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CHARACTERIZATION OF SINGLE-STRANDED DNA BINDING PROTEINS IN RAT GLIAL NUCLEI

By

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ABSTRACT

CHARACTERIZATION OF SINGLE-STRANDED DNA BINDING PROTEINS IN RAT GLIAL NUCLEI

BY

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Single-stranded DNA binding proteins are those proteins which preferentially bind single-stranded DNA as opposed to double-stranded DNA and are known to be involved in recombination and amplification of DNA. To examine their potential involvement in selective induction of neurogenic tumors, nuclei were isolated from target glia and non-target liver of carcinogenically sensitive Sprague-Dawley (SD) and resistant Berlin-Druckrey-IV (BD-IV) rats of various ages as well as rapidly proliferating glioma cells. Nuclei were fractionated into chromatin, a pre-ribosomal RNA protein complex, heterogeneous nuclear ribonucleoprotein complex (hnRNP), and nucleoplasm. SSBs were isolated therefrom, quantitated, and characterized by electrophoresis. A comparison of the contents of SSBs relative to RNA and their electrophoretic profiles between chromatin and hnRNP revealed that SSBs of liver chromatin were mainly those which were associated with RNA. However, it was found that glial chromatin, particularly that of juvenile rats, was enriched with SSBs and contained a heterogeneous series of SSBs which were not found in liver chromatin and presumably associated with chromosomal DNA. Some of these SSBs were found to be enriched in glial chromatin of SD rats as compared with that

of BD-IV rats. High mobility group proteins (HMG) 1 and 2 constituted major SSB components in the nucleoplasm and a greater amount of these HMG's were found in juvenile glia, as compared to adult glia as well as juvenile and adult liver. Fractionation and isolation of glial SSBs and determination of their biological functions may contribute to the further understanding of the role these proteins play in the selective induction of neurogenic tumors.

This dissertation is dedicated to my parents Jed and Pam, who were kind enough to support me throughout all of my costly academic endeavors.

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I. INTRODUCTION

Single-stranded DNA binding proteins (SSBs) are those proteins which bind preferentially to single-stranded DNA (ssDNA) as opposed to double-stranded DNA (dsDNA) under selected conditions (Chase and Williams, 1986). These proteins have also been referred to as DNA unwinding proteins (Alberts and Frey, 1970), DNA melting proteins (Jensen et al., 1976), and DNA helix-destabilizing proteins (Alberts and Sternglanz, 1977). In bacteriophages SSBs are necessary for DNA replication, recombination, and function to destabilize dsDNA (Alberts and Frey, 1970), and stimulate DNA polymerase activity (Huberman, 1971). Prokaryotic SSBs are essential for DNA recombination (ssDNA dependent ATPase activity), cell division, mutagenesis, prophage induction, hybridization of ssDNA, and ATP-dependant unwinding of dsDNA (see Chase and Williams, 1986 and Lohman et al., 1988 for reviews). However, the existence of mammalian SSBs has not been established as previously isolated SSBs have turned out to be actually proteins of intermediate metabolism (Richter et al., 1986) or degraded products of heterogeneous nuclear ribonucleoprotein (hnRNP) particles (Pandolfo et al., 1985; Valentini et al., 1985; Kumar et al., 1986; Merrill et al., 1987). This is despite the fact that these proteins unwind dsDNA, stimulate

DNA polymerase-α (Herrick and Alberts 1976a and b), and in some cases function as ssDNA-dependent ATPases (Hsieh et al., 1986). Rat liver mitochondrial SSB P16 (Pavco and Van Tuyle, 1985) believed to be analogous to Xenopus mitochondrial 15,500 M_r SSB (Mignotte et al., 1985) which has been shown to share amino acid sequence homology with E. coli SSB, may be the exception. This would not be surprising since mitochondria are thought evolutionarily to represent prokaryotic cells which have been captured by the cytoplasm of eukaryotic cells (Attardi et al., 1975; Slater, 1981). Well characterized high mobility group proteins (HMG) 1 and 2 preferentially bind ssDNA (Isackson et al., 1979) and depending on conditions these proteins can serve to either stabilize or destabilize dsDNA, and to stimulate DNA polymerase-α and ß (Butler et al., 1985; Bonne et al., 1982; Duguet and deRecondo, 1978). Although it is generally believed that HMG 1 and 2 are associated with chromatin (Johns, 1982), evidence exists which suggests that these proteins may actually be localized to the nucleoplasm (Comings and Harris, 1976b; Conner and Comings, 1981).

Many proteins isolated from rat glial nuclei via DNA affinity chromatography have yet to be characterized and may represent true mammalian SSBs associated with DNA (Heizmann et al., 1982). Unlike proteins of intermediate metabolism, these proteins have been shown to be eluted from ssDNA at high salt concentrations. The possibility that these glial

proteins are arising from hnRNP particles cannot be excluded at the present time. One goal of this study was to determine the origins of rat glial SSBs.

Another aspect of this study is to explore the possible role glial SSBs may play in the neoplastic process. In the rat, the greatest proliferation of neuroglial cells occurs after birth (Gilmore, 1971). A single exposure of pregnant rats to N-ethyl-N-nitrosourea (ENU) induces tumors in the offspring which are mostly of glial or Schwann cell origin (Koestner et al., 1972). Repair of the promutagenic lesion O^6-ethylguanine in the brain (target tissue) is slow, while in liver (nontarget tissue) repair of the promutagenic lesion O^6-ethylguanine is rapid (Goth and Rajewski, 1974; Chang et al., 1980). Alkylation of the O^6 position of guanine by ENU results in a point G->A transition (Singer, 1975; Pegg, 1983) and thus O^6-alkylguanine can lead to nucleic acid polymerase reactions which result in the misincorporation of dTMP instead of the expected dCMP (Pegg, 1983). This DNA adduct is efficiently repaired by the suicidal methyltransferase enzyme which reverts O^6-alkylguanine residues back to guanine by transferring the alkyl group to its own cysteine residue and thereby inactivating the enzyme. Neuro-carcinogenic susceptibility varies among rat stains. For example, Berlin-Druckrey-IV (BD-IV) rats are least susceptible to transplacental N-ethyl-N-nitrosourea (ENU) tumor induction in the brain, while BD-IX (and Sprague-Dawley) rats are most

susceptible (Druckrey et al., 1970), despite similar persistence of O^6-ethylguanine in brain DNA of these two rat strains (D'Ambrosio et al., 1986). It appears as though an additional factor(s) other than repair of O^6-ethylguanine is involved in differences in carcinogenic susceptibility between these two rat strains.

The chemically induced sequence of processes resulting in carcinogenesis via abnormal expression of the cellular onc gene is likely to involve gene rearrangement and amplification. Trent et al. (1986) have identified amplification and expression of the cellular oncogene c-myc in double minute containing cells from a patient with glioblastoma multiforme. They also show that amplification is associated with rearrangement of the c-myc gene in these human brain tumors. Certain cell lines derived from tumors (neuroblastomas and glioblastomas) induced by transplacental exposure to ENU show amplification of the neu transforming gene (Schechther et al. 1984). Amplification of the proto-oncogene N-myc is a common event in vivo and is associated with advanced stages of tumor growth of neuroectodermal origin (neuroblastomas and retinoblastomas) (Kohl et al. 1984; Brodeur et al. 1984). Liberman et al. (1985) have shown that certain tumors of glial origin (glioblastoma multiforme) exhibit a 6-60 fold amplification of copies of the EGF receptor gene. It thus seems likely that proteins involved with rearrangement and amplification of the genome play

important roles in neoplastic transformation. It seems plausible that proteins such as SSBs which are closely associated with processing of DNA could play a role in the neoplastic process.

The present study characterizes rat SSBs in an attempt to examine their possible involvement in neuro-carcinogenic susceptibility. Cell types examined include carcinogenic target glia and non-target liver of susceptible SD and resistant BD-IV rats as well as rapidly proliferating glioma cells. Nuclei have been fractionated into chromatin, a pre-messenger RNA-protein complex, a pre-ribosomal RNA-protein complex, and nucleoplasm. SSBs have been isolated via DNA affinity chromatography, quantitated, and further characterized by electrophoresis.

REVIEW OF THE LITERATURE

STATUS of MAMMALIAN SSBs

Relationship to hnRNP derivatives. Herrick and Alberts (1976a) have employed DNA affinity chromatography in the isolation of SSBs from unfractionated calf thymus tissue. Extracts were loaded onto the affinity columns at 0.05 M NaCl and eluted with 2 M NaCl in a stepwise manner or via a NaCl gradient. Three protein species of M_r 24,000, 33,000 (low salt eluting), and 33,000 (high salt eluting) were isolated. The

24,000 M_r SSB termed UP1 (calf-unwinding protein 1) was further purified. Based on native and SDS electrophoresis UP1 is a symmetric monomer with an isoelectric point of 7.8 under nondenaturing conditions. UP1 is located in the cytosol in at least 800,000 copies per cell and it is speculated that, since UP1 displays preferential affinity for ssDNA, much of the protein can be bound to ssRNA at any instant *in vivo*. Also, this protein, unlike the T4 gp 32 and *E. coli* SSB depresses the T_m of natural DNA (*Clostridium*).

Two possible pathways for the melting of native DNA by UP1 have been suggested. In the first, double-stranded DNA would breathe to form small stretches of single-stranded coil. UP1 would then bind and hold the single strands apart. The second pathway, which the authors favored, involved UP1 binding to some double-helical form, which then denatured into single strands with UP1 bound to it. Although UP1 binds preferentially to ssDNA, it also has an affinity for dsDNA, which is at least 10 times weaker than its attraction to ssDNA (Herrick and Alberts, 1976b). UP1 does not promote renaturation and based on its ability to melt more DNA than it can directly cover, UP1 binds noncooperatively to DNA. Although T4 gp 32 did not melt poly [r(AU)] or catalyze t-RNA renaturation as UP1 does, T4 gp 32 and SSB from *E. coli* can bind to RNA under some conditions. These findings, along with the fact that UP1 is located in the non-chromatin cell fraction, suggested to Herrick and Alberts (1976b) that helix-

destabilizing proteins in general may be involved in transcription and/or translation. They further speculated that these SSBs may be found to be associated with nuclear ribonucleoprotein particles. However, based on the fact that these proteins destabilize DNA and stimulate DNA polymerase-α, Herrick and Alberts (1976b) concluded that UP1 is most likely associated with DNA.

UP1 stimulates calf thymus DNA polymerase-α by 10 fold, while higher amounts of UP1 inhibit the polymerase (Herrick et al., 1976). UP1 does not stimulate calf DNA polymerase-ß (Herrick and Alberts, 1976b). UP1 is also reported to consist of 195 amino acid residues, a blocked NH_2 terminus, and a single N^GN^G-dimethylarginine residue near its COOH terminus (Williams et al., 1985b). The unusual amino acid residue dimethylarginine is also present in histones, HMG proteins, ribosomal proteins, and hnRNP particles. As discussed below, UP1 is actually a proteolytic product of hnRNP A protein.

It is known that antibodies raised against calf thymus SSBs react to 40S hnRNP core proteins from HeLa cells (Valentini et al., 1985). The electrophoretic patterns of the hnRNP particles also matches the electrophoretic patterns of the calf thymus SSBs, and following partial tryptic digestion, HeLa cell SSB and hnRNPs produce immunoreactive fragments of the same molecular weight and isoelectric point. Further studies reveal that calf thymus SSBs are actually specific

proteolytic products of hnRNP core proteins (Pandolfo et al., 1985). Antibodies have been produced in mice against purified calf thymus UP1 (24,000-26,000 M_r) and a strong homology between the hnRNP proteins and UP1 exists based on peptide mapping and partial amino acid sequencing. Antibodies to UP1 also react with hnRNP core proteins of M_r 32,000-38,000 in western blots of HeLa cell sonicates. In vitro proteolysis of HeLa cell crude extracts results in the increased generation of polypeptides of 24,000-28,000 M_r, which in turn react with calf thymus SSBs to result in a decrease in hnRNP protein C. Further digestion results in the production of a 24,000 M_r band which appears to be UP1. Pandolfo et al., (1985) has partially purified the trypsin-like protease that cleaves the hnRNP protein to smaller (24,000-28,000 M_r) SSBs. They also point out that hnRNP core proteins do not stimulate DNA polymerase-α, while once cleaved to 24,000 M_r, they do. Kumar et al., (1986) partially purified and sequenced the core hnRNP proteins A_1 and A_2 and showed that the primary structure of the SSB UP1 is nearly identical to that of the N-terminal domain of HeLa core hnRNP protein A1. The degree of homology is 89% with the only difference being the interchange of lysine and arginine. It has also been suggested, based on amino acid sequencing, that UP1 represents specifically the NH_2-terminal two thirds of the 32,000 hnRNP protein since the NH_2-terminus of UP1 is blocked (Merrill et al., 1987). The COOH-terminal region of UP1 is also extremely glycine-rich

(45%)(Merrill et al., 1987). Calf thymus UP2 (39,500 M_r) has a high degree of sequence homology with UP1 (Merrill et al., 1986), and also probably represents proteolytic products of 40S hnRNPs.

Two additional calf thymus SSBs of M_r 48,000 and 61,000 have been isolated via chromatography on ds and ssDNA affinity columns, and chromatography on hydroxylapatite (Sapp et al., 1985). Extracts were loaded onto the DNA affinity columns at 0.05 M NaCl and nonspecifically bound proteins were eluted with 0.4 M NaCl, while the M_r 48,000 and 61,000 proteins were eluted with 2 M NaCl. These proteins bind in a noncooperative manner to ssDNA. The two proteins are immunologically and biochemically related to each other as well as to calf thymus UP1, and it is believed that the 48,000 M_r protein is a proteolytic product of the 61,000 M_r protein. UP1 is hypothesized to be derived from the 48,000 or 61,000 M_r proteins and changes in pH between 6.5-8.0 do not change the binding of these proteins to ssDNA (Sapp et al., 1985). Both proteins stimulate DNA synthesis catalyzed by mammalian DNA polymerase-α in the presence of activated (DNA containing a large number of primer sites and short template regions) calf thymus DNA as primer-template. Most likely both of these proteins are derived in turn from hnRNP proteins.

Cobianchi et al. (1978) have also isolated a SSB from calf thymus. Extracts were applied to DNA affinity columns at 0.05 M NaCl and the single-stranded column was eluted with

0.25 M, 0.5 M, and 1.0 M NaCl in a stepwise manner. Proteins eluting with 1.0 M NaCl were further purified. The SSB isolated has a M_r of 18,000-20,000 in SDS polyacrylamide gels and like UP1, stimulates DNA polymerase-α on activated calf thymus DNA, denatured calf thymus DNA, and denatured poly [d(A-T)] templates at protein/DNA ratios of 2:1 or lower. DNA polymerase-β is only slightly stimulated by this SSB. Based on its similar properties to UP1, this protein also most likely represents degraded hnRNP products.

The structure of a newborn rat brain helix-destabilizing protein has been examined using cDNA cloning (Cobianchi et al., 1986). Using a synthetic oligonucleotide probe corresponding to a 5 amino-acid sequence in the N-terminal region of the calf helix-destabilizing protein UP1, a cDNA library of newborn rat brain poly (A^+)RNA in a phage was screened. Positive clones were screened with a second probe corresponding to a 5 amino acid-sequence in the C-terminal region of calf UP1 and one of these positive clones was selected for study. In the cDNA a 988-residue open reading frame predicts a 34,215 dalton protein of 320 amino acids. Of the 195 amino acid residues of UP1, residues 2 through 196 of the rat brain protein are identical to UP1. A 124 amino acid sequence in the C-terminal portion of the rat brain protein is not present in the purified calf UP1 protein and the amino acid content of the 124 amino acid residues consists of 11% asparagine, 15% serine, and 40% glycine. Since this

protein shares high homology to UP1, it is likely that it is also degraded hnRNP products.

Dreyfuss et al. (1988) have reviewed hnRNP in eukaryotic cells. MRNA in eukaryotic cells arise from primary gene transcripts called heterogeneous nuclear RNAs (hnRNAs). HnRNAs are differentiated from other RNAs based on size, nuclear location, and the fact that the RNA polymerase that transcribes them is antibiotic sensitive (Dreyfuss et al., 1988). HnRNAs give rise via RNA polymerase II (Pederson, 1983) to pre-mRNA (Darnell, 1982) which contains 5' cap structures (M^7Gpp,an inverted guanosine cap), 3' polyadenylated tails, and intervening sequences that are later spliced out (introns) (Dreyfuss et al., 1988). This hnRNA is then translocated through nuclear pores and spliced mRNAs accumulate in the cytoplasm. HnRNP particles which consist of hnRNA with specific proteins bound to it can be sedimented in sucrose gradients as heterodispersed material between 30s and 250s (Billings and Martin, 1978; Choi and Dreyfuss, 1984) and at least 75% of hnRNA is associated with the 30s particle which as a monomer accommodates 500 $\pm$ 100 nucleotides of hnRNA (Choi et al., 1986). Following mild ribonuclease hydrolysis hnRNP particles are shown to sediment from 30-250s (Pederson, 1974). HnRNP is the form in which hnRNA exists in the nucleus (Samarina et al., 1968; Pederson, 1974; Kish and Pederson, 1975; Brunel and Lelay, 1979). The mechanism of transition from hnRNP (nuclear) to mRNPs (protein associated with

cytoplasmic mRNA) is unknown (Choi et al., 1986). What is known is that no proteins have been found that were bound to both mRNA and hnRNP and it is possible that hnRNP proteins actually process the pre-mRNA.

