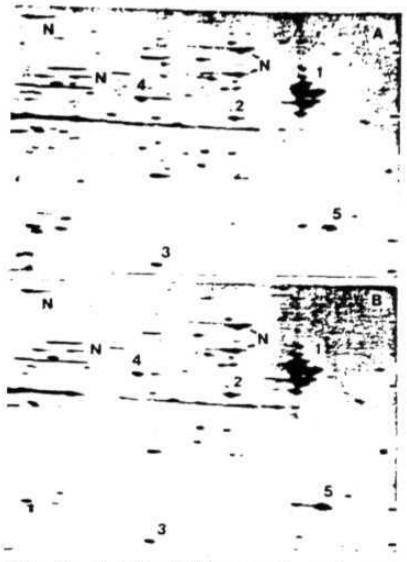

Fig 2: 2-DE of Neuronal membrane proteins. N indicates protein(s) specific to neurons.

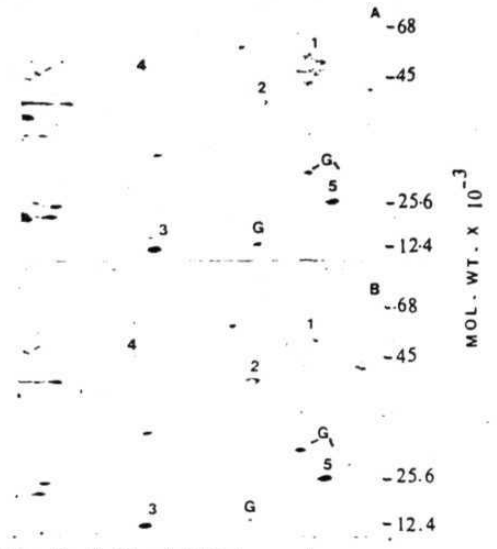

Fig 3: 2-DE of Glial membrane proteins. G indicates protein(s) specific to glia.

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 gel 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

This work is supported by the CSIR. New Delhi, project No. 9(226)86-EMR-II. We thank Prof.P.R.K.Reddy, Dean, School of Life Sciences, for the facilities.

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From "2D-PAGE", C1991) Academic Press, |.|. 131-133, Edt. by Dunn M.I. Burdett P. E and Trand C. M.

DNA BINDING PROTEIN CHARACTERIZATION USING-2DE

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1 INTRODUCTION

In brain, functional and cellular complexity is enormous [1] probably it is a consequence of brain specific gene expression which involves the mediation of DMA binding proteins(DBPs). Very DBPs have thus been characterized and to understand the basis of b specific functions, it is necessary to identify and characterize DNA binding proteins involved. The DBPs in higher mammals exist very scanty amounts and their identification is complicated [3] they follow development dependent expression. In the present study describe the identification and partial characterization of two binding proteins using 2-DE followed by ultra sensitive si staining. He suggest that the 2-DE can be potentially exploited identifying rare and scarce DNA binding proteins.

2 MATERIALS AND METHODS

Ten day old Histar strain albino ruts of both sexes weighing about gms were used for the experiments. Nuclei were isolated from rat b as described earlier [4] and DNA free nuclear protein extract prepared following ammonium sulphate fractionation and polyethy glycol precipitation as described [3]. Double strand (ds) and sir strand (ss) DNA-cellulose chromatography was performed as descr: [5]. Various protein fractions were electrophoresed on SDS-I according to Laemmli with modifications as suggested by Thomas Kornberg [6]. Two dimensional electrophoresis was done as descr by O'Farrel [7] followed by ultra sensitive silver staining [4].

