STUDIES ON HELIX STABILISING DNA BINDING PROTEIN FROM THE THERMOPHILIC ARCHAEON *Sulfolobus acidocaldarius*

A Thesis Submitted for the Degree of

DOCTOR OF PHILOSOPHY

By

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To my parents

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Certificate

This is to certify that the thesis entitled **Studies on helix stabilising DNA binding protein from the thermophilic archaeon *Sulfolobus acidocaldarius*** is based on the results of the work done by **Ms Gauri Benegal** for the degree of **Doctor of Philosophy** under my supervision. This work has not been submitted for any degree or diploma of any University or Institution.

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Declaration

I hereby declare that the work presented in this thesis entitled **Studies on helix stabilising DNA binding protein from the thermophilic archaeon *Sulfolobus acidocaldarius*** is entirely original and was carried out by me in the Department Animal Sciences, University of Hyderabad, under the supervision of **Prof. P. R. K. Reddy** and **Prof. T. Suryanarayana. I** further declare that to the best of my knowledge this work has not been submitted before for the award of degree or diploma from any University or Institution.

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ABBREVIATIONS

A_{260}	absorbance at 260 nm
A_{280}	absorbance at 280 nm
ATP	adenosine tri phosphate
bp	base pairs
BSA	bovine serum albumin
CM	carboxy methyl
dsDNA	double stranded DNA
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetate
g	centrifugal field number (number of times g)
GTP	guanosine tri phosphate
GMP	guanosine mono phosphate
HSNP-	Helix stabilising nucleoid protein
IgG	Immunoglobin G
kDa	kilo daltons
NP-40	Nonidet P-40 (non ionic detergent)

mM	milli molar
nm	nano meters
Poly (U)	poly ribouridylic acid
Poly (A)	poly riboadenylic acid
PMSF	phenyl methyl sulfonyl fluoride
PAGE	poly acrylamide gel electrophoresis
Pi	inorganic phosphate
RNA	Ribonucleic acid
RNase	Ribonuclease
RPM	revolutions per minute
ssDNA	single stranded DNA
SDS	sodium dodecyl sulphate
TEMED	N, N, N',N'- tetramethylethylenediamine
Tris	tris (hydroxy methyl) aminomethane

CHAPTER 1

INTRODUCTION

The present thesis deals with a nucleoid associated helix stabilizing DNA binding protein HSNP-C from the thermoacidophilic archaeon *Sulfolobus acidocaldarius.* Its role in condensation and compaction of DNA were studied which would help us in understanding the organisation and packaging of DNA in the organism and archaea in general. This protein possesses multifunctions like condensation of DNA, promotion of renaturation of complementary single stranded DNA, mononucleotide binding and ribonuclease (RNase) activity.

HSNP-C is from the 7 kDa group of DNA binding proteins from *Sulfolobus acidocaldarius.* This group comprises of five proteins that have a similar size with a molecular mass of 7 kDa (7a-7e) and are closely related in their amino acid sequence (Choli *et ah,* 1988b). In the present study a comparative study of three of these proteins 7c, 7d (HSNP-C) and 7e were done with respect to nucleic acid binding, nucleotide binding and RNase activity.

A brief account of archaea, histone like proteins from bacteria and archaea, HMG proteins is given in the following pages.

1.1 Archaea :

The archaea have been defined at molecular level as a third primary kingdom consisting of methanogens, the extreme halophiles and sulfur dependent extreme thermophiles. Woese and colleagues (Woese *et al.,* 1990) used the 16S ribosomal RNA a molecular chronometer for evolutionary relatedness of organisms and showed that at a nucleotide sequence level, the archaea are different from the bacteria and eukaryotes. Henceforth, organisms have been divided into three primary groupings viz. the bacteria, eukarya and archaea (Fox *et al.,* 1980; Woese *et al.,* 1990).

Woese's classification had no obvious way to establish which had given rise to which or whether all the three had emerged from a common primitive ancestor *progenote.*

Iwabe (1989) and Gorgaten *et al.,* (1989), provided a root for the universal tree giving us scope to reason about the evolution of major cell types. The rooting thus showed that archaea and eukarya are sister groups. The first branch of the tree of life separated the archaeal/eukaryote lineage from that leading to bacteria. In effect, archaea are the closest living prokaryote relatives of the eukaryotes (Brown *et al.,* 1995). Archaea were previously referred to as archaebacteria (Woese and Fox, 1977) since they resemble bacteria phenotypically. But phylogenetically they showed no resemblance to the bacteria. On the contrary they showed phylogenetic closeness to the eukaryotes (Woese *et al*., 1990).