The 30s hnRNP particle is composed of anywhere from 75-80% protein to 85-90% protein, depending upon the type of determination employed (Billings and Martin, 1978). Direct determinations on pelleted or ethanol precipitated 30s RNP have given a composition of about 90% protein and 10% RNA. The 40s hnRNP particles in HeLa cells have been identified and characterized (Beyer et al., 1977) and 6 lower molecular weight polypeptides have been identified as the protein constituents of the 40s ribonucleoprotein complex. The 3 groups of closely spaced doublet proteins are termed group A (M_r 32,000 A_1/34,000 A_2), group B (M_r 36,000 B_1/37,000 B_2) and group C (M_r 42,000 C_1/44,000 C_2). The pI of group A proteins, 9.2(A_1) and 8.4(A_2), and B_1 are basic, while for B_2, C_1 and C_2 the pIs are acidic. The group A proteins are major nuclear proteins constituting 60% of the total hnRNP particle protein mass. The A and B group hnRNP proteins dissociate from hnRNA at 150 mM NaCl while the C proteins dissociate at 750 mM NaCl (Beyer et al., 1977). Others have isolated 35s hnRNP particles from HeLa cells and identified 9 core proteins (Wilk et al., 1985). Cross-species conservation was also demonstrated via peptide mapping of proteins A_1 and A_2 from bovine and human cells.

Two functions have been proposed for hnRNP proteins. The first is in the packaging of hnRNA, such that the hnRNA can be compacted into the nucleus (Dreyfuss et al., 1988) and the second is in splicing (removal of introns) of the pre-mRNA complex . (Choi et al., 1986). Monoclonal antibodies generated against hnRNP C_1 (41,000 M_r) and C_2 (43,000 M_r) proteins inhibit the splicing or removal of intervening sequences from pre-mRNA in vitro and prevent the formation of mature mRNA (Choi et al., 1986). Also, hnRNA is highly nuclease sensitive and the 60s complex with which C proteins are associated forms a multicomponent splicing complex (Frendewey and Keller, 1985; Brody and Abelson, 1985). Various hnRNP proteins have been sequence (Adam et al., 1986; Sachs et al., 1986; Grange et al., 1987; Kumar et al., 1986; Swanson et al., 1987; Haynes et al., 1987).

The total nuclear RNP fraction consists of the proteins associated with the hnRNA, snRNA, and pre-rRNA particles (Mattaj, 1984). The pre-ribosomal ribonucleoprotein particle (45S) is composed of 61% protein and 39% RNA (Kumar and Warner, 1972). The precursor rRNA (45S) is synthesized in the fibrillar portion of the nucleolus from which the RNA moves to the granular portion on the RNP particle (Olson and Busch, 1978) and then out to the cytoplasm (18S and 28S)(Warner and Soeiro, 1967). Other proteins known to contain methylated arginine residues in addition to hnRNP proteins (Beyer et al., 1977; Boffa et al., 1977; Karn et al., 1977) include histones

(Paik and Kim, 1980), HMG 1 and 2 (Boffa et al., 1979), and nucleolar proteins C23 (Lischwe et al., 1982; Lischwe et al., 1985b). It is believed that C23 is a component of the pre-ribosomal RNP particle (Olson and Thompson, 1983) and glycine and dimethylarginine clusters present in this 34,000 M_r protein may be characteristic of RNA-associated proteins (Lischwe et al., 1985a).

Rat brain ribonucleoproteins have been examined and shown to be released from RNA after treatment with 0.25 M and 0.4 M NaCl (Stevenin and Jacob, 1974). Electron microscopic examination of hnRNP particles from brain reveal folded strands with long fibrils containing regions of varying widths and densities (Stevenin et al., 1976). Brain hnRNA treated with ribonuclease release polypeptides ranging from 23,000-150,000 M_r (Stevenin et al., 1977). At low ribonuclease concentrations the hnRNP particles accumulate at 35-45S and proteins in the 30,000-38,000 M_r range are released. At higher ribonuclease concentrations, the total amount of protein at 35-45S decreases, however, proteins of M_r 30,000-38,000 predominate and at very high enzyme concentrations the particles are almost all hydrolyzed. Stevenin et al. (1977) also report that sequences of up to 200-300 nucleotides are protected from ribonuclease hydrolysis by the ribonucleoproteins. Based on characteristics of the hnRNP particles following ribonuclease treatment, investigators conclude that the native particles are polyparticles made up

of variable numbers of monoparticles (Samarina et al., 1968; Pederson, 1974). Gallinaro-Matringe et al. (1975) have examined hnRNP particles isolated from rat brain nuclei via a linear sucrose gradient centrifugation method and found 45 proteins to be associated with particles greater than or equal to 60S. A group of easily released species, with 75% and 95% being removed by 0.25 M NaCl and 0.7 M NaCl exists. This group contains 8 proteins between 29,000 and 39,000 M_r and the phosphorylated proteins bind more tightly to the RNA than do the nonphosphorylated proteins.

Relationship to enzymes of intermediary metabolism. Several mammalian SSBs have been shown to actually be enzymes of intermediary metabolism such as lactic dehydrogenase (LDH) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Helix destabilizing protein-1 (HDP-1) has been isolated from unfractionated mouse myeloma cells (Planck and Wilson, 1980). The extract was loaded onto DNA affinity columns at 50 mM NaCl (pH 8.8) and eluted from both ssDNA columns with 2 M NaCl. HDP-1, which preferentially binds ssDNA, is heterogeneous with respect to M_r (24,000-33,000), with the major species being of 27,000 M_r. HDP-1 possesses a homologous primary structure based on tryptic peptide mapping, lowers the T_m of poly [d(A-T)], and binds noncooperatively to ssDNA. Although 75% of HDP-1 is localized to the nucleus, it is not chromatin-associated. This protein is also distinct from high mobility

group proteins (HMG) 1 and 2, histones, and P8 SSB. Fragments of 19,000 to 24,000 M_r are produced following tryptic hydrolysis of HDP-1. In the presence of ssDNA, a 22,000 M_r fragment (22HDP) is produced by tryptic hydrolysis, while in the absence of ssDNA, a 19,000 M_r fragment (19HDP) predominates with both fragments binding ssDNA, suggesting that the NH_2-terminal end of HDP-1 is not necessary for binding to ssDNA. These investigators also point out that since HDP-1 recognizes RNA, some role in ribonucleoprotein metabolism may exist for this protein. HDP-1 is known to stimulate its homologous DNA polymerase-α (Detera et al., 1981) and amino acid sequencing of UP-1 and HDP-1 reveals a high degree of sequence homology between these two helix destabilizing proteins (Williams et al., 1985a). Antibody probing shows that HDP-1 actual has an M_r of 36,000-38,000 and during isolation proteolysis results in the formation of the 27,000 M_r protein and smaller fragments (Planck and Wilson, 1985). This mouse myeloma protein has been identified as a LDH (A) isoenzyme (Sharief et al., 1986). The protein possess LDH activity and rabbit antiserum prepared against it cross reacts with purified LDH-A isoenzyme. It has been suggested that the binding of these enzymes of intermediary metabolism to ssDNA is due to a fortuitous affinity for ssDNA via their nucleotide binding site (NAD for G3PDH or NADH for LDH) (Richter et al., 1986). However, it is also interesting to note that these proteins are eluted from the ss matrix at a very low salt concentration and

therefore, may be suspected as binding nonspecifically to ssDNA. A 34,000 M_r SSB has been isolated from a rat pheochromocytoma cell line (PC 12) (Biocca et al., 1984). Extracts were loaded onto DNA affinity columns at 50 mM NaCl and then subjected to ds/ssDNA affinity chromatography and proteins eluted with 2 M NaCl. The 34,000 M_r protein constitutes 0.5% of the total soluble proteins and consists largely of acidic vs basic amino acid residues. Fifteen percent of the protein is phosphorylated and this protein has an isoelectric point of 8.0. Synthesis of this 34,000 SSB is progressively inhibited following arrest of mitosis and neurite outgrowth induced by nerve growth factor. The authors suggest that control of synthesis of this SSB is related to activation of differentiation induced by nerve growth factor in this neoplastic cell line. This SSB has also turned out to be an enzyme of intermediary metabolism, LDH (Calissano et al., 1985). It cross-reacts with antibodies raised against rabbit LDH and antibodies raised against the 34,000 SSB also react with rabbit LDH. Furthermore, it was shown through immunocytochemical localization techniques and quantitative electron microscopy that LDH-SSB is located in the nucleus of various cell types, including enriched rat cerebellar glial cells and rat fibroblasts (Cattaneo et al., 1985). Since treatment with DNase resulted in a reduction in nuclear staining and treatment with RNase did not, this protein is most likely associated with chromatin in vivo and hence the

possibility of involvement of LDH-SSB in some nuclear functions exists.

A SSB termed P8 has been isolated from mouse 3T6 cells and human diploid fibroblasts (Tsai and Green, 1973). Cells were extracted and loaded on to a ssDNA affinity column at 0.05 M NaCl. P8 was subsequently eluted with 0.2 M NaCl. P8 fails to bind to dsDNA, however, since this protein elutes from ssDNA at such a low salt concentration it is questionable as to whether or not this represents a true SSB. Indeed, P8 has been purified to homogeneity and shown to be G3PDH (Perucho et al., 1977). The amino acid composition of P8 is very similar to that of G3PDH and the P8 protein possesses a high G3PDH activity.

Both SSB-37 and SSB-35 preferentially bind ssDNA (Grosse et al., 1986). SSB-35 stimulates DNA polymerase-α primer complex five fold whereas SSB-37 inhibits primase-initiated replication. Also, SSB-37 binds cooperatively to ssDNA and possess G3PDH activity, while SSB-35 possess LDH activity. The true identities of these two SSBs were confirmed by amino acid sequencing.

A rat liver SSB of M_r 30,000, isolated by ds/ssDNA affinity chromatography (loaded at 0.05 M NaCl) and ultragel AcA44 filtration has been identified as LDH-5 based on similar (148 of the 157 residues) amino acid compositions and HPLC tryptic peptide maps (Williams et al., 1985a). The specific activities of LDH and this helix-destabilizing protein are

proteins are arising from hnRNP particles cannot be excluded at the present time. One goal of this study was to determine the origins of rat glial SSBs.

Another aspect of this study is to explore the possible role glial SSBs may play in the neoplastic process. In the rat, the greatest proliferation of neuroglial cells occurs after birth (Gilmore, 1971). A single exposure of pregnant rats to N-ethyl-N-nitrosourea (ENU) induces tumors in the offspring which are mostly of glial or Schwann cell origin (Koestner et al., 1972). Repair of the promutagenic lesion O^6-ethylguanine in the brain (target tissue) is slow, while in liver (nontarget tissue) repair of the promutagenic lesion O^6-ethylguanine is rapid (Goth and Rajewski, 1974; Chang et al., 1980). Alkylation of the O^6 position of guanine by ENU results in a point G->A transition (Singer, 1975; Pegg, 1983) and thus O^6-alkylguanine can lead to nucleic acid polymerase reactions which result in the misincorporation of dTMP instead of the expected dCMP (Pegg, 1983). This DNA adduct is efficiently repaired by the suicidal methyltransferase enzyme which reverts O^6-alkylguanine residues back to guanine by transferring the alkyl group to its own cysteine residue and thereby inactivating the enzyme. Neuro-carcinogenic susceptibility varies among rat stains. For example, Berlin-Druckrey-IV (BD-IV) rats are least susceptible to transplacental N-ethyl-N-nitrosourea (ENU) tumor induction in the brain, while BD-IX (and Sprague-Dawley) rats are most

susceptible (Druckrey et al., 1970), despite similar persistence of O^6-ethylguanine in brain DNA of these two rat strains (D'Ambrosio et al., 1986). It appears as though an additional factor(s) other than repair of O^6-ethylguanine is involved in differences in carcinogenic susceptibility between these two rat strains.

The chemically induced sequence of processes resulting in carcinogenesis via abnormal expression of the cellular onc gene is likely to involve gene rearrangement and amplification. Trent et al. (1986) have identified amplification and expression of the cellular oncogene c-myc in double minute containing cells from a patient with glioblastoma multiforme. They also show that amplification is associated with rearrangement of the c-myc gene in these human brain tumors. Certain cell lines derived from tumors (neuroblastomas and glioblastomas) induced by transplacental exposure to ENU show amplification of the neu transforming gene (Schechther et al. 1984). Amplification of the proto-oncogene N-myc is a common event in vivo and is associated with advanced stages of tumor growth of neuroectodermal origin (neuroblastomas and retinoblastomas) (Kohl et al. 1984; Brodeur et al. 1984). Liberman et al. (1985) have shown that certain tumors of glial origin (glioblastoma multiforme) exhibit a 6-60 fold amplification of copies of the EGF receptor gene. It thus seems likely that proteins involved with rearrangement and amplification of the genome play

important roles in neoplastic transformation. It seems plausible that proteins such as SSBs which are closely associated with processing of DNA could play a role in the neoplastic process.

The present study characterizes rat SSBs in an attempt to examine their possible involvement in neuro-carcinogenic susceptibility. Cell types examined include carcinogenic target glia and non-target liver of susceptible SD and resistant BD-IV rats as well as rapidly proliferating glioma cells. Nuclei have been fractionated into chromatin, a pre-messenger RNA-protein complex, a pre-ribosomal RNA-protein complex, and nucleoplasm. SSBs have been isolated via DNA affinity chromatography, quantitated, and further characterized by electrophoresis.

REVIEW OF THE LITERATURE

STATUS of MAMMALIAN SSBs

Relationship to hnRNP derivatives. Herrick and Alberts (1976a) have employed DNA affinity chromatography in the isolation of SSBs from unfractionated calf thymus tissue. Extracts were loaded onto the affinity columns at 0.05 M NaCl and eluted with 2 M NaCl in a stepwise manner or via a NaCl gradient. Three protein species of M_r 24,000, 33,000 (low salt eluting), and 33,000 (high salt eluting) were isolated. The

24,000 M_r SSB termed UP1 (calf-unwinding protein 1) was further purified. Based on native and SDS electrophoresis UP1 is a symmetric monomer with an isoelectric point of 7.8 under nondenaturing conditions. UP1 is located in the cytosol in at least 800,000 copies per cell and it is speculated that, since UP1 displays preferential affinity for ssDNA, much of the protein can be bound to ssRNA at any instant *in vivo*. Also, this protein, unlike the T4 gp 32 and *E. coli* SSB depresses the T_m of natural DNA (*Clostridium*).

Two possible pathways for the melting of native DNA by UP1 have been suggested. In the first, double-stranded DNA would breathe to form small stretches of single-stranded coil. UP1 would then bind and hold the single strands apart. The second pathway, which the authors favored, involved UP1 binding to some double-helical form, which then denatured into single strands with UP1 bound to it. Although UP1 binds preferentially to ssDNA, it also has an affinity for dsDNA, which is at least 10 times weaker than its attraction to ssDNA (Herrick and Alberts, 1976b). UP1 does not promote renaturation and based on its ability to melt more DNA than it can directly cover, UP1 binds noncooperatively to DNA. Although T4 gp 32 did not melt poly [r(AU)] or catalyze t-RNA renaturation as UP1 does, T4 gp 32 and SSB from *E. coli* can bind to RNA under some conditions. These findings, along with the fact that UP1 is located in the non-chromatin cell fraction, suggested to Herrick and Alberts (1976b) that helix-

destabilizing proteins in general may be involved in transcription and/or translation. They further speculated that these SSBs may be found to be associated with nuclear ribonucleoprotein particles. However, based on the fact that these proteins destabilize DNA and stimulate DNA polymerase-α, Herrick and Alberts (1976b) concluded that UP1 is most likely associated with DNA.

UP1 stimulates calf thymus DNA polymerase-α by 10 fold, while higher amounts of UP1 inhibit the polymerase (Herrick et al., 1976). UP1 does not stimulate calf DNA polymerase-ß (Herrick and Alberts, 1976b). UP1 is also reported to consist of 195 amino acid residues, a blocked NH_2 terminus, and a single N^GN^G-dimethylarginine residue near its COOH terminus (Williams et al., 1985b). The unusual amino acid residue dimethylarginine is also present in histones, HMG proteins, ribosomal proteins, and hnRNP particles. As discussed below, UP1 is actually a proteolytic product of hnRNP A protein.