3 RESULTS

Nuclear proteins were chromatographed on dsDNA-cellulose column the column was washed successively with buffers of step-1 increasing NaCl concentrations (0.1, 0.2, 0.6, 1.0 and 2.0 Several minor proteins were eluted in 0.1 and 0.2 M NaCl buffer they were ignored as proteins bound to DNA cellulose below 200 mM ; concentration are not significantly binding type [8]. The I fractions of 2M NaCl eluates were analysed by SDS-PAGE (Fig.1) and proteins denoted DBP-1 and DBP-2 were identified. In order to cl whether or not DBP-1 and DBP-2 are specific for double stranded or, single stranded DNA, the 2M NaCl eluates from dsDNA cellul chromatography were chromatographed on a ssDNA-cellulose column. proteins were found only in flow through; but not in salt elu indicating preferential affinity of these proteins to native DNA. dimensional electrophoresis allowed further elmract <Ti on of DI and DBP *V.* having molecular weights 50kDa and 48kDa, with isoelect Points (pI) 5.3 and 5.2 respectively.

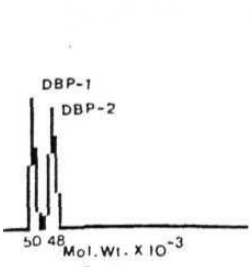

Fig 1

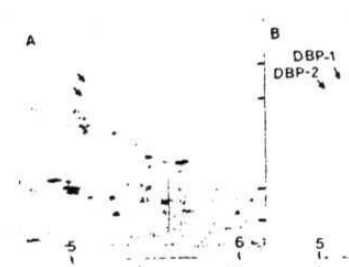

Fig 2

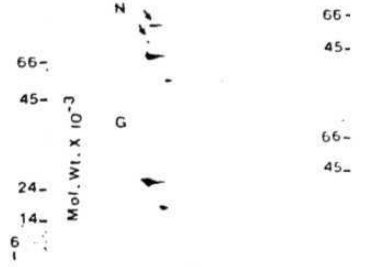

Fig 3

Fig 1: Scan of SDS-PAGE of 2M NaCl eluate of dsDNA cellulose column. Fig 2: 2-DE of total Nuclear proteins (A) from brain and DBP-1 & DBP-2 (B). Fig 3: 2-DE of PEG pelletable proteins from Neuronal (N) and Glial (G) nuclei showing the presence of DBP-1 & 2 only in Neurons.

4 DISCUSSION

The interaction of proteins with genome is usually brought about by DNA binding regulatory proteins [9]. In order to search for such regulatory proteinn of cell nucleus, we chose to analyse UNA binding proteins in the cerebral cortex of rut, using a combination of methods such as DNA **affinity** chromatography nnd 2-DE followed by silver staining. He have identified two proteins DBP-1 and DBP-2 with preferential affinity to bind to dsDNA (Fig 2b). The isoelectric point and relative molecular mass of these proteins were derived' from 2-DE. He could not observe these polypeptides in nuclear preparations **from** 1 and 2 month rnt brains (data not shown). Hence we presume DBP-1 and DBP-2 are **present** in very curly stages of brain development and occur in substantially low amount. Further, separation of nuclei into neuronal and glial indicated the presence of these proteins only in neurons (Fig 3). The functions of DBP-1 nnd DBP-2 are not **clearly** known at r»resen1. but ns these proteins are prominent at 10 days of post-natal age, that too, only in neurons, we suspect they night be involved in neuronal **differentiation** as a transition **from** proliferating precursor cells to non-dividing terminally differentiated neurons takes place *nl* this stage of brain development [10]. The results from UMA affinity chromatography and 2-DE suggests that DBP-1 and DBP-2 might be involved in development dependent neuronal gene expression in brain function.

5 ACKNOWLEDGEMENTS

This work was supported by CSIR, New Delhi (No. 9(226)86-EMR-II). We thank Prof. P. K. R. Reddy, Dean, School of Life Sciences for facilities.