Archaea lack certain features originally used to define prokaryotes (such as peptidoglycan). But they do share a number of complex features with eubacteria such as the presence of a single circular chromosome which contains genes arranged in polycistronic operons (Keeling *et al.,* 1994). Certain molecular features that suggest a special relationship between archaea and eukaryotes were the presence of N-linked glycoproteins (Mescher *et al.,* 1976), the lack of formylmethionine (White and Bailey St, 1972), shared resistance to various antibiotics and the presence of tRNA introns (Zillig, 1987). The striking similarity between eukaryotic and archaeal transcriptional machinery also reinforces the primitive nature of archaea.

1.1.1 Divisions of Archaea :

The members of archaea have distinct and characteristic living habitats. Some are strict anaerobes, some need high salinity, some are sulfur dependent and some need high temperature for their growth and survival. Phenotypically archaea are divided into three groups (i) methanogens (ii) halophiles and (iii) sulfur dependent thermophiles.

1.1.1.1 Methanogens : This group comprises of organisms that are not restricted to extreme environments. They are found in a variety of anaerobic habitats like sediments, sludge digestors, the guts of insects, the large bowel of man and animals. They are obligate methane producers.

***1.1.1.2** Halophiles :* They predominantly are found in salt lakes and are responsible for reddening and rotting of salted meat and fish.

1.1.1.3 Sulfur dependent organisms : Most, but not all of the organisms in this group are thermophilic. They grow at low pH and generate energy by metabolising sulfur.

Phylogenetically, archaea have been divided into two sub kingdoms viz., Crenarchaeota comprising of the sulfur dependent extreme thermophiles and the other Euryarchaeota consisting of methanogens, extreme halophiles, extreme thermophiles and sulfur reducing organisms. As revealed by the ribosomal RNA sequence comparisons, the archaea form a coherent phylogenetic unit that is equivalent in phylogenetic status to the bacteria. The division of archaea into Crenarchaeota and Euryarchaeota was based on 16S rRNA cataloguing and similarities or differences with respect to eukarya / bacteria (Wheelis *et al.*, 1992). 16S rRNA targeted oligonucleotide probes were used to identify members of these archaeal sub kingdoms. (Burggraf *et al.*, 1994).

1.2 Biochemistry and Molecular Biology of Archaea :

Cell envelope of different lineages of archaea exhibit distinct chemical and structural diversity. Their cell walls lack peptidoglycan or muramic acid and ester linked lipids (Kandler and Konig, 1985; Langworthy, 1985) which separate them most distinctly from bacteria and eukarya.

The archaeal genome size resembles that of bacterial genome which is 1.0 X 10^9 to 2.4 X 10^9 daltons (Klein and Schnorr, 1984). Archaea exhibit a broad spectrum of chromosomal G + C contents ranging from 21 to 61 mol% for methanogens (Balch *et al.*, 1979) and 46 to 60 mol% G + C for the other groups like *Thermoproteales*, *Sulfolobales*.

It was shown that halobacteria contain extrachromosomal covalently closed, circular DNA's of relatively low G + C content (Bailey and Morton, 1978).

Recently the complete 1.66 megabase pair genome sequence of an autotrophic archaeon *Methanococcus jannaschii* was determined. Most of the genes involved in transcription, translation and replication in *Methanococcus jannaschii* were found to be similar to those found in eukaryotes. Of the 1738 predicted protein coding regions, only 38% could be assigned cellular role (Bult *et al.,* 1996). In another project, over 300 kilo base-pair of 3 mega base-pair genome of the thermophilic archaeon *Sulfolobus solfataricus* has been sequenced. It was found to have one open reading frame of at least 100 amino acids per kilo base-pair of the sequence and 50% of these open reading frames have been related with known genes through database searches. The partially sequenced genome of *Sulfolobus solfataricus* also suggests clustering of genes by function. (Charlebois *et al.,* 1996).

Eukaryal and sulfur dependent archaeal rRNAs are found to be highly modified than the other archaeal groups and bacteria. Archaeal 5S rRNAs are diverse and are found to share similarities with both eukarya and bacteria. Like eukarya, 5S rRNA from the sulfur dependent archaea have triphosphorylated 5' termini whereas the methanogens and halophiles have 5' terminal monophosphate like the bacteria. The 5S rRNA from *Sulfolobus* and *Pyrodictum occultum* were found to contain two modified nucleosides - N-4 acetyl cytidine (ac4c) and N-4 acetyl-2'-0 methyl cytidine (ac4Cm) (Bruenger *et al.,* 1993). Archaea] tRNAs show a wide variety of modified bases like Cm, m^1G, $m^2{}_2G$, m^1A, m^5C and also thiolated nucleosides (Fox *et al.,* 1980; Gupta and Woese, 1980; Best, 1978; Kilpatrick and Walker; 1981). Archaeal tRNA lacks formyl methionyl tRNA as in the case of eukarya (Bailey and Morton, 1978).