It is known that antibodies raised against calf thymus SSBs react to 40S hnRNP core proteins from HeLa cells (Valentini et al., 1985). The electrophoretic patterns of the hnRNP particles also matches the electrophoretic patterns of the calf thymus SSBs, and following partial tryptic digestion, HeLa cell SSB and hnRNPs produce immunoreactive fragments of the same molecular weight and isoelectric point. Further studies reveal that calf thymus SSBs are actually specific

proteolytic products of hnRNP core proteins (Pandolfo et al., 1985). Antibodies have been produced in mice against purified calf thymus UP1 (24,000-26,000 M_r) and a strong homology between the hnRNP proteins and UP1 exists based on peptide mapping and partial amino acid sequencing. Antibodies to UP1 also react with hnRNP core proteins of M_r 32,000-38,000 in western blots of HeLa cell sonicates. In vitro proteolysis of HeLa cell crude extracts results in the increased generation of polypeptides of 24,000-28,000 M_r, which in turn react with calf thymus SSBs to result in a decrease in hnRNP protein C. Further digestion results in the production of a 24,000 M_r band which appears to be UP1. Pandolfo et al., (1985) has partially purified the trypsin-like protease that cleaves the hnRNP protein to smaller (24,000-28,000 M_r) SSBs. They also point out that hnRNP core proteins do not stimulate DNA polymerase-α, while once cleaved to 24,000 M_r, they do. Kumar et al., (1986) partially purified and sequenced the core hnRNP proteins A_1 and A_2 and showed that the primary structure of the SSB UP1 is nearly identical to that of the N-terminal domain of HeLa core hnRNP protein A1. The degree of homology is 89% with the only difference being the interchange of lysine and arginine. It has also been suggested, based on amino acid sequencing, that UP1 represents specifically the NH_2-terminal two thirds of the 32,000 hnRNP protein since the NH_2-terminus of UP1 is blocked (Merrill et al., 1987). The COOH-terminal region of UP1 is also extremely glycine-rich

(45%)(Merrill et al., 1987). Calf thymus UP2 (39,500 M_r) has a high degree of sequence homology with UP1 (Merrill et al., 1986), and also probably represents proteolytic products of 40S hnRNPs.

Two additional calf thymus SSBs of M_r 48,000 and 61,000 have been isolated via chromatography on ds and ssDNA affinity columns, and chromatography on hydroxylapatite (Sapp et al., 1985). Extracts were loaded onto the DNA affinity columns at 0.05 M NaCl and nonspecifically bound proteins were eluted with 0.4 M NaCl, while the M_r 48,000 and 61,000 proteins were eluted with 2 M NaCl. These proteins bind in a noncooperative manner to ssDNA. The two proteins are immunologically and biochemically related to each other as well as to calf thymus UP1, and it is believed that the 48,000 M_r protein is a proteolytic product of the 61,000 M_r protein. UP1 is hypothesized to be derived from the 48,000 or 61,000 M_r proteins and changes in pH between 6.5-8.0 do not change the binding of these proteins to ssDNA (Sapp et al., 1985). Both proteins stimulate DNA synthesis catalyzed by mammalian DNA polymerase-α in the presence of activated (DNA containing a large number of primer sites and short template regions) calf thymus DNA as primer-template. Most likely both of these proteins are derived in turn from hnRNP proteins.

Cobianchi et al. (1978) have also isolated a SSB from calf thymus. Extracts were applied to DNA affinity columns at 0.05 M NaCl and the single-stranded column was eluted with

0.25 M, 0.5 M, and 1.0 M NaCl in a stepwise manner. Proteins eluting with 1.0 M NaCl were further purified. The SSB isolated has a M_r of 18,000-20,000 in SDS polyacrylamide gels and like UP1, stimulates DNA polymerase-α on activated calf thymus DNA, denatured calf thymus DNA, and denatured poly [d(A-T)] templates at protein/DNA ratios of 2:1 or lower. DNA polymerase-β is only slightly stimulated by this SSB. Based on its similar properties to UP1, this protein also most likely represents degraded hnRNP products.

The structure of a newborn rat brain helix-destabilizing protein has been examined using cDNA cloning (Cobianchi et al., 1986). Using a synthetic oligonucleotide probe corresponding to a 5 amino-acid sequence in the N-terminal region of the calf helix-destabilizing protein UP1, a cDNA library of newborn rat brain poly (A^+)RNA in a phage was screened. Positive clones were screened with a second probe corresponding to a 5 amino acid-sequence in the C-terminal region of calf UP1 and one of these positive clones was selected for study. In the cDNA a 988-residue open reading frame predicts a 34,215 dalton protein of 320 amino acids. Of the 195 amino acid residues of UP1, residues 2 through 196 of the rat brain protein are identical to UP1. A 124 amino acid sequence in the C-terminal portion of the rat brain protein is not present in the purified calf UP1 protein and the amino acid content of the 124 amino acid residues consists of 11% asparagine, 15% serine, and 40% glycine. Since this

protein shares high homology to UP1, it is likely that it is also degraded hnRNP products.

Dreyfuss et al. (1988) have reviewed hnRNP in eukaryotic cells. MRNA in eukaryotic cells arise from primary gene transcripts called heterogeneous nuclear RNAs (hnRNAs). HnRNAs are differentiated from other RNAs based on size, nuclear location, and the fact that the RNA polymerase that transcribes them is antibiotic sensitive (Dreyfuss et al., 1988). HnRNAs give rise via RNA polymerase II (Pederson, 1983) to pre-mRNA (Darnell, 1982) which contains 5' cap structures (M^7Gpp,an inverted guanosine cap), 3' polyadenylated tails, and intervening sequences that are later spliced out (introns) (Dreyfuss et al., 1988). This hnRNA is then translocated through nuclear pores and spliced mRNAs accumulate in the cytoplasm. HnRNP particles which consist of hnRNA with specific proteins bound to it can be sedimented in sucrose gradients as heterodispersed material between 30s and 250s (Billings and Martin, 1978; Choi and Dreyfuss, 1984) and at least 75% of hnRNA is associated with the 30s particle which as a monomer accommodates 500 $\pm$ 100 nucleotides of hnRNA (Choi et al., 1986). Following mild ribonuclease hydrolysis hnRNP particles are shown to sediment from 30-250s (Pederson, 1974). HnRNP is the form in which hnRNA exists in the nucleus (Samarina et al., 1968; Pederson, 1974; Kish and Pederson, 1975; Brunel and Lelay, 1979). The mechanism of transition from hnRNP (nuclear) to mRNPs (protein associated with

cytoplasmic mRNA) is unknown (Choi et al., 1986). What is known is that no proteins have been found that were bound to both mRNA and hnRNP and it is possible that hnRNP proteins actually process the pre-mRNA.

The 30s hnRNP particle is composed of anywhere from 75-80% protein to 85-90% protein, depending upon the type of determination employed (Billings and Martin, 1978). Direct determinations on pelleted or ethanol precipitated 30s RNP have given a composition of about 90% protein and 10% RNA. The 40s hnRNP particles in HeLa cells have been identified and characterized (Beyer et al., 1977) and 6 lower molecular weight polypeptides have been identified as the protein constituents of the 40s ribonucleoprotein complex. The 3 groups of closely spaced doublet proteins are termed group A (M_r 32,000 A_1/34,000 A_2), group B (M_r 36,000 B_1/37,000 B_2) and group C (M_r 42,000 C_1/44,000 C_2). The pI of group A proteins, 9.2(A_1) and 8.4(A_2), and B_1 are basic, while for B_2, C_1 and C_2 the pIs are acidic. The group A proteins are major nuclear proteins constituting 60% of the total hnRNP particle protein mass. The A and B group hnRNP proteins dissociate from hnRNA at 150 mM NaCl while the C proteins dissociate at 750 mM NaCl (Beyer et al., 1977). Others have isolated 35s hnRNP particles from HeLa cells and identified 9 core proteins (Wilk et al., 1985). Cross-species conservation was also demonstrated via peptide mapping of proteins A_1 and A_2 from bovine and human cells.

Two functions have been proposed for hnRNP proteins. The first is in the packaging of hnRNA, such that the hnRNA can be compacted into the nucleus (Dreyfuss et al., 1988) and the second is in splicing (removal of introns) of the pre-mRNA complex . (Choi et al., 1986). Monoclonal antibodies generated against hnRNP C_1 (41,000 M_r) and C_2 (43,000 M_r) proteins inhibit the splicing or removal of intervening sequences from pre-mRNA in vitro and prevent the formation of mature mRNA (Choi et al., 1986). Also, hnRNA is highly nuclease sensitive and the 60s complex with which C proteins are associated forms a multicomponent splicing complex (Frendewey and Keller, 1985; Brody and Abelson, 1985). Various hnRNP proteins have been sequence (Adam et al., 1986; Sachs et al., 1986; Grange et al., 1987; Kumar et al., 1986; Swanson et al., 1987; Haynes et al., 1987).

The total nuclear RNP fraction consists of the proteins associated with the hnRNA, snRNA, and pre-rRNA particles (Mattaj, 1984). The pre-ribosomal ribonucleoprotein particle (45S) is composed of 61% protein and 39% RNA (Kumar and Warner, 1972). The precursor rRNA (45S) is synthesized in the fibrillar portion of the nucleolus from which the RNA moves to the granular portion on the RNP particle (Olson and Busch, 1978) and then out to the cytoplasm (18S and 28S)(Warner and Soeiro, 1967). Other proteins known to contain methylated arginine residues in addition to hnRNP proteins (Beyer et al., 1977; Boffa et al., 1977; Karn et al., 1977) include histones

(Paik and Kim, 1980), HMG 1 and 2 (Boffa et al., 1979), and nucleolar proteins C23 (Lischwe et al., 1982; Lischwe et al., 1985b). It is believed that C23 is a component of the pre-ribosomal RNP particle (Olson and Thompson, 1983) and glycine and dimethylarginine clusters present in this 34,000 M_r protein may be characteristic of RNA-associated proteins (Lischwe et al., 1985a).

Rat brain ribonucleoproteins have been examined and shown to be released from RNA after treatment with 0.25 M and 0.4 M NaCl (Stevenin and Jacob, 1974). Electron microscopic examination of hnRNP particles from brain reveal folded strands with long fibrils containing regions of varying widths and densities (Stevenin et al., 1976). Brain hnRNA treated with ribonuclease release polypeptides ranging from 23,000-150,000 M_r (Stevenin et al., 1977). At low ribonuclease concentrations the hnRNP particles accumulate at 35-45S and proteins in the 30,000-38,000 M_r range are released. At higher ribonuclease concentrations, the total amount of protein at 35-45S decreases, however, proteins of M_r 30,000-38,000 predominate and at very high enzyme concentrations the particles are almost all hydrolyzed. Stevenin et al. (1977) also report that sequences of up to 200-300 nucleotides are protected from ribonuclease hydrolysis by the ribonucleoproteins. Based on characteristics of the hnRNP particles following ribonuclease treatment, investigators conclude that the native particles are polyparticles made up

of variable numbers of monoparticles (Samarina et al., 1968; Pederson, 1974). Gallinaro-Matringe et al. (1975) have examined hnRNP particles isolated from rat brain nuclei via a linear sucrose gradient centrifugation method and found 45 proteins to be associated with particles greater than or equal to 60S. A group of easily released species, with 75% and 95% being removed by 0.25 M NaCl and 0.7 M NaCl exists. This group contains 8 proteins between 29,000 and 39,000 M_r and the phosphorylated proteins bind more tightly to the RNA than do the nonphosphorylated proteins.

Relationship to enzymes of intermediary metabolism. Several mammalian SSBs have been shown to actually be enzymes of intermediary metabolism such as lactic dehydrogenase (LDH) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Helix destabilizing protein-1 (HDP-1) has been isolated from unfractionated mouse myeloma cells (Planck and Wilson, 1980). The extract was loaded onto DNA affinity columns at 50 mM NaCl (pH 8.8) and eluted from both ssDNA columns with 2 M NaCl. HDP-1, which preferentially binds ssDNA, is heterogeneous with respect to M_r (24,000-33,000), with the major species being of 27,000 M_r. HDP-1 possesses a homologous primary structure based on tryptic peptide mapping, lowers the T_m of poly [d(A-T)], and binds noncooperatively to ssDNA. Although 75% of HDP-1 is localized to the nucleus, it is not chromatin-associated. This protein is also distinct from high mobility

group proteins (HMG) 1 and 2, histones, and P8 SSB. Fragments of 19,000 to 24,000 M_r are produced following tryptic hydrolysis of HDP-1. In the presence of ssDNA, a 22,000 M_r fragment (22HDP) is produced by tryptic hydrolysis, while in the absence of ssDNA, a 19,000 M_r fragment (19HDP) predominates with both fragments binding ssDNA, suggesting that the NH_2-terminal end of HDP-1 is not necessary for binding to ssDNA. These investigators also point out that since HDP-1 recognizes RNA, some role in ribonucleoprotein metabolism may exist for this protein. HDP-1 is known to stimulate its homologous DNA polymerase-α (Detera et al., 1981) and amino acid sequencing of UP-1 and HDP-1 reveals a high degree of sequence homology between these two helix destabilizing proteins (Williams et al., 1985a). Antibody probing shows that HDP-1 actual has an M_r of 36,000-38,000 and during isolation proteolysis results in the formation of the 27,000 M_r protein and smaller fragments (Planck and Wilson, 1985). This mouse myeloma protein has been identified as a LDH (A) isoenzyme (Sharief et al., 1986). The protein possess LDH activity and rabbit antiserum prepared against it cross reacts with purified LDH-A isoenzyme. It has been suggested that the binding of these enzymes of intermediary metabolism to ssDNA is due to a fortuitous affinity for ssDNA via their nucleotide binding site (NAD for G3PDH or NADH for LDH) (Richter et al., 1986). However, it is also interesting to note that these proteins are eluted from the ss matrix at a very low salt concentration and

therefore, may be suspected as binding nonspecifically to ssDNA. A 34,000 M_r SSB has been isolated from a rat pheochromocytoma cell line (PC 12) (Biocca et al., 1984). Extracts were loaded onto DNA affinity columns at 50 mM NaCl and then subjected to ds/ssDNA affinity chromatography and proteins eluted with 2 M NaCl. The 34,000 M_r protein constitutes 0.5% of the total soluble proteins and consists largely of acidic vs basic amino acid residues. Fifteen percent of the protein is phosphorylated and this protein has an isoelectric point of 8.0. Synthesis of this 34,000 SSB is progressively inhibited following arrest of mitosis and neurite outgrowth induced by nerve growth factor. The authors suggest that control of synthesis of this SSB is related to activation of differentiation induced by nerve growth factor in this neoplastic cell line. This SSB has also turned out to be an enzyme of intermediary metabolism, LDH (Calissano et al., 1985). It cross-reacts with antibodies raised against rabbit LDH and antibodies raised against the 34,000 SSB also react with rabbit LDH. Furthermore, it was shown through immunocytochemical localization techniques and quantitative electron microscopy that LDH-SSB is located in the nucleus of various cell types, including enriched rat cerebellar glial cells and rat fibroblasts (Cattaneo et al., 1985). Since treatment with DNase resulted in a reduction in nuclear staining and treatment with RNase did not, this protein is most likely associated with chromatin in vivo and hence the

possibility of involvement of LDH-SSB in some nuclear functions exists.

A SSB termed P8 has been isolated from mouse 3T6 cells and human diploid fibroblasts (Tsai and Green, 1973). Cells were extracted and loaded on to a ssDNA affinity column at 0.05 M NaCl. P8 was subsequently eluted with 0.2 M NaCl. P8 fails to bind to dsDNA, however, since this protein elutes from ssDNA at such a low salt concentration it is questionable as to whether or not this represents a true SSB. Indeed, P8 has been purified to homogeneity and shown to be G3PDH (Perucho et al., 1977). The amino acid composition of P8 is very similar to that of G3PDH and the P8 protein possesses a high G3PDH activity.

Both SSB-37 and SSB-35 preferentially bind ssDNA (Grosse et al., 1986). SSB-35 stimulates DNA polymerase-α primer complex five fold whereas SSB-37 inhibits primase-initiated replication. Also, SSB-37 binds cooperatively to ssDNA and possess G3PDH activity, while SSB-35 possess LDH activity. The true identities of these two SSBs were confirmed by amino acid sequencing.

A rat liver SSB of M_r 30,000, isolated by ds/ssDNA affinity chromatography (loaded at 0.05 M NaCl) and ultragel AcA44 filtration has been identified as LDH-5 based on similar (148 of the 157 residues) amino acid compositions and HPLC tryptic peptide maps (Williams et al., 1985a). The specific activities of LDH and this helix-destabilizing protein are

also similar. The protein elutes from ssDNA-cellulose at a low salt concentration (0.15 M NaCl) and decreases the melting temperature of poly [d(A-T)-d(A-T)]. Since pre-incubation of this LDH-SSB protein with NADH blocks its binding to ssDNA, these workers suggest that NADH and ssDNA may be binding to the same site on the protein.

