DISCUSSION
Brain is a complex organ with cellular, regional and functional heterogeneity (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 embryonic 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 \textit{Lamin Bl}. The pI of the protein also matches the acidic pI of \textit{lamin} Bl. All the \textit{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 \textit{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 \textit{possibility}. The precise role of different lamins is not clearly known in chromatin conformation

48
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 isoelectric 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 between 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 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 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 argument 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 α and β polymerase activity. Further, when DNA polymerase α and β 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 heterogeneity 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 occurrence of 56 ssDBP in brain is speculative because of the complexity of brain. The protein is less abundant and based on its isolation, it can be categorized into a rare DNA binding regulatory 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 heterogeneity. 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.