The archaeal ribosomes are similar in size to bacterial ribosomes (70S) and smaller than 80S eukaryotic ribosomes (Douglas *et al.,* 1980). The rRNA components of archaeal ribosomes are similar to those found in bacterial ribosomes (Vinstentin *et al.,* 1972). Electron microscopic studies show that the 30S subunit of archaebacterial ribosomes contains an additional structure called the archaeal 'bill' (Lake *et al.,* 1982). The ribosomes from Euryarchaeota resemble bacteria in protein content with few or no

proteins greater than 30,000 daltons in mass. On the contrary, the ribosomes from Crenarchaeota resemble the eukaryotes in having a much higher protein content with many proteins greater than 30,000 daltons in mass (Cammerano *et al,* 1986). The archaeal ribosomal proteins show more sequence homology to that of their eukaryal counterparts (Matheson, 1985). Ribosomes from Sulfolobales were found to be insensitive to inhibitors of protein synthesis (Sanz *et al,* 1994). The archaeal ribosomes lack binding site for bacterial 70S antibiotic inhibitors like chloramphenicol or streptomycin. Instead, they have binding sites for eukaryal 80S inhibitors such as anisomycin.

An intron was found within the 23S rRNA gene of sulfur dependent thermoproteale *Desulfurococus mobilis* (Kjems and Garrette, 1985; 1988; 1991). This archaeal intron was later shown to encode a site specific DNA endonuclease that contains the LAGLIDADG motif and shares many properties with *intein* endonucleases in other kingdoms. (Dalgaard *et al,* 1994). Two tRNA genes in *Sulfolohus solfataricus* were found to contain intervening sequences (Kaine *et al,* 1983). Intervening sequences were also found in the gene encoding DNA polymerase from *Thermococcus litoralis* (Perler *et al,* 1992). Archaeal elongation factor EF2, like the eukaryotic counterpart, has a structural domain that can be ADP-ribosylated by Diphtheria toxin (Kessel and Klink, 1980; Pappenheimer *et al.,* 1983). Inhibition of DNA synthesis in a few methanogens was obtained by adriamycin. The DNA dependent RNA polymerase from archaea was found to be insensitive to transcriptional inhibitors like rifampicin and α-amanitin (Zillig *et al.,* 1978; Prangishvilli *et al,* 1982). In archaeal systems elongation factors EF2 was found to be insensitive to kirromycin and also pulvomycin that usually inhibit bacterial elongation factor EF-Tu (Cammarano *et al.*, 1982)

A novel type II DNA topoisomerase (reverse gyrase) which can introduce positive superhelical turns into DNA was detected in extracts of *Sulfolobus acidocaldarius.* This enzyme was found to be active at temperatures above 55°C and required ATP and Mg^{+2} (KiKuchi and Asai, 1984; Bouthier *et al,* 1990; Confalonieri *et al,* 1993). Later, a DNA topoisomerase II was purified to homogenity from *Sulfolobus shibatae.* This enzyme possessed a unique ability to relax both positively and negatively supercoiled DNA

(Bergerat *et al,* 1994). An ATP dependent topoisomerase was also isolated from the archaeon *Sulfolobus acidocaldarius* which could relax only negatively supercoiled DNA at high temperature. Yet, another topoisomerase was isolated from *Sulfolobus* (Strain B12) which shared common characteristics with *Escherichia coli* DNA topoisomerase II (Assiari, 1994). A DNA topoisomerase III was purified from *Desulfurococcus amylolyticus* which was found to be ATP independent relaxing topoisomerase (Slesarev *et al.*, 1991).

The archaeal DNA dependent RNA polymerases show striking homology immunologically and structurally with the eukaryotic polymerases (Huet *et al.,* 1983). Archaeal transcription machinery was found to be similar to RNA polymerase I, II and HI systems of eukaryotes (Baumann *et al.,* 1995b). Of the 13 subunits of the *Sulfolobus acidocaldarius* enzyme, the three largest (B, A' and A") subunits are homologues of eubacterial β and (3' but are still much closer in sequence to the largest subunits found in each eukaryotic RNA polymerase I, II and III (Huet *et al.*, 1983; Berghofer *et al.*, 1988; Zillig *et al.,* 1989). Of the ten smaller proteins, six are homologues of the eukaryote specific subunits shared in some combination among the eukaryotic RNA polymerases. Langer *et al* (1995) also reviewed work on the consensus archaeal promoter which is similar in sequence to its eukaryotic counterpart