Relationship of rat liver SSBs to HMG proteins. A 25,000 M_r protein has been isolated from regenerating rat liver (Duguet and deRecondo, 1978). The extract was loaded onto ds/ssDNA affinity columns at 0.05 M NaCl and after rinsing with 0.15 M NaCl to remove nonspecifically bound proteins, the SSBs were eluted with 0.4, 0.8, and 1.4 M NaCl in a stepwise fashion. Proteins eluting with 0.4 M NaCl were subjected to phosphocellulose chromatography and from this matrix, the purified 25,000 M_r protein was eluted. This SSB is present at about 1 x 10^6 copies per cell and the amount of 25,000 M_r protein present in the nucleus is comparable to that of the cytosol, making the intracellular location of the protein difficult to determine. This protein also appears to be independent of regeneration and capable of lowering the melting point of poly [d(A-T)]. This 25,000 M_r protein unwinds double-stranded poly (A)-poly (U) and binds to supercoiled SV40 DNA, despite its poor affinity for dsDNA. Incorporation of deoxyribonucleotide by DNA polymerase-α in the presence of ss template is stimulated 3-fold by the 25,000 M_r protein and

the protein also stimulates the activity of homologous rat liver DNA polymerase-ß (DNA repair function) by 2-3 fold. It does not possess any deoxyribonuclease or ATPase activities and subsequently has been isolated from regenerating rat liver (HD25) and normal rat liver (S25). The S25 protein is present at 1 x 10^6 copies/cell, but unlike HD25 which exists at low ionic strength as a tetramer, S25 can only be isolated in a monomeric form. The pattern of proteolytic fragments is the same for HD25 and S25 between the basic species of the two, since in S25 the acidic band is found to be minor. The elution pattern from phosphocellulose is the same between these two proteins. However, unlike HD25, in the presence of S25, destabilization of poly [d(A-T)]-d(T-A)] does not occur, but rather stabilization is reported. Furthermore, S25 is found to inhibit DNA polymerase-α, in contrast to HD25. A slight stimulation of DNA polymerase-ß is detected in the presence of poly [d(A-T)]-d(T-A)]. DNA polymerase-ß activity is not inhibited by S25 on a poly (dC)-oligo (dG) template. HD25 which contains the acidic subspecies is only synthesized in dividing cells, and can unwind dsDNA and stimulate homologous DNA polymerases-α and ß, while the same protein synthesized by normal rat liver (S25) does not possess these functions, since it contains only the basic subspecies. It was later shown that S25 induces a decrease in the linking number of DNA as a function of the protein/DNA ratio, such that it appears negatively supertwisted after removal of the

protein (Duguet et al., 1981). Electron microscopy reveals that complexes of S25 with SV40 are beaded structures that resembled minichromosomes. Various analyses have shown that the true identity of S25 and HD25 is actually the nonhistone protein HMG 1 (Bonne et al., 1982). HMG proteins are discussed at the latter part of this dissertation.

Survey of other mammalian SSBs. An examination of mouse acities cells has uncovered a SSB of 30,000-35,000 M_r in the combined cytosolic and chromatin fractions (Otto et al., 1977). Extracts were loaded onto ds/ssDNA affinity columns linked in tandem and proteins eluted from the single-stranded column in a stepwise manner with 0.25 M, 0.5 M and 1 M NaCl. Proteins eluting between 0.25 M and 0.5 M NaCl were pooled and further purified. The isolated SSB binds RNA and stimulates DNA polymerase-α up to a protein/DNA ratio of 6. At higher ratios, the protein inhibits the polymerase-α. The binding site size of this SSB was 6-10 nucleotides long and following phosphorylation of the protein, although binding to ssDNA did not change, stimulation of the DNA polymerase-α was inhibited. Further characterization of this protein has not been performed and thus the true nature of this protein remains speculative.

SSB-48 (M_r 48,000) has been isolated from mammalian Novikoff hepatoma cells (Koerner and Meyer, 1983). Extracts were subjected to a number of fractionation steps including

ssDNA affinity chromatography. The extract was loaded onto single-stranded column at a low salt concentration (20 mM Tris-HCl (pH 8.0) - 5mM ß mercaptoethanol - 1mM EDTA - 10% glycerol). The single-stranded column was eluted with a linear 0 to 0.35 M NaCl gradient and SSB-48 eluted at 0.12 M NaCl. This protein binds cooperatively to ssDNA, stimulates DNA polymerase B, and destabilizes dsDNA. SSB-48 exists as a globular monomer and the amino acid composition reveals a low lysine and arginine content (unlike histones), minimal cysteine (like other SSBs), and a high content of serine and glutamic acid (unlike other SSBs). However, since SSB-48 was not isolated by first passing the extract over a double-stranded DNA matrix and is eluted from the ssDNA column at such a low salt concentration, it is questionable as to whether or not this protein actually represents a true single-strand specific binding protein. Another protein, termed R protein (M_r 33,000-35,000), isolated via extraction of the lipoprotein fraction of spermocyte enriched nuclei, was isolated in the absence of a double-stranded DNA column and protein extracts were loaded onto the ssDNA affinity column at a low salt concentration of 2.5-5.0 mM KCl (Mather and Hotta, 1977). SSBs were eluted with 2 M NaCl and further purification was performed on an ion exchange resin. R-protein promotes reannealing of denatured DNA strands in the presence of Mg^{+2}, while in the absence of $Mg+^{2}$ this protein promotes unwinding of duplex DNA. When phosphorylated, R-

protein losses its ability to bind to or reanneal denatured DNA. This same protein is found in a reduced amount in rat liver. Although no further characterization of this protein has taken place, most likely does not represent a true SSB since a dsDNA column was not employed in its isolation and a low salt concentration was used in loading the protein on the ssDNA column.

HeLa cell C1 factor has been partially purified from postmicrosomal supernatant solutions via ds/ssDNA affinity chromatography and other procedures (Novak and Baril, 1978). C1 was loaded onto the DNA affinity columns at 50 mM NaCl and eluted from the ssDNA matrix with 0.4 M NaCl. This protein has a M_r between 30,000-35,000, stimulates HeLa cell DNA polymerase-α by 15-30 fold, and only moderately stimulates HeLa DNA polymerase-β and gamma, rat liver DNA polymerase-α and β, E. coli DNA polymerase I, and B. subtilis DNA polymerase III. C1 also does not unwind poly [d(A-T)] and native Clostridium perfringens DNA. This protein has not been further characterized and whether or not it represents a true SSB or degraded hnRNP products is unknown.

The intermediate filament protein vimentin has been shown to bind rRNA (Traub and Nelson, 1982) and ssDNA (Traub et al., 1983). Affinity of vimentin for RNA is abolished at 125 mM KCl, while for ssDNA at 220 mM KCl a 50% binding efficiency is present and at 300 mM KCl approximately 25% of the vimentin still binds the DNA. This is still, however, a low amount of

salt. The cooperative binding of vimentin to ssDNA is optimum at 200 mM KCl and vimentin also binds dsDNA. Despite the low salt conditions necessary to inhibit the binding of vimentin to ssDNA and the fact that this protein binds to dsDNA, these workers hypothesize that vimentin may be involved in transcription. Also, a 68,000 and a 145,000 M_r neurofilament protein isolated from porcine spinal cord have considerably higher affinity for denatured DNA than for rRNA (Traub et al., 1985). Native DNA is a weak competitor for the proteins and the binding of these proteins to ssDNA is cooperative.

Rat brain SSBs. Heizmann et al. (1982) have examined total nuclear SSBs in differentiating rat brain cortex and cerebellar neurons. Isolated nuclei were extracted with 0.6 M KCl and proteins were ^{14}C labeled by reductive methylation. This extract was then desalted by Sephadex G-25 chromatography, fractionated via sequential ds/ssDNA affinity chromatography, with the extract being loaded onto the DNA affinity columns at 0.05 M NaCl (pH 8.8). These columns were separated, eluted with 2 M NaCl, proteins eluting from the ssDNA column pooled (based on radioactivity), precipitated with trichloroacetic acid (20% w/v final), and centrifuged. The protein pellet was then dissolved in lysis buffer and subjected to two-dimensional gel electrophoresis. In prenatal cortical neurons, the SSB pattern is described as being flat and streaky, becoming more accentuated over time up to 7 days

(cortical neurons essentially stop dividing at birth). Proteins of pI 6.1-7.0, M_r 32,000-43,000 and pI 7.2 to greater than 8, M_r 35,000-45,000 show pronounced developmental fluctuations as do two perinatal proteins of pI 7.1-7.4/M_r 60,000 present only at fetal stages and pI 7.4-7.9/M_r 66,000 protein appearing rapidly after birth. In cerebellar neurons, proliferating to nonproliferating and thus undifferentiated to differentiated neurons extend over a span of 3 weeks starting at postnatal day 3 and ending on postnatal day 20. Alterations take place in a region defined by pI 6.1-7.2/M_r 30,000-45,000. At postnatal day 14, 30 clear spots appear whereas at prenatal day 3, 4 poorly defined streaks are present. Of special interest to these investigators are two microheterogeneous proteins of M_r 35,000 and 38,000 pI 7.1 to > 8.0 which appear in both cortex and cerebellar neurons following the arrest of cell division in each cell type. Developmental studies were not performed on glial SSBs since with this cell type the transition from a proliferative to a nonproliferative state extends over a long period and overlaps with the time of differentiation. However, in whole glial nuclei a SSB protein of pI 5.4/M_r 48,000 is present exclusively in this cell type and a SSB of pI 6.5-7.3/M_r 44,000 is found exclusively in the liver. The SSB of pI 7.2 to > 8/M_r 35,000 and 38,000 present in neurons, are also found to be present in the glial and liver nuclei. Based on these findings it is postulated that at least some of the developmental changes

observed reflect fluctuations in SSBs which may be involved with control of cell proliferation or gene expression. Whether these proteins are arising from DNA or whether they represent hnRNP products was not addressed.

Rat liver mitochondrial SSB. Rat liver mitochondrial SSB P16 has been purified and characterized (Pavco and Van Tuyle, 1985). Extracts were mixed by gentle rotation with ssDNA agarose at 0.5 M NaCl (pH 7.4) and the ssDNA agarose was then packed into a column and protein eluted with 7% (wt/vol) NH_4OH pH 12.2. The peak absorbance was pooled, concentrated, and subjected to alkaline CsCl isopycnic gradient centrifugation (final pH 13.3) to separate the protein from DNA. Gradients were manually fractionated from the top into 1 ml fractions, adjusted to pH 7 with 1.0 M HCl, and dialyzed against 0.2 M NaCl(pH 7.4). The partial amino acid composition of P16 reveals that the highly charged nature of this protein is due to the high content of Asx, Glx, and Arg. However, it is assumed that about half of the glutamic acid and aspartic acid residues reported are in the amide form prior to hydrolysis since the isoelectric point of P16 has previously shown to be 7.6-7.8 (Van Tuyle and Pavco, 1981). Tryptophan, cysteine, and proline were not included in the compositional analysis. A protease-resistant fragment of M_r 6,000 that retains the capacity to bind ssDNA is produced by digestion of DNA-bound P16 with proteinase K. In addition to the high content of the

charged amino acids Asx, Glx, and Arg, several amino acids were present in the 6,000 M_r fragment in disproportionately high concentrations and include serine, glycine, tyrosine, phenylalanine, arginine, and a single histidine residue. The aromatic amino acids tyrosine and phenylalanine are directly involved in the binding of T4 gp 32 to ssDNA. When P16 is incubated with rRNA, ssDNA, and dsDNA, there is no indication that P16 binds to either dsDNA or RNA and direct electron microscopic examination of complexes prepared by mixing purified P16 with ssDNA reveals thickened irregular fibers characteristic of protein-associated ssDNA complexes. The strong affinity of P16 for ssDNA was exemplified by the harsh method required to elute P16 from the ssDNA column (NH_4OH, pH 12.2) as 8 M urea and 5 M NaCl are only partially effective. Greater than 90% of this SSB is localized to the mitochondria with only trace amounts being present in nuclear and cytoplasmic fractions. It has also been reported by these investigators that P16 is synthesized on cytoplasmic ribosomes since cycloheximide, but not chloramphenicol inhibits translation of this protein. The protein is then transported by an unknown mechanism into the mitochondria. P16 functions to stabilize D-loop structures by protecting the nascent strand of the D-loop from branch migration upon parental strand cleavage <u>in vitro</u> (Van Tuyle and Pavco, 1981). P16 also binds exclusively to the displaced ss of normal and expanded D-loops and to the single strand gap segments of

molecules with the characteristics of B-gapped circles (Van Tuyle and Pavco, 1984). Forty-nine P16 molecules are present per mitochondrial DNA in the bound population composed predominantly of D-loop DNA and it was concluded that P16 functions in all stages of the asymmetrical replication cycle of mitochondrial DNA (Van Tuyle and Pavco, 1984). P16 is believed to be analogous to Xenopus mitochondrial 15,500 M_r SSB (Mignotte et al., 1985) which shares amino acid sequence homology with E. coli SSB (Mignotte et al., 1988), making P16 a true mammalian SSB. This would not be surprising since mitochondria are thought evolutionarily to represent prokaryotic cells which have been captured by the cytoplasm of eukaryotic cells (Attardi et al., 1975; Slater, 1981).

SSDNA-DEPENDENT ATPASE ACTIVITIES in MAMMALIAN CELLS

Single-stranded DNA dependent ATPase activities isolated from prokaryotic cells bind ssDNA and play pivotal roles in DNA recombination via the formation of D-loops (Riddles and Lehman, 1985; Julin et al., 1986). This type of ATPase activity has been isolated from a number of mammalian and even human cells and adds further credence to the existence of SSBs in mammalian cells.

Mammalian ssDNA-dependent ATPases. This activity found in monkey tissue culture cells has been shown to be different from the T-antigen ATPase activity based on its elution from

phosphocellulose, low salt/high affinity for ATP relationship, and stimulation of the ATPase activity by specifically ssDNA (Brewer et al., 1983). The ATPase activity is dependent on ssDNA and a divalent cation (Mg^{+2}, Mn^{+2}, or Ca^{+2}). Also, superhelical (Form I) SV40 DNA is a substrate for ATPase binding, while relaxed Form I nicked circular (Form II), and double-stranded linear SV40 DNAs are not.

A ssDNA-dependent ATPase activity has been isolated from mouse myeloma (Hachmann and Lezius, 1976). This enzyme has a M_r of 100,000 as determined from its sedimentation coefficient and a pI of 6.5. The ssDNA-dependent ATPase catalyzes the conversion of ATP to ADP and orthophosphate. Although ribonucleotides are also hydrolyzed, the K_m values for these substrates are much higher. UTP, CTP, and dCTP are inhibitory at higher concentrations and divalent cations, Mg^{+2} or Mn^{+2}, are required for the reaction. The enzyme exhibits a broad pH optimum from pH 5.5 to 8.0 and is most active between 0 and 150 mM KCl, with 250 mM KCl being 50% inhibitory. This ATPase is also dependent on the presence of ssDNA, with the rate of ATP hydrolysis increasing as a function of an increasing DNA concentration. Natural RNA, ss and ds synthetic polyribonucleotides do not to activate the enzyme.

A DNA-dependent ATPase activity has also been isolated from bovine retropharyngeal lymphocytes (Otto, 1977). Through competition experiments it is evident that all 3 ATPases

cleave ATP and dATP preferentially over other nucleotides and all activities are dependent on the presence of ssDNA and Mg^{+2} ions. The K_m and optimum ionic strength varies between the three ssDNA-dependent ATPases. Also, ATPase I is recovered only from proliferating lymphocytes, ATPases II and III are recovered from G_o-arrested cells, and ATPases II and III activities are also isolated in a 5 fold increase from proliferating versus resting lymphocytes. The author suggests that these enzymes may play a role in the replication process and recombination.

Another ssDNA-dependent ATPase activity has been fractionated from Novikoff hepatoma cells (a mammalian acities cell tumor) (Thomas and Meyer, 1982). The M_r of the ATPase is 65,000 and based on sedimentation studies the protein is believed to exist as a dimer of two equally-sized and probably identical subunits. This enzyme hydrolyses ATP or dATP to ADP plus Pi without the production of AMP. The ATPase requires a divalent cation (Mg^{+2} or Mn^{+2}), has a broad pH optimum of 7.0 to 10.0, and is unaffected by salt up to 20 mM. The rate of hydrolysis of ATP is linear up until approximately 50% of the substrate is hydrolyzed and ATPase activity is directly proportional to the amount of enzyme added up to 200 units/25 ul assay. ATP and dATP are utilized equally as well by the ATPase, while other ribonucleoside triphosphates and dexoyribonucleoside triphosphates are poorly utilized. The enzyme also has an absolute requirement for a polynucleotide

effector with both duplex DNA and ds calf thymus DNA supporting the reaction. Single-stranded DNA supports the reaction most efficiently and synthetic polynucleotides with the exception of poly (dT) are less effective than denatured DNA. High concentrations of SSBs and ADP inhibit the ATPase activities. The DNA-dependent ATPase activity is able to stimulate DNA polymerase ß, but not α and upon addition of SSBs, polymerase ß is stimulated to a greater extent than could be observed with the two individual proteins. It is suggested that this protein plays a role in DNA repair.