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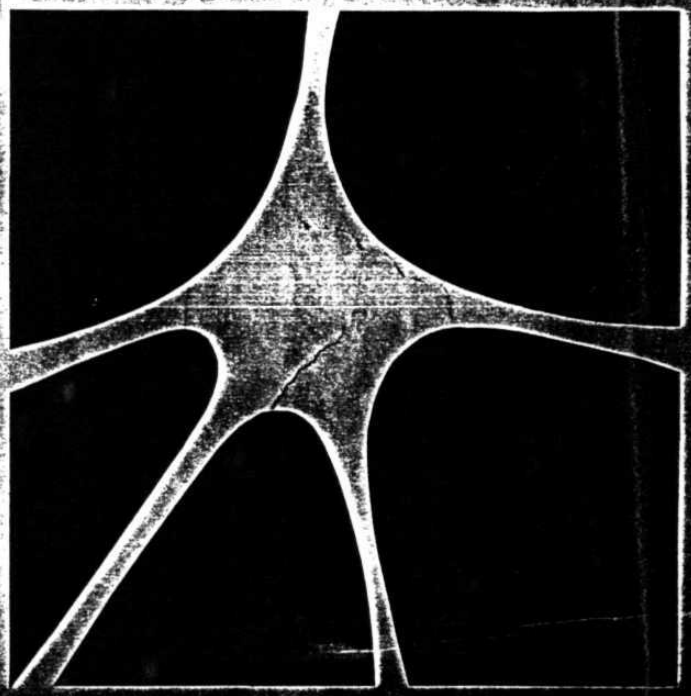

VOLUME 59 • SUPPLEMENT • 1992

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A

INDUCTION OF HEAT SHOCK PROTEIN LIKE POLYPEPTIDE IN FETAL MOUSE BRAINS EXPOSED TO OCHRATOXIN A.

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Ochratoxin A (OA; 3mg/kg), a mycotoxin and a potent teratogen, was injected intraperitoneally to pregnant mice on day 11 of gestation (day 1 = plug day), and neurochemical changes in fetal brains were examined. OA treatment produced intrauterine growth retardation as well as microencephaly and reductions in tissue weights and DNA contents of fetal brains. The level of lipid peroxide in fetal brains was increased markedly on the 3rd and 4th day after injection. The significant increment of superoxide dismutase activity was shown on the 1st and 2nd day after treatment. An acidic heat shock protein(HSP)-like polypeptide with molecular weight of approximately 30kD was induced in fetal brains 36 hours after injection, although HSP70 was not produced. These results suggest that HSP-like polypeptide may be induced by accelarations of free radical formation by the treatment with OA, indicative of response to stress such as cell death in developing brain.

B

CELL ADHESIVE ACTIVITY OF PROTEIN ZERO. T.Yazaki*, M.Miura*, H.Asou*, Y.Kotake*, K.Kitamura** & K.Uyemura*. *Dept. of Physiol., Sch. of Med., Kaita Univ., Tokyo 160 & **Saitama Med. Sch., Saitama 350-04, Japan.

Protein zero (P0), a member of immunoglobulin superfamily, plays a role in adhesion of PNS myelin. Cell adhesion activity of P0-expressing C6 glioma (C6P0) cells, after P0cDNA transfection, was examined. By immuno-histological staining and Western blotting, expression of P0 was confirmed at the cell surface. Homophilic adhesion of C6P0 cells was observed by the DiI-DiO cell staining method. C6P0 cells showed a stronger ability to form large particles from dissociated cells, compared to control cells. Competition assay of cell adhesion, by the addition of synthetic peptides of the P0 sequence, revealed that peptide 90-96 caused 50% inhibition of aggregation. Also the P0-glycopeptide (91-96) suppressed cell adhesion markedly. These results suggest that this region of glycopeptide in P0 is quite important for cell adhesion. P0 has a single oligosaccharide of a hybrid N-glycoside type, with microheterogeneity due to sulfate, sialic acid and glucuronic acid contents. Further analysis of these regions may elicit the functional cell adhesive domain of P0.