Human ssDNA-dependent ATPases. Cobianchi et al. (1978) have isolated a ssDNA-dependent ATPase from the human heteroploid cell line EUE. The activity is dependent on ssDNA, has an optimum pH of 6, and requires Mg^{+2} or Ca^{+2} for activity. Mn^{+2} is ineffective in promoting the ATPase activity. Based on gel-filtration and sedimentation in glycerol gradients, a M_r of 110,000 has been estimated for this enzyme. The enzyme is reported to split ATP into ADP and Pi, without the production of AMP and to bind equally as well to ds as opposed to ssDNA, with hydrolysis of ATP only occurring in the latter situation. This protein is unable to unwind DNA-RNA structures, but can at least partially unwind dsDNA from the 3' end. It also does not stimulate DNA polymerase-α on activated (gapped) DNA. However, it can do so on poly [d(A-T)] and supercoiled DNA.

Another ssDNA-dependent ATPase activity has been isolated

from human KB cell nuclei (Boxer and Korn, 1980). The single protein species possessing the ssDNA-dependent ATPase activity has a M_r of 75,000 as determined by SDS polyacrylamide gel electrophoresis and a pI of 8.5 by two-dimensional electrophoresis. The enzyme converts ATP to ADP and Pi without the generation of an AMP and the rate of ATP hydrolysis is known to be directly proportional to the enzyme concentration. Although no other NTPs or dNTPs are hydrolyzed by the enzyme, all of them appear to be competitive inhibitors of the reaction, with ADP and AMP being the most potent inhibitors. The ATPase requires a divalent cation (Mg^{+2}, Mn^{+2}, or Ca^{+2}) for activity and exhibits a pH optimum of 6.8-8.8. The ATPase is resistant to salt up to concentrations of 200 mM and denatured calf thymus DNA, closed circular single-stranded M13 DNA, poly (dT), poly (dA), and poly (dC) are all equally effective in supporting the ATPase activity. Polyribonucleotides and yeast RNA are essentially unable to support the ATPase activity, as is intact dsDNA. Both singly nicked PM2 form II DNA and blunt-ended duplex DNA molecules generated by Hae II restriction endonuclease digestion support ATP hydrolysis. Also, no unwinding activity has been demonstrated by the enzyme, however the enzyme is known to stimulate incorporation of dNMPs by DNA polymerase-α and β in the presence of ATP. It has been concluded that this DNA-dependent ATPase activity most likely plays a role in DNA replication.

A DNA-recombinogenic activity has been isolated in human skin fibroblasts from patients suffering from Bloom's syndrome (Kenne and Ljungquist, 1984). Bloom's syndrome represents a chromosomal instability syndrome in which patients are believed to be defective in DNA repair and replication. An increase in all forms of cancer, especially leukemias and various carcinomas, are known to be associated with this syndrome. A filter binding assay, used to measure recombinogenic activity, involved joint molecule formation from supercoiled DNA and fragments of homologous viral DNA binding to a nitrocellulose filter. The recombinogenic protein catalyzes the homologous pairing of a single-stranded fragment and the complementary strand in duplex DNA producing a D-loop. Treatment with trypsin results in a loss of recombinase activity which indicates that the activity is due to a protein and replacement with non-homologous DNA results in a loss of activity. Requirements for the formation of D loops include Mg^{+2} and ATP for this enzyme. Inhibition of the reaction is produced with even low concentrations of NaCl (30 mM NaCl) and gel filtration and sucrose gradient centrifugation reveals a protein of M_r 15,000-20,000. Normal fibroblasts display a lower recombinogenic activity than is found in the Bloom's syndrome fibroblasts, suggesting that the increase in cancers associated with Bloom's syndrome may be due to an increase in this recombinogenic activity. The recombinogenic activity is believed to be analogous to that

of the E. coli recA protein's ability to induce D-loop formation in DNA.

Evidence that homologous recombination is mediated by extracts prepared from a human bladder carcinoma has been obtained (Kucherlapati et al., 1985). Two non-complementing and non-reverting deletion plasmids of a phosphotransferase gene conferring resistance to neomycin were incubated with cell extracts. This mixture was used to transform recombination-deficient recA E. coli cells. A 100 to 1000 times greater recombination frequency is observed with the use of the carcinoma extract as opposed to without it. This recombination activity is dependent on riboadenosine 5'-triphosphate, Mg^2, and dNTPs. Also, examination of DNA from neomycin resistant colonies shows that a substantial proportion of the plasmids are dimers. This coupled with the fact that recA deficient strains of E. coli are unable to form dimer molecules suggests that homologous recombination has taken place. It was concluded that mammalian somatic cells in culture have the ability to catalyze homologous recombination in vitro.

Another recombinase has been partially purified from human B lymphoblasts (Hsieh et al., 1986). Recombinase activity was followed by monitoring formation of joint molecules. The incubation of linear duplex DNA and homologous single-stranded circular DNA in the presence of the recombinase activity results in the formation of a product

judged to be identical by agarose gels to that of the product of the purified recA protein when incubated under the same conditions. Since the product does not form upon incubation of each DNA substrate along with the recombinase activity, it was concluded that the product represents joint molecules. Strand transfer is unaffected by RNAses, suggesting that RNA does not play a role in joint molecule formation. Joint molecule formation is dependent on Mg^{+2} and ATP, while ATP analogs such as AMP inhibit formation. Exonuclease and reannealing do not play a role in joint molecule formation and the recombinase does not carry out any DNA synthesis. The recombinase does carry out strand transfer in a homology-dependent manner as non-homologous DNA substrates fail to produce joint molecules. Electron microscopy reveals a 1:1 stoichiometry of circular single-stranded DNA and linear double-stranded DNA as the major product of strand transfer. It is known that the human recombinase, like recA protein, exhibits a stoichiometric dependence on single-stranded DNA in forming joint molecules by strand transfer. A displaced strand exists for ssDNA, but does not interfere with the formation of joint molecules. Also, the recombinase initiates strand transfer at the free ends of linear duplex DNA and strand displacement proceeds exclusively in a 3' to 5' direction, while the recA protein carries out strand displacement in a 5' to 3' direction. The authors conclude that <u>in vitro</u> this enzyme is involved in recombination.

The ability of mammalian cells to participate in homologous recombination has also been examined by Rauth et al. (1986). Double-stranded pSV2 Neo deletion mutants were mixed with single-stranded MSX phage DNA and transfected with a human bladder carcinoma cell line by the calcium phosphate coprecipitation method. Cells containing the wild-type Neo gene were selected for by growth on antibiotic containing media. Recombination was recovered and for molecular analysis similar sets of substrates were cotransfected into monkey cos cells. DNA isolated from the transfected cells was used to transform recA deficient E. coli and recombination measured as a function of kanamycin resistant colonies. Passage of the intact Neo gene confers kanamycin resistance to the bacteria, and this is achieved following passage of the DNA through cos cells. The number of recombinants recovered is higher when ds deletion mutant plasmid substrates are used as opposed to ssDNA plasmids. To test the ability of single-stranded DNA to participate in recombination, ssDNA was mixed with circular or linear ds pSV2 Neo deletion mutant phage and incubated with mammalian nuclear extracts. Both combinations result in recombinant products (kanamycin resistant colonies) and ssDNA is at least as efficient as its ds counterpart in generating recombinants. The presence of nicks in ss and especially dsDNA substrates results in increased frequencies of recombination as compared to the unnicked substrates. These investigators point out that the mammalian recombination

enzymes are expected to all be DNA-binding proteins and this being the case, addition of excess non-homologous DNA would be expected to inhibit the homologous pairing reaction. This is indeed the case, with non-homologous dsDNA inhibiting the reaction better than ssDNA which has only a slight affect under the conditions they employed. It was concluded that this enzyme is similar to the recA protein and is responsible for ssDNA functioning in homologous recombination by mammalian cells in vivo or by nuclear extracts in vitro.

HMG 1 and 2

General properties. High mobility group proteins 1 and 2 (HMG 1 and 2) represent a class of nonhistone proteins thought to be associated with chromatin (Johns, 1982). Some properties of these two HMG proteins have been reviewed by Johns (1982) and include extractability from chromatin with 0.35 M NaCl, solubility in 2% (w/v) trichloroacetic acid, and these proteins have molecular weights of approximately 26,500 for HMG 1 and 26,000 for HMG 2. Both proteins are composed of 25% basic and 25% acidic amino acids and contain a high amount of proline (7% or more). Amino acid sequencing has also shown that HMG 1 and 2 are highly homologous, with the carboxyl half of HMG 1 containing an unbroken run of 41 glutamic acid and aspartic acid residues, and the carboxyl half of HMG 2 containing a 41 residue peptide with a continuous sequence of

35 glutamic acid and aspartic acid residues. Two-dimensional electrophoresis of HMG 1 from calf thymus reveals a smear (since at neutral pH HMG 1 partially aggregates and precipitates) from pI 6.0-7.7 with some discrete spots, while HMG 2 has been shown to have 5 subfractions of pI 7.0-8.4 (Johns, 1982). HMG 1 and 2 are also selectively released from nuclei following treatment with DNase I under conditions which also preferentially degrade DNA sequences involved in the production of polyadenylated mRNA, suggesting that HMG 1 and 2 are associated with active genes (Levy et al., 1977; Einck and Bustin, 1985) and are structural proteins in active chromatin (Johns, 1982). Furthermore, HMG 1 and 2 have been shown to stimulate transcription by RNA polymerase (Stoute and Marzluff, 1982; Tremethick and Molloy, 1986; Tremethick and Molloy, 1988; Watts and Molloy, 1988; and Yang-Yen and Rothblum, 1988) and HMG 1 mediates nucleosome assembly in vitro (Bonne-Andre et al., 1984). HMG 1 inhibits the formation of Z DNA in negatively supercoiled plasmid DNA (Waga et al., 1988) and binds A-T DNA sequences (Elton et al., 1987).

Calf thymus HMG 1 and 2 have been reported to bind preferentially to ssDNA as opposed to dsDNA when 0.2 M NaCl extracts are loaded onto DNA affinity columns linked in tandem (Isackson et al., 1979). At lower salt concentrations (0.05 M and 0.1 M NaCl) these proteins bind to the dsDNA column. It has been reported that HMG 1 elutes from dsDNA affinity

columns at 0.08 M NaCl, and although 0.2 M NaCl removes all of the HMG 1 and 2 from dsDNA affinity columns, this salt concentration does not completely remove these proteins from chromatin (Isackson et al., 1981). These investigators speculate that approximately ≥50% of HMG 1 and 2 proteins remain bound to ss regions present in chromatin and indeed mouse myeloma nucleosomes have been shown to contain 35-40% ssDNA with stoichiometric amounts of HMG 1 and 2 when isolated from unfractionated nuclei (Jackson et al., 1979). Bidney and Reeck (1978) have also shown that HMG 1 and 2 bind preferentially to ssDNA. These workers employed 0.75 M NaCl in the extraction of chromatin from hepatoma cells and subjected the extract to Sepharose Cl-2B and Bio-Rex 70 chromatographies. Fractions were then pooled and loaded at 0.05 M NaCl onto ds/ssDNA affinity columns linked in tandem and two proteins eluting in a stepwise manner with 0.5 M NaCl were examined. These investigators conclude that HMG 1 and 2 which bind preferentially to the ssDNA affinity column are analogous to calf thymus HMG 1 and 2 proteins based on their electrophoretic mobility, salt concentration (0.35 M NaCl) at which they dissociate from chromatin, solubility in 2% TCA, insolubility in 10% TCA, and amino acid composition. Russnak et al. (1988) through the use of band competition assays have shown that Friend erythroleukemic mouse cell HMG 1 binds A+T-rich sequences 3' to the coding regions of various genes (dsDNA). These investigators also report that HMG 1 does not

bind ssRNA containing these high affinity binding sites as determined by the absence of formation of ribonucleoprotein complexes.

Destabilization of native DNA. It has been suggested that since HMG 1 and 2 preferentially bind ssDNA, it is likely that these proteins would be able to unwind or destabilize dsDNA (Isackson et al., 1979). Indeed, it has been shown, based on analysis of amino acid composition, electrophoretic mobility, and tryptic peptide mapping that S25 and HD25 proteins isolated from normal and regenerating rat liver respectively, are in fact HMG 1 (Bonne et al., 1982). As discussed earlier, HD25 destabilizes dsDNA and stimulates DNA polymerase-α and β, while S25 does not (Duguet and deRecondo, 1978 and Duguet et al., 1977). These investigators included among other things, Mg^{+2} in their DNA melting assay. S25 has subsequently been shown to interact with supercoiled DNA to form a beaded structure (Bonne et al., 1980). In the DNA of SV40, negative superhelical turns equivalent to the number of beaded structures observed are introduced by the S25 or HMG 1 protein from normal rat liver (Duguet et al., 1981). HMG 1 and 2 have also been shown by another group of workers to change the ds structure of DNA (Javaherian et al., 1978) and to destabilize the ds helix of DNA (Javaherian et al., 1979). Reports from these workers suggest that in the absence of NaCl, HMG 1 and 2 actually stabilizes DNA. However, with the addition of NaCl

from 0.025 M to 0.075 M and the proper protein to DNA ratio, HMG 1 and 2 facilitates DNA melting. At a high ratio of HMG 1 to DNA (>2), scattering increases considerably, making the spectrophotometric analysis of DNA melting difficult. These investigators did not include Mg^{+2} in their thermal denaturation buffer. Several other investigators also report HMG 1 capable of unwinding duplex DNA (Brown and Anderson, 1986; Sheflin and Spaulding, 1989).

While some investigators reported that HMG 1 and 2 unwinds DNA, others have presented data to the contrary. Yu et al. (1977) have isolated HMG 1 and 2 from calf thymus by 0.35 M NaCl extraction of chromatin and via spectrophotometric analysis of DNA melting show an increase in the melting temperature of DNA following the addition of HMG 1 and 2 proteins. Mg^{+2} was not included in their melting buffer.

In an attempt to determine whether or not HMG 1 destabilizes or stabilizes dsDNA, Butler et al. (1985) have examined the interactions of HMG 1 with DNA. These investigators prepared HMG 1 using standard techniques and via UV absorbance spectroscopy they report finding that in ionic concentrations of less than 25-30 mM Na^{+}, HMG 1 does indeed stabilize DNA against thermal denaturation. Above 30 mM Na^{+} it appears as though HMG 1 is a helix-destabilizing protein since addition of HMG 1 decreases the temperature at which DNA alone melts. However, the authors state that interpretation of the denaturation profile is difficult due

to turbidity above the transition temperature caused by protein denaturation and aggregation. By heating HMG 1 alone it was demonstrated that this protein under goes thermal denaturation between 55-65°C, which lead to the increase in hyperchromicity observed. These workers conclude that the apparent destabilization of DNA by HMG 1 found by others is in fact an artifact caused by aggregation of the protein with resultant light scattering above 56°C. A similar conclusion has been reached by Marekov et al. (1984). Neither group of workers employed Mg^{+2} in their melting buffer.

Makiguchi et al. (1984) reported that HMG 1 and 2 isolated from pig thymus chromatin were Mg^{+2} binding proteins and have shown spectrophotometrically that these proteins unwind dsDNA in the presence of Mg^{+2} (or Ca^{+2}) cation, at low protein-to-DNA ratios (<0.05). This destabilization is not due to the removal of Mg^{+2} from DNA by these two proteins. Furthermore, at higher protein-to-DNA ratios (>0.5) and in the presence of Mg^{+2} HMG 1 and 2 increase the melting temperature of DNA and thus stabilize DNA. The melting temperature of the control DNA used was 56° C, suggesting aggregation of HMG proteins is not responsible for the increase in hyperchromicity, since the DNA melts at less than 56° C following the addition of HMG 1 and 2.

Cellular location. It is generally believed that HMG 1 and 2 are bound to active chromatin or nucleosomes (Weisbrod and

Weintraub, 1979; Johns, 1982; Einck and Bustin, 1985). However, several investigators whose work led to this conclusion failed to first separate the nucleoplasm fraction from the chromatin fraction while isolating HMG 1 and 2 proteins. As suggested by Peterson and McConkey (1976), if nuclei are not first washed with 0.075 M NaCL prior to chromatin isolation, cross contamination of chromatin by nucleoplasmic HMG proteins can occur. This appears to be the case in several reports localizing HMG 1 and 2 to nucleosomes (Goodwin et al., 1977; Seyedin and Kistler, 1979; Jackson et al., 1979). Smith et al. (1978) have immunized rabbits with purified pig thymus HMG 1 and 2 and produced antisera which reacts with HMG 1 and 2 from pig thymus. This antisera does not react with any of the calf thymus HMG proteins nor does it react with pig thymus HMG 14 and 17. However, the antisera does react nonspecifically with histones 2A, H2B, H3 and H4. Thus, positive immunochemical staining of chromatin by the HMG 1 and 2 antisera may reflect the presence of histones rather than HMG 1 and 2, especially since the ratio of HMG to DNA in nuclei is only 0.03:1 and 1:1 for histones to DNA (Johns, 1982). Some staining of the nuclear membrane and nucleolus also occurs and is deemed artifactual.

Using red cell mediated microinjection, Rechsteiner and Kuehl (1979) introduced ^{125}I-labeled HMG 1 into the cytoplasm of HeLa and bovine fibroblasts and through autoradiography have shown the rapid movement of HMG 1 into the nucleus. It

also appears to these investigators that HMG 1 concentrates in condensed chromosomes of miotic cells. However, since visualization was performed at 1000X (light microscope) instead of with an electron microscope, it is less convincing that HMG proteins are actually associated with chromatin and just not present in the surrounding nucleoplasm. These investigators do, however, demonstrate that the association of HMG 1 with nuclei is dynamic as ^{125}I-HMG 1 moves out of the nucleus and through the cytoplasm of one cell into the cytoplasm and then to the nucleus of another cell.

HMG 1 has been isolated from the nucleoplasm of mouse liver nuclei previously washed in a 0.075 M NaCl - EDTA solution (Comings and Harris, 1976b). After centrifugation, HMG 1 was identified in the supernatant based on M_r and electrophoretic mobility and extraction of the pelleted chromatin with 0.35 M NaCl did not produce any significant amounts of HMG 1. Also, since only trace amounts of HMG 1 were found in the cytoplasm, it was concluded that this protein truly is enriched in the nucleoplasm, and not the chromatin fraction of rat liver.

Conner and Comings (1981) have purified HMG 1 from the 0.075 M NaCl - EDTA wash of mouse liver nuclei. The molecular weight, migration in two-dimensional electrophoretic gels, and the first 9 residues of the NH_2-terminal region are identical to the calf thymus HMG 1 protein. Also, both the calf and mouse HMG proteins are soluble in 2% TCA and the mouse HMG 1

protein cross-reacts with anti HMG 1 antibody produced against calf thymus HMG 1 protein. Examination of the rest of the amino acid sequence reveals that the mouse HMG 1 protein is more acidic, has a higher content of serine, glutamic acid, and aspartic acid, and a lower content of lysine than the calf thymus protein. A curious finding is that although the mouse liver HMG 1 protein can be isolated from the nucleoplasm following 0.075 M NaCl washing of chromatin, the calf thymus HMG 1 protein cannot be isolated in this manner as it remains bound to chromatin up to 0.35 M NaCl. These investigators speculate that if these proteins are indeed bound to chromatin, the structure of chromatin may determine their extractability. Liver, which is metabolically more active than thymus is expected to have more of its chromatin in an active extended conformation, which may be more accessible to solvents. Another explanation may involve the amount of ssDNA present in the chromatin of the two cell types. If more ssDNA is present in the thymic chromatin than the liver chromatin, then the thymus HMG is more apt to have a greater affinity for the chromatin and thus bind to it _in vivo_, while the liver HMG 1 protein would be present in the nucleoplasm _in vivo_ if the liver chromatin has less ssDNA regions. In any event, these investigators conclude that HMG 1 is present in the nucleoplasmic fraction of liver nuclei and chromatin fraction of calf thymus nuclei.

Matthew _et al._ (1979) have analyzed rabbit thymus HMG

proteins isolated from nucleosomes via micrococcal nuclease treatment of whole nuclei. They conclude that up to 50% of HMG 1 and 2 is weakly bound to chromatin since these two proteins can be removed from nuclei with 0.14 M NaCl. Mathew et al. (1979) speculate that 2 populations each of HMG 1 and 2 exist, one population which is fairly loosely bound and can be readily removed from chromatin and a second population which is firmly bound and not readily removed from chromatin with the saline-EDTA wash. It is also possible that the HMG 1 and 2 are actually binding to ssDNA regions on chromatin either endogenously present or induced during disruption of nuclei. The amount of ss regions may determine how much HMG is present in the nucleoplasm verses bound to chromatin. HMG 1 may also be binding to H1 in chromatin. It has been reported that HMG 1, but not 2, isolated from calf thymus nuclei washed with 0.075 M NaCl -EDTA and extracted with 0.35 M NaCl binds H1 and can be eluted from each of the three H1 subfractions with 0.05-0.15 M NaCl (Yu and Spring, 1977). It appears as though a combination of these binding properties of HMG 1 and 2 are at play in the localization of HMG proteins in the nucleus.

HMG 1 has been isolated in both the nuclei and the cytosol of cultured hepatoma cells (Isackson et al., 1980). Cytosol and nuclei were separated from each other via sucrose-low salt homogenization and centrifugation. Following extraction with 0.35 M NaCl, in the case of chromatin, HMG

proteins were isolated from both fractions via ds/ssDNA cellulose affinity chromatography. Extracts were loaded at 0.2 M NaCl and HMG 1 was eluted from the ssDNA column with 0.3 M NaCl and subjected to Sephadex G-100 and ammonium sulfate fractionation. Sequential ds/ssDNA affinity chromatography was then again employed. The purified protein isolated from the cytosol has the same SDS and acid-urea electrophoretic mobilities as nuclear HMG 1 isolated from both calf thymus and hepatoma cells. Another cytosolic protein eluting from the ssDNA affinity column with 0.5 M NaCl has been identified as HMG 2 based on its elution and electrophoretic mobility. Amino acid analysis of the cytosolic protein and HMG isolated from hepatoma nuclei show a very similar sequence and the cytosolic protein reacts with antibodies generated against calf thymus HMG 1 protein.

Gordon et al. (1981) have studied the binding of rat embryonic skeletal muscle to nuclei and found when nuclei are isolated via mechanical disruption of cells, only 30-40% of the total HMG 1 and 2 are recovered from nuclei, while the majority is recovered from the cytosol. Cytoplasmic extracts prepared from cells enucleated via cytochalasin B treatment, a nonmechanical cell fractionation method, shows only 10% of HMG 1 present in the cytoplasm, while 90% is recovered from the nuclei. These findings suggest that HMG 1 leaks out of nuclei into the cytoplasm during mechanical isolation of nuclei and these investigators conclude that the majority of

HMG 1 and 2 is superficially located in chromatin.

Proliferating and nonproliferating tissues. The amount of HMG 1 and 2 present in proliferating and nonproliferating rat organs, including liver and brain in the latter group, has been quantitated (Seyedin and Kistler, 1979). It was demonstrated that HMG 2 is recovered in a much lower amount from nonproliferating tissue, as opposed to proliferating tissue. These investigators were able to identify a reciprocal relationship between HMG 2 and H1 in every organ they examined, with HMG 2 being recovered in small amounts from nonproliferating tissues and H1 being recovered in large amounts from nonproliferating tissues. HMG 1 does not show marked organ variability from proliferating verses nonproliferating tissue. High levels of HMG 2 were found in proliferating tissues such as bone marrow, testis, small intestine mucosa, thymus, and spleen. These workers conclude that HMG 2 plays a role in cell replication.

Seyedin et al. (1981) have also shown that levels of HMG 1 and 2 are decreased in mouse neuroblastoma cells that have been induced to differentiate by serum deprivation. When cell growth is inhibited, HMG 1 and 2 levels are not affected in either HeLa or neuroblastoma cells even though H1 accumulates. This suggests that HMG 1 and 2 are not correlated to mitotic rate. Once again, induction of irreversible morphologic differentiation in neuroblastoma cells with dibutyryl cyclic

AMP or dimethylsulfoxide treatment decreases the levels of HMG 1 and 2, suggesting that loss of these two proteins may be related to a commitment of these cells to differentiation. Goodwin et al. (1985) have found an HMG-like protein (HMG I) to be increased in proliferating fibroblasts, decreased in rat liver, and increased in fibroblasts transformed with avian sarcoma virus. HMG I has recently been sequenced (Lund et al., 1987). Mosevitsky et al. (1989) reported HMG 1 and 2 to be high in hepatic and brain tissues in the cytosol and found HMG 1 and 2 in increased amounts in nuclei from tissues containing undifferentiated cells (lymphoid and testis).

III. MATERIALS AND METHODS

Cells and Tissues

F98 glioma cells cloned from an anaplastic glioma produced in a CD Fisher rat by transplacental exposure to N-ethyl-N-nitrosourea (Ko and Koestner, 1980; Ko et al., 1980), were grown in a spinner flask in Eagle's minimum essential medium supplemented with 5 % fetal bovine serum, 4 mM glutamine, 100 units/ml penicillin, and 50 ug/ml streptomycin at 37°C. Cells were harvested by centrifugation, washed with the culture medium, and stored at -70°C until used.

A breeding pair of BD-IV rats was a generous gift of Dr. G. Stoica (Department of Pathology, Texas A&M University, College Station, Texas) and pregnant SD rats (18 day gestation) were obtained from Charles River Laboratories Inc.(Portage, MI). All animals were kept in a 12-hour light: 12-hour dark cycle and fed a standard diet and water ad libitum. Following spontaneous vaginal delivery, juvenile (4-8-day-old) animals were euthanatized via CO_2, decapitated and cortices stripped of vessels and meninges and quickly frozen in dry ice. Cortices were stored at -20°C until utilized. Livers were obtained in a similar manner. Adult rat brains (Sprague-Dawley) were purchased frozen from Pel-Freez Biologicals (Rogers, AR) and rapidly stripped of vessels,

meninges, and cerebellum at the time of use.

Isolation of Nuclei

Glioma cells (20 mg DNA) were suspended in 60 ml of buffer A (0.32 M sucrose - 10 mM Tris-HCl [pH 7.4]- 5 mM $MgCl_2$ - 1% Triton X-100 - 0.5 mM dithiothreitol [DTT] - 1 mM phenylmethanesulfonyl fluoride [PMSF]- 1 uM leupeptin - 1 uM pepstatin - 4% isopropanol) and homogenized in a Dounce homogenizer with a loose fitting pestle. After 10 minutes in ice, the homogenate was centrifuged 750 x g for 10 minutes. The sediment (nuclei) was washed by resuspension and centrifugation in 35 ml of buffer A and stored frozen at -30°C.

Glial nuclei were isolated from 20 rat cerebral cortices according to the procedure of Thompson (1973). Briefly, stripped cortices were thawed, homogenized in a solution containing 2 M sucrose - 1 mM $MgCl_2$ - 1 uM leupeptin - 1 uM pepstatin - 1 mM PMSF - 4 % isopropanol and centrifuged for 1 hour at 22,000 rpm in a Beckman SW 28 rotor. The supernatant was discarded and the pellet resuspended in 2.4 M sucrose - 1 mM $MgCl_2$ - 1 uM leupeptin - 1 uM pepstatin - 1 mM PMSF - 4 % isopropanol. One ml of a solution containing 1.5 M sucrose - 1 mM $MgCl_2$ - 1 uM leupeptin - 1 uM pepstatin - 1 mM PMSF - 4 % isopropanol was carefully layered on top of 4 ml of the suspension. Following centrifugation at 30,000 rpm in a Beckman SW 55 rotor for 30 minutes, the 1.5 M layer

was removed. The resulting pellet (glial nuclei) was washed in buffer A by resuspension and centrifugation (2000 x g, 10 min.), and stored frozen at -70°C.

Livers (10 g) were homogenized with a motor-driven glass-Teflon homogenizer in 60 ml of a solution containing 0.25 M sucrose - 25 mM KCl - 5 mM $MgCl_2$ - 10 mM Tris-HCl (pH 7.4) - 1 mM PMSF - 1 uM leupeptin - 1 uM pepstatin - 4% isopropanol and filtered through 2 layers of Miracloth. The homogenate was then mixed with 2 volumes of the homogenizing media containing 2.3 M sucrose and nuclei were sedimented through a 5 ml layer of the 2.3 M sucrose solution by centrifugation at 25,000 rpm for 1 hour in a Beckman SW 28 rotor. Liver nuclei thus obtained were washed with 30 ml of buffer A by resuspension and centrifugation (1,500 x g, 10 minutes), and stored frozen at -30°C until use.

Isolation of Nuclear Fractions

Isolated nuclei (4-5 mg DNA) were homogenized in 35 ml of a solution containing 150 mM NaCl - 10 mM Tris-HCl (pH 8.0) - 1 mM EDTA - 0.5 mM DTT - 1 mM PMSF - 1 uM leupeptin - 1 uM pepstatin -4% isopropanol (buffer B) using a Dounce homogenizer. After centrifugation at 1500 x g for 10 minutes, chromatin was prepared from the sediment and the nucleoplasmic and total RNP fractions were isolated from the supernatant.

To obtain chromatin, the sediment was successively washed with 35 ml of buffer B, sedimented at 1500 x g for 10 minutes,

and then with 35 ml of 10 mM Tris-HCl (pH 8.0) - 1 mM EDTA - 0.5 mM DTT - 1 mM PMSF - 1 uM leupeptin - 1 uM pepstatin - 4% isopropanol (buffer C) by resuspension and centrifugation (1500 x g, 10 min.). The resulting sediment was homogenized by hand using a Teflon homogenizer in 35 ml of buffer C and centrifuged at 17,500 x g for 20 minutes.

To separate the nucleoplasm and the total RNP fraction, the supernatant obtained after centrifugation of the nuclear homogenate (as above) was centrifuged at 17,500 x g for 20 minutes and the resulting sediment discarded. The supernatant was then centrifuged for 3 hours at 40,000 rpm in a Beckman Ti 70.1 rotor, yeilding the sediment representing the total RNP fraction and the supernatant representing the nucleoplasmic fraction. The nucleoplasmic fraction was dialyzed against 2 liters of buffer D (20 mM Tris-HCl, pH 8.8 - 200 mM NaCl - 0.1 mM DTT - 1 mM EDTA - 5% glycerol) for 16 hours, centrifuged 17,500 x g for 20 minutes to remove insoluble materials, and subjected to DNA affinity chromatography.

Isolation of the hnRNP fraction from F98 glioma cells was performed according to the procedure of Kish and Pederson (1978). Nuclei (20 mg DNA) were sonicated in 10 mM Tris-HCl (pH 7.4) - 10 mM NaCl - 1.5 mM $MgCl_2$ - 1 mM PMSF - 1 uM leupeptin - 1 uM pepstatin, layered on top of the same buffer containing 30 % sucrose and centrifuged at 5,000 rpm, for 15 min in a Beckman SW 28 rotor. The material remaining on top

of the 30 % sucrose was removed and layered on top of a discontinuous sucrose gradient consisting of 60 % sucrose (20 ml), 45 % sucrose (2 ml), 10 % sucrose (8 ml), in the same buffer. After centrifugation at 26,000 rpm for 90 min. in a Beckman SW 28 rotor the 45 % layer containing the hnRNP fraction was removed and concentrated by precipitation in 67 % ethanol at -30° C.

Protein Extraction and DNA Affinity Chromatography

Chromatin, hnRNP, or total RNP fractions were homogenized in 2 M NaCl - 10 mM Tris-HCl (pH 8.0) - 1 mM EDTA - 0.75 mM DTT - 1.5 mM PMSF - 1 uM leupeptin - 1 uM pepstatin - 6% isopropanol using a motor-driven glass-Teflon homogenizer and centrifuged at 35,000 rpm for 17 hours in a Beckman Ti 70.1 rotor. The supernatant was dialyzed against two changes of buffer D for 20 hours and the small amount of precipitate formed during dialysis was removed by centrifugation at 17,500 x g for 20 minutes. The extract thus obtained was loaded at a flow rate of 4 ml/h onto a dsDNA cellulose column (1 x 7 cm) previously treated with S_1 nuclease (Isackson et al., 1979), linked in tandem to a ssDNA agarose column (1 x 7 cm), such that the extract flowed first over the ds matrix and then over the ss matrix (Herrick and Alberts, 1976). The columns were then washed in tandem with buffer D, separated and individually eluted with buffer D containing 2 M NaCl at a flow rate of 6 ml/h. Chromatographic fractions were

exhaustivley dialyzed against either H_2O or 0.2 M ammonium bicarbonate (pH 8.7), lyophilized and subjected to electrophoretic analysis.