C

IDENTIFICATION OF A 38 kDa DNA-BINDING PROTEIN IN RAT BRAIN

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A single strand DNA-binding protein from rat brain was purified using DNA-cellulose chromatography and further confirmed by non-radioactively labelled filter binding assay. The protein is eluted at 0.2M NaCl concentration and has an absorbtion maxima at 265 nm. Further characterization was done by two-dimensional electrophoresis (2DE) followed by ultrasensitive silver staining and it revealed that the protein had a relative molecular mass of 38 kDa and an isoelectric point (pI) of 5.3. The protein was found to have no significant effect on the Tm of calf thymus DNA, suggesting no helix winding or unwinding properties. Since the protein was eluted at 0.2M concentration, we suspect a regulatory role, or it could possibly be involved in DNA repair, replication or transcriptional events in the brain. Further studies are being carried out to reveal its tissue specificity and functional significance in brain.

D

LIVER 94 K MEKA PROTEIN INTERACTS WITH Go$\beta\gamma$

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We have baan reported that MEKA protein forms hetero-trimeric complex with transducin β and γ subunits. It is interesting to investigate whether MEKA or MEKA-like proteins present in non-retinal tissues. One cDNA clone isolated froa a bovine liver cDNA library has identical nucleotide sequence to tha retinal MEKA cDNA. By th« PCR analysis, we found the existence of MEKA or MEKA-like mRNAs in the liver and brain RNAs aa in the retina Expression of MEKA-like proteins in the liver and brain was also confirmed by the western blot analysis. Than. wa purified three MEKA-like proteins froa liver soluble fraction for reconstitution study with brain Ooa and Go$\beta\gamma$. Although CTT> binding to the Goo was stimulated by the Go$\beta\gamma$, this stlaulaory effect waa raducad in tha presence of 94 K MEKA-like protein. Purified isoform of two 35 K MEKA-like proteins revealed no effect on the GIP binding

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β-AMYLOID PROTEIN BLOCKADE OF D-ASPARTATE REUPTAKE: ENHANCEMENT OF EXCITOTOXICITY? Westphalen R I. and Dodd P. R. *Clinical Research Centre. Royal Brisbane Hospital Foundation, Bancroft Centre, Herston Q4029,AUSTRALIA*

Senile plaques are s pathological marker of Alzheiaer Disease (AD). Since the deposition of the β- amyloid protein forms senile plaques, much wort has focussed on a relationship between β-amyloid protein and neuropathology. Independent of this arc reports that excitatory amino acids (EAAs) are involved in the pathogenesis of AD, due to their excitotoxic (EA. neurotoxicity) properties. These proposals have recently merged in the study by Mattson *et al.* (1992), who found that the portion of the β-amyloid protein that mediates cell death (amino acids 25-35: Yankner *et al.*, 1990), enhances glutamate excitotoxicity. Cell death may also be enhanced by blocking sodium-dependent glutamate uptake (Rosenburg *et al.*, 1992). The present study investigated whether β25-35 amyloid interferes with the glutamate transport protein. Our preliminary studies showed that 5 uM synthetic β25-35 amyloid reduced (76.9 ± 2.4% of control) the ability of D-aspartate to displace high-affinity sodium-dependent D-[^{3}H]aspartate binding to human cortical synaptic membranes. The lack of change in the affinity of D-aspartate in displacement (Control: 140.6 ± 57.8 nM; β25-35: 108 5 ± 17.6 nM) suggests that the β25-35 amyloid protein blockade may be of a non-competitive nature

Mattson *et al.* (1992) *J. Neurosci.* **12**(1), 56-61.
Rosenburg *et al.* (1992) *J Neurosci* **12**(2376-389.
Yankner *et al* (1990) *Science* **250**, 279-282.

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HOW TO MAKE THE CARBOXYLATED POLYSTYRENE BEADS UNIFORM ?

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for the attachment of antigen or antibody to then in the production of immunoreagents. Traditional method can not do it wall. I have obtained very uniform carboxylated polyatyrene beads by adding acrylic acid into the reacting system after it had been reacting for 1.5 hours, as illustrated by electron microscope. If polystyrene and acrylic acid wara added at the same time, very different siza of the beads would ba formed.