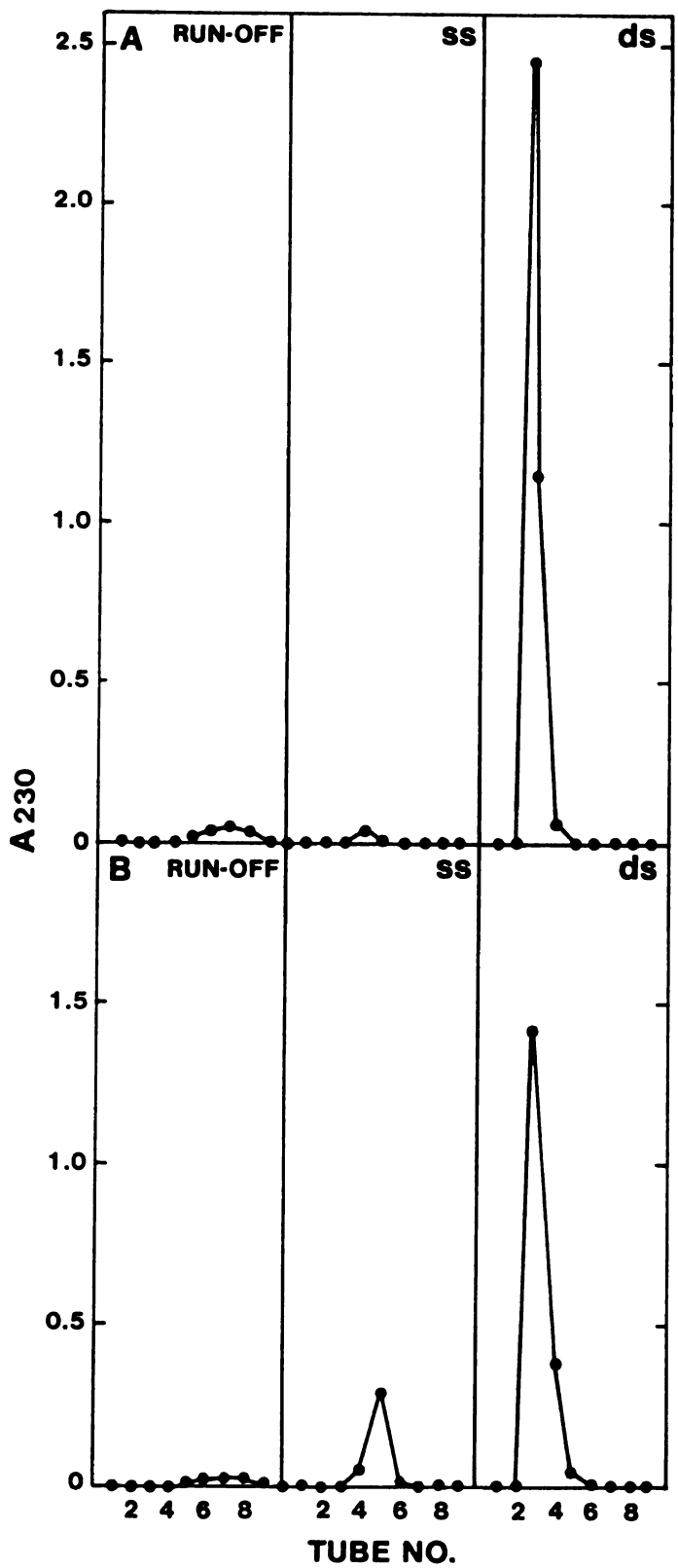
Electrophoretic Analysis of Protein

Single-dimension sodium dodecyl sulfate (SDS) polyacrylamide (11%) gel electrophoresis was performed as described by Laemmli (1970). Acetic acid-2.5 M urea polyacrylamide (15 %) gel electrophoresis was carried out according to Panyim and Chalkey (1969). For two-dimensional electrophoresis, electrofocusing in the first dimension was carried out according to the method of Takami and Busch (1979) and the second dimension gel was run as described by Laemmli (1970) at an 8.5% acrylamide concentration.

Chemical Determinations

Protein was determined using the Coomassie protein assay reagent (Pierce Chemical Company). RNA was determined on the 0.3 M NaOH-hydrolyzable (37°C,60 min.) material using an A_{260} of 1 mg/ml of hydrolyzed RNA equal to 32.2 (Fleck and Munro, 1962). DNA was determined on the hot 5% perchloric acid-hydrolyzable material either by the diphenylamine reaction (Burton, 1956) or by using an A_{260} of 1 mg/ml of hydrolyzed DNA equal to 28.

Figure 1: Double-stranded (ds) and single-stranded (ss) DNA affinity chromatography of proteins from F98 glioma chromatin. Extract was first loaded at 0.05 M NaCl in buffer D and eluted from each column separately with 2 M NaCl (A). Fractions containing protein eluting from the ds DNA column were then pooled, dialyzed against 0.2 M NaCl in buffer D and reapplied at this salt concentration to the DNA affinity columns and eluted with 2 M NaCl (B). The run-off fraction represents protein binding to neither ds nor ss DNA columns.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RUN-OFF
ss
ds
B
RUN-OFF
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ds
A 230
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2.0
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2 4 6 8
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IV. RESULTS

Nuclei of carcinogenic target glia and non-target liver of sensitive SD and resistant BD-IV rats at various ages were fractionated into chromatin and nucleoplasm. SSBs were then isolated from these nuclear fractions, quantitated, and characterized by electrophoresis. Rapidly proliferating glioma cells were utilized to obtain nuclear RNP complexes and to identify SSBs associated with RNA.

SSBs of Chromatin

Chromatin was extracted with 2 M NaCl and proteins were then applied to DNA affinity columns linked in tandem such that the extract first flowed over the dsDNA column and then the ssDNA column. Application of protein extracts at 0.05 M NaCl (cf. Herrick and Alberts, 1976a) resulted in very little recovery of protein from the ssDNA column (Fig. 1-A) and most proteins bound to the dsDNA column. When proteins binding to the dsDNA column at 0.05 M NaCl were reapplied to the DNA affinity columns at 0.2 M NaCl, all the histones bound to the dsDNA column and a considerable amount of SSBs were recovered from the ssDNA column (Fig. 1-B). Based on this finding, all extracts were routinely loaded at 0.2 M NaCl. It was found that the omission of protease inhibitors (PMSF, leupeptin, and

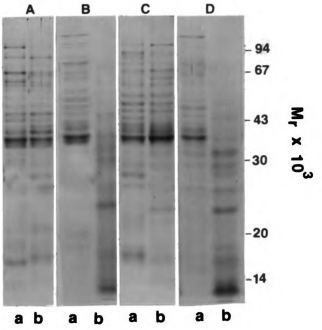


Figure 2: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of single-stranded DNA binding proteins isolated from chromatin of various cell types. The protein samples (7-10 ug) from F98 glioma (A), 6-day-old glia (B), adult glia (C), and adult liver (D) isolated in the presence (a) or absence (b) of protease inhibitors were applied onto 11 % slab gels.

pepstatin) during isolation resulted in a decrease in high molecular weight protein bands and a corresponding increase in low molecular weight protein bands (Fig. 2), suggesting that proteins were proteolytically degraded. As seen in this figure, proteolysis was more extensive in chromatin of juvenile glia and liver as compared to adult glia and the glioma. Proteolysis apparently occurred during the extraction of chromatin with 2 M NaCl. Omission of these inhibitors during the preparation of nuclei and chromatin resulted in no change in electrophoretic profiles.

The contents of SSBs relative to chromosomal DNA and RNA in various cell types are summarized in Table 1. Juvenile glia had the highest amount of SSBs present among the cell types examined. The adult glia chromatin contained less SSBs than the juvenile glia chromatin, whereas juvenile and adult liver had a comparable amount of SSBs in chromatin. The SSB content of the glioma chromatin was similar to that of liver chromatin. No quantitative difference was found in SSBs of glia chromatin obtained from juvenile BD-IV and SD rats.

The two-dimensional electrophoretic profiles of SSBs isolated from chromatin of the various cell types are presented in Fig. 3. Juvenile and adult glia (Fig. 3-B and -C) revealed a more heterogeneous population of SSBs as compared with juvenile liver (Fig. 3-D). The juvenile liver profile was similar to the glioma profile (Fig. 3-A). Every protein component present in liver chromatin appeared to be

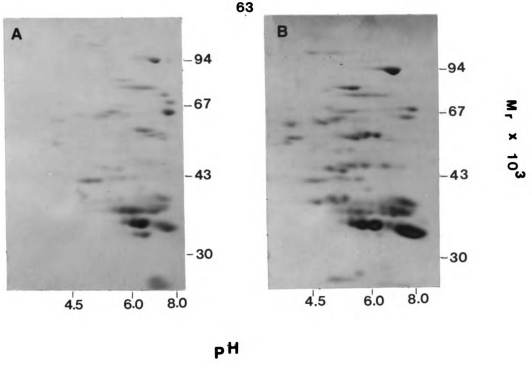
Table 1. Quantitation of single-stranded DNA binding proteins in chromatin.

Cell type & Age	n[a]	Weight Ratio[b]		
		RNA / DNA	SSB / DNA	SSB / RNA
Glia, 5-8d old	2	0.15 ± 0.02	0.21 ± 0.02	1.44 ± 0.36
Glia, adult	3	0.17 ± 0.03	0.15 ± 0.02	0.88 ± 0.08
Liver, 4-6d old	2	0.082 ± 0.00	0.046 ± 0.0071	0.56 ± 0.09
Liver, adult	2	0.10 ± 0.01	0.048 ± 0.001	0.47 ± 0.06
F98 glioma	3	0.17 ± 0.06	0.082 ± 0.045	0.46 ± 0.10

[a] number of trials

[b] mean ± standard deviation

Figure 3: Two-dimensional gel electrophoresis of single-stranded DNA binding proteins isolated from chromatin of various cell types. The protein samples (20-25 ug) isolated from chromatin of F98 glioma (A), 5-8-day-old glia (B), adult glia (C), and 4-6 day old liver (D) were separated according to isoelectric point by isoelectric focusing in the first dimension and according to molecular weight by SDS electrophoresis in the second dimension.


A
B
C
D
94
67
43
30
4.5
6.0
8.0
pH
Mr x 10^3

present in glial chromatin. However, additional proteins of acidic pH and M_r 33,000-67,000 were found to be associated with glial chromatin while being absent or present in much lower amounts in liver chromatin. Comparison of two-dimensional electrophoretic profiles of BD-IV and SD glial chromatin (Fig. 4) reproducibly identified four proteins of acidic pH and M_r 50,000-67,000 (arrows) as being more abundant in SD glia than BD-IV glia.

SSBs of RNP

Centrifugation of the post-chromatin supernatant of glioma nuclei at 100,000 x g (3 hours) yielded a particulate fraction containing RNA (referred to as total RNP). The amount of RNA recovered in this fraction was found to be approximately one third that of chromosomal DNA. The protein /RNA ratio was found to be 1.79 (Table 2), which is similar to the value reported for pre-ribosomal RNA-protein complexes of HeLa cells (Kumar et al., 1972). Glia and liver nuclei yielded very little material corresponding to this fraction. HnRNP isolated from glioma cells on the other hand, exhibited a protein/RNA ratio of 8.97 (Table 2), which is similar to the value obtained for pre-messenger RNA-protein complexes similarly isolated from hepatoma cells (Kish and Pederson, 1978). The RNA/DNA ratio of 4.62 of the hnRNP fraction indicates some contamination by chromatin fragments. The total RNP and hnRNP fractions were extracted with 2 M NaCl

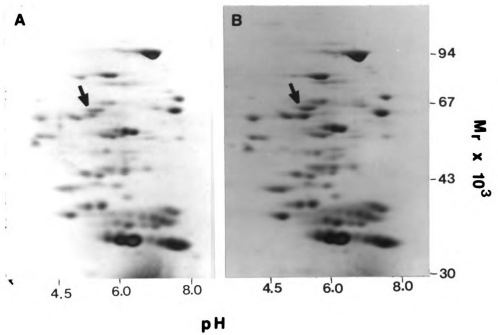


Figure 4: Two-dimensional electrophoresis of single-stranded DNA binding proteins isolated from glial chromatin of 5-day-old BD-IV (A) and SD (B) rats. The protein samples (23-24 ug) were analyzed as described in the legend to Fig. 3. Arrows indicate an area where quantitative differences between the two rat strains was noted.

Table 2. Quantitation of single-stranded DNA binding proteins in nuclear ribonucleoprotein particles.

Nuclear fraction	Weight Ratio[a]		
	RNA / DNA	Protein / RNA	SSB / RNA
Total RNP	31.6 ± 0.75	1.68 ± 0.29	0.057 ± 0.010
HnRNP	4.62 ± 0.79	8.97 ± 0.85	0.433 ± 0.04

[a] mean ± standard deviation for n=3 trials each

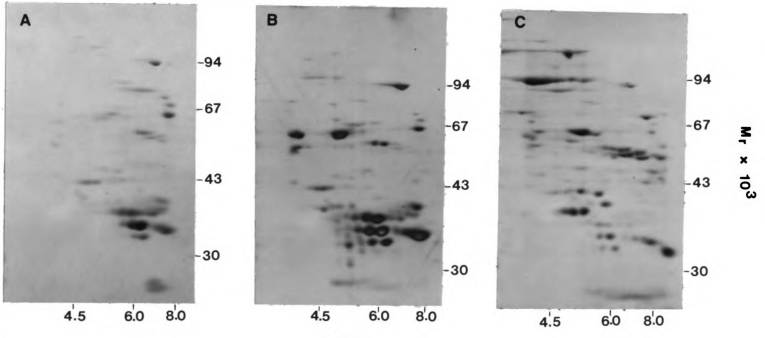


Figure 5: Two-dimensional electrophoretic profiles of single-stranded DNA binding proteins isolated from various nuclear fractions of F98 glioma cells. The protein samples (20-25 ug) obtained from chromatin (A), heterogeneous nuclear ribonucleoprotein particles (B), and total ribonucleoprotein particles (C) were analyzed as described in the legend to Fig. 3.

and SSBs isolated therefrom by DNA affinity chromatography. As shown in Table 2, a greater amount of SSBs were found in hnRNP than total RNP. The SSB/RNA ratio of hnRNP (0.43) resembled that (0.46) of glioma chromatin.

Two-dimensional electrophoresis revealed the hnRNP fraction (Fig. 5-B) to be different from the total RNP fraction (Fig. 5-C). Some of the proteins found in the hnRNP fraction were also present in the total RNP fraction. The total RNP fraction contained acidic proteins of higher molecular weight not present in the hnRNP fraction. The hnRNP fraction contained essentially all the protein components found in the chromatin fraction of the glioma (Fig. 5-A), with an additional series of proteins not found in the chromatin fraction.

SSBs of Nucleoplasm

Examination of the UV absorption spectrum of the nucleoplasmic fractions gave an $A_{280/260\ nm}$ of approximately 1.2, indicating that this fraction was essentially free from nucleic acids. Therefore, the nucleoplasmic fractions were directly dialyzed against a buffer containing 0.2 M NaCl and fractionated by DNA affinity chromatography. As seen in Table 3, glial nuclei were enriched with nucleoplasmic proteins as compared with liver nuclei. Results also showed that the nucleoplasm of juvenile glia contained almost two times more SSBs than that of juvenile liver. The content of

Table 3. Quantitation of nucleoplasmic single-stranded DNA binding proteins.

Cell type & Age	n[a]	Relative to chromosomal DNA[b]	
		Total protein	SSB
Glia, 5d old	3	0.26 ± 0.012	0.033 ± 0.0062
Glia, adult	2	0.23 ± 0.021	0.007 ± 0.0017
Liver, 4d old	2	0.12 ± 0.021	0.016 ± 0.004
Liver, adult	4	0.10 ± 0.026	0.011 ± 0.002
F98 glioma	2	0.34 ± 0.17	0.016 ± 0.001

[a] number of trials

[b] mean ± standard deviation

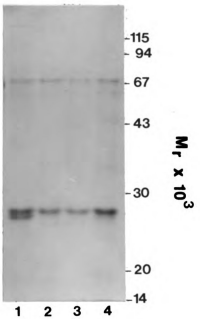


Figure 6: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of single-stranded DNA binding proteins isolated from nucleoplasm of various cell types. The protein samples (0.8-1.8 ug) of F98 glioma cells (1), 6-day-old glia (2), adult glia (3), and adult liver (4) were loaded onto an 11 % slab gel.

nucleoplasmic SSBs decreased with increasing age in both glia and liver. Nucleoplasm of glioma cells had approximately the same amount of SSBs as the nucleoplasmic fraction of juvenile liver.

Electrophoretic analysis revealed the nucleoplasm (Fig. 6) of glia and liver to have one band of M_r 25,500 and a minor band of M_r 67,000. In contrast, the glioma profile revealed two major bands of M_r 25,500 and 25,000. These low molecular weight proteins in the glioma nucleoplasm were found to be soluble in 0.5 M perchloric acid. Electrophoretic mobilities of these proteins relative to histones in either acid-urea gel (Fig. 7-A) or SDS gel (Fig. 7-B) electrophoresis suggested that they were HMG 1 and 2. It thus appears that the nucleoplasm of liver and glia contained mainly HMG 1, while the nucleoplasm of the rapidly proliferating glioma contained both HMG 1 and 2. Densitometer scanning of the acid-urea gel revealed that the amount HMG 1 and 2 in nucleoplasm was approximately 40 times greater than that in chromatin. No difference in the amount or in the electrophoretic profile of nucleoplasmic SSBs was noted between juvenile glia of SD and BD-IV rats.

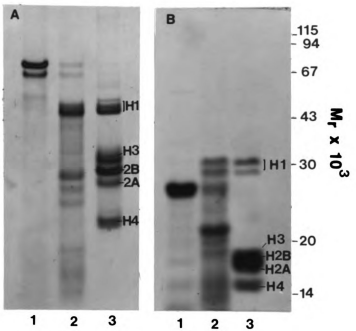


Figure 7: Electrophoretic analysis of 0.5 M perchloric acid-soluble proteins obtained from chromatin and nucleoplasmic fractions of F98 glioma cells. Chromatin (lane 2) and nucleoplasm (lane 1) were treated with 0.5 M perchloric acid (30 min., in ice). The acid soluble proteins were then precipitated with 20 % trichloroacetic acid and washed successively with acetone-HCl and acetone. The protein samples obtained from nuclei equivalent to 160 ug DNA were analyzed by electrophoresis either on a SDS polyacrylamide (11%) gel (B) or an acetic acid-urea polyacrylamide (15%) gel (A). Lane 3, calf thymus histones (20 ug).

V. DISCUSSION

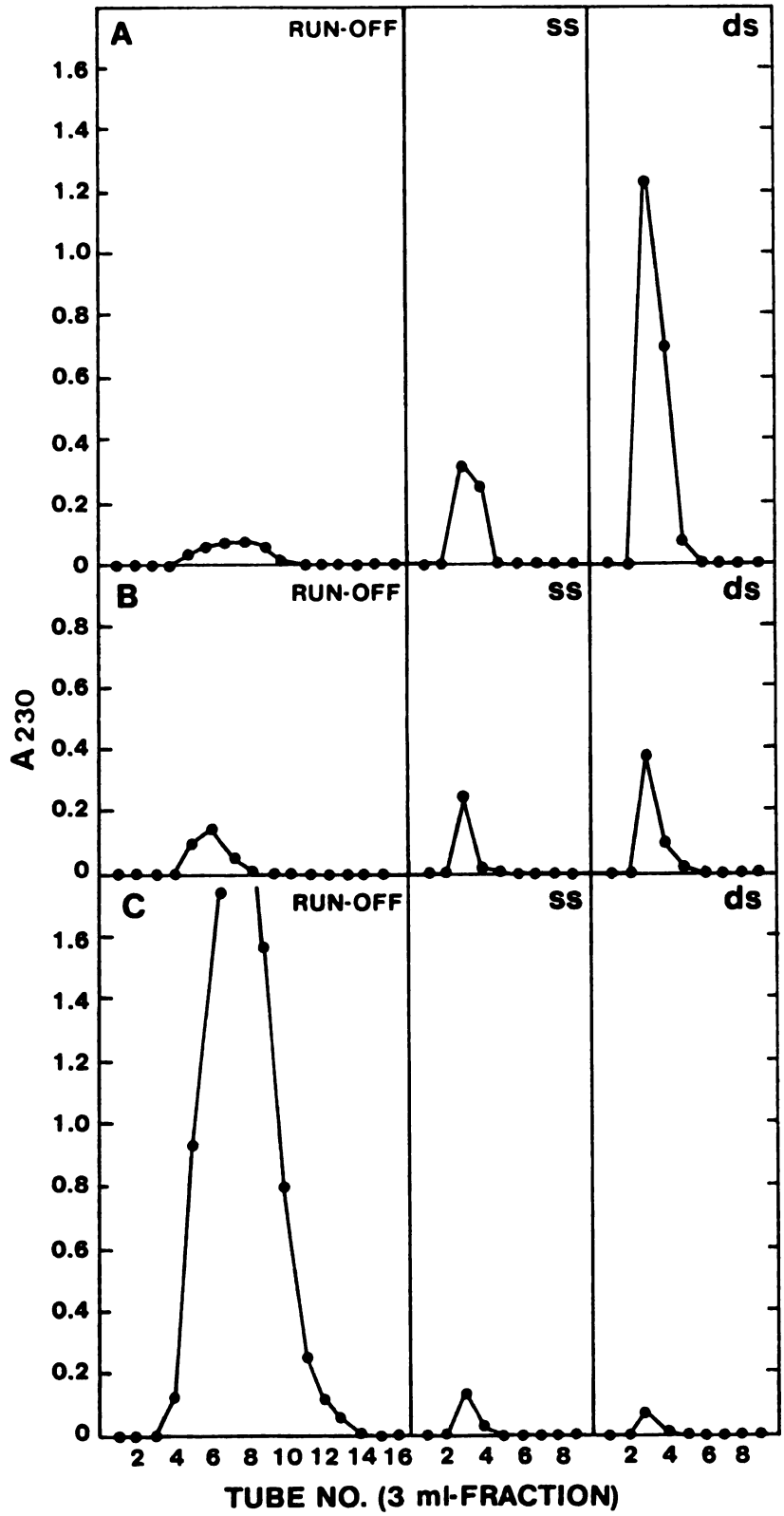
The present study has shown that the content of SSBs in glial chromatin isolated from juvenile rats is several times higher than that of liver chromatin isolated from rats of the same age. While juvenile and adult liver chromatin contained similar amounts of SSBs, the SSB content in glial chromatin decreased with increasing age (Table 1). Electrophoretic analyses have further shown that SSBs of the glial chromatin were more heterogeneous than those of the liver chromatin (Fig. 3). Valentini et al. (1985) and Pandolfo et al. (1985), upon examination of HeLa cells and calf thymus, have shown that mammalian SSBs mainly arise from the hnRNP fraction. It has been noted in this study that the SSB content relative to RNA of liver chromatin (Table 1) was similar to that of the hnRNP fraction obtained from the rapidly proliferating glioma cells (Table 2). This together with the similarity of the electrophoretic profiles between SSBs of liver chromatin and those of the hnRNP fraction (Fig. 2 and Fig. 3) suggests that SSBs of liver chromatin were mainly associated with newly synthesized RNA. The higher SSB/RNA ratio as well as the presence of additional protein components in chromatin of glial cells suggests that glial chromatin contains additional SSBs which were presumably associated with DNA.

As has been shown in Figs. 6 and 7, HMG proteins were the major constituents of SSBs in the nucleoplasm. While rapidly proliferating cells (glioma) contained HMG 1 and 2 in approximately equal proportions, as others have reported (Seydin and Kistler, 1979; Einck and Bustin, 1985), nucleoplasmic SSBs of glia and liver consisted mainly of HMG 1. The nucleoplasm of juvenile glia was found to contain approximately twice as much SSBs as the nucleoplasm of juvenile liver (Table 3). In both glia and liver the content of SSBs appeared to decrease with increasing age.

It thus seems clear that chromatin of glia, a target of N-nitrosourea tumor induction, contains more SSBs which have been identified to be associated with DNA than does chromatin of non-target liver. Furthermore, several components of such SSBs were enriched in glial chromatin of carcinogenically susceptible SD rats as compared with that of carcinogenically resistant BD-IV rats (Fig. 4). Carcinogenesis is a complex process and is known to involve amplification and recombination as well as the loss of particular genes. SSBs are likely to play a crucial role in such events. Fractionation and determination of the biological functions of these glial SSBs may contribute to the further understanding of the role these proteins play in selective induction of neurogenic tumors.

Appendix

Figure A1: DNA affinity chromatography of extracts obtained from chromatin (A), total ribonucleoprotein particle (B), and nucleoplasmic fractions (C) of F98 glioma cells. Extracts were loaded onto ds/ss DNA columns linked in tandem at 0.2 M NaCl and eluted separately from each column with 2 M NaCl. The run off represents protein binding to neither DNA affinity column.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SS
ds
B
RUN-OFF
SS
ds
C
RUN-OFF
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TUBE NO. (3 ml-FRACTION)

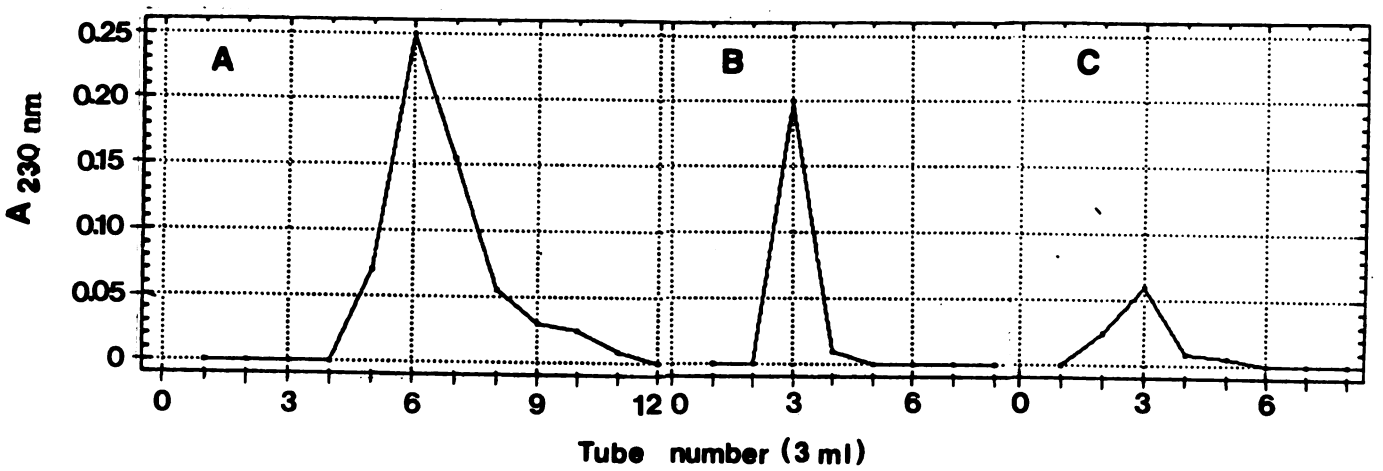

Figure A2: DNA affinity chromatography of extracts obtained from heterogeneous nuclear ribonucleoprotein particles of F98 glioma cells. Extracts were loaded and eluted from the DNA affinity columns as described in Fig. A1. A, protein binding to neither DNA column; B, protein eluting from the ss DNA column; C, protein eluting from the ds DNA affinity column.

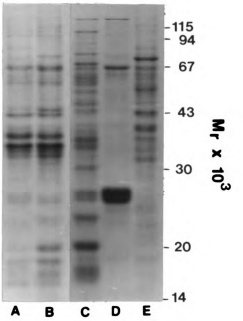

Figure A3: Single-dimension SDS polyacrylamide electrophoreses of SSBs isolated from chromatin (A), hnRNP (B), total RNP (C), nucleoplasm (D), and cytosol (E) of F98 glioma cells. The protein samples were analyzed on 11% slab gels.

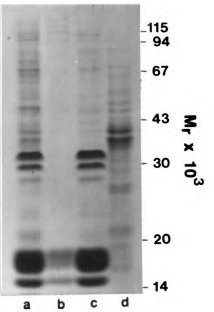

Figure A4: Single-dimension electrophoretic analysis of F98 glioma chromatin extract after DNA affinity chromatography. (a) Total proteins (10 ug) applied onto DNA affinity columns at 0.2 M NaCl, (b) proteins (2.0 ug) precipitated after dialysis prior to loading on affinity columns, (c) proteins (10 ug) eluting from dsDNA column, and (d) proteins (9.0 ug) eluting from ssDNA column were analyzed on SDS-polyacrylamide gels. Total proteins consisted of mostly histones H1, H3, H2B, H2A, and H4, while the precipitate after dialysis contained mostly H3 and H4. Histones bound to the ds column.

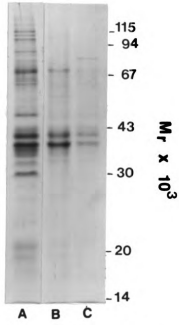

FIGURE A5: Dissociation of single-stranded DNA binding proteins from F98 glioma chromatin. Chromatin was extracted in a stepwise fashion with 0.6 M NaCl (A), 1.2 M NaCl (B), and 2.0 M NaCl (C). Extracts were then subjected to DNA affinity chromatography and analyzed by electrophoresis. Proteins derived from an equivalent amount of chromosomal DNA were analyzed on SDS-polyacrylamide gels.

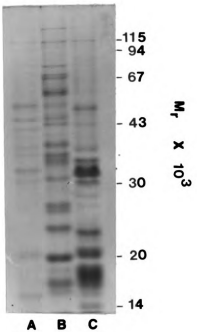

Figure A6: Single-dimension electrophoretic analysis of total RNP extract of F98 glioma cells after DNA affinity chromatography. Proteins (2.9 ug) binding to neither DNA affinity column (A), proteins (9.6 ug) eluted from the ss DNA column (B), and proteins (8.0 ug) eluted from the ds DNA column (C) were analyzed on SDS-polyacrylamide gels.

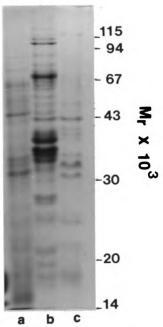

Figure A7: Single-dimension electrophoretic analysis of F98 glioma hnRNP extract after DNA affinity chromatography. Proteins (6.9 ug) binding to neither DNA affinity column (a), proteins (14 ug) eluted from the ss DNA column (b), and proteins (2.9 ug) eluted from the ds DNA column (c) were analyzed on SDS-polyacrylamide gels.

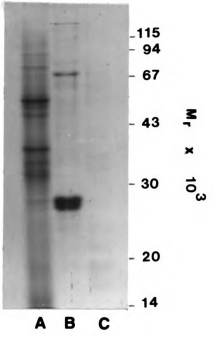

Figure A8: Single-dimension electrophoretic analysis of the nucleoplasmic fraction of the F98 glioma after DNA affinity chromatography. Proteins (16 ug) binding to neither DNA affinity column (A), proteins (3.4 ug) eluted from the ss DNA column (B), and proteins (2.6 ug) eluted from the ds DNA column (C) were analyzed on SDS-polyacrylamide gels.

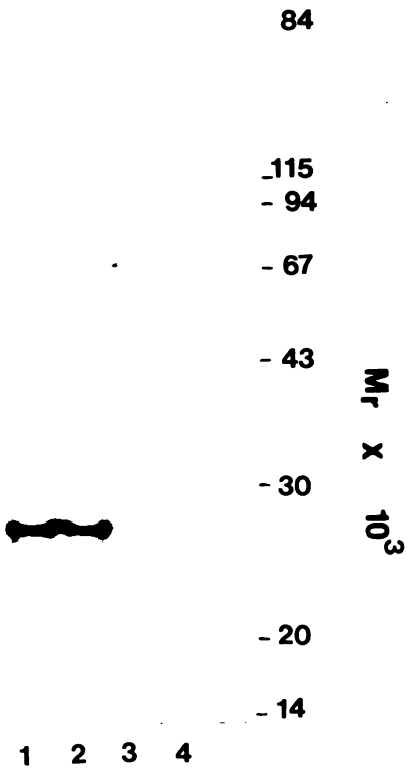

Figure A9: Single-dimension electrophoretic analysis of nucleoplasmic fractions obtained from juvenile glia of Berlin-Druckrey-IV and Sprague-Dawley rats. Lane 1 (BD-IV), proteins eluted from the ss DNA column; lane 2 (SD), proteins eluted from the ss DNA column; lane 3 (BD-IV), proteins eluted from the ds DNA column; lane 4 (SD), proteins eluted from the ds DNA column. Electrophoresis was run on SDS-polyacrylamide gels.

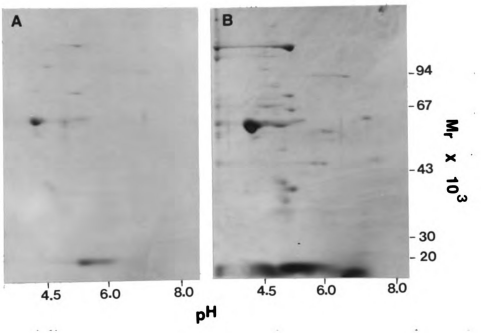

Figure A10: Two-dimensional electrophoresis of SSBs from nucleoplasm. Protein samples (4.5 ug) from adult liver (A) and the protein sample (19 ug) from F98 glioma cells (B) were separated by isoelectric focusing in the first dimension and according to molecular weight by SDS electrophoresis in the second dimension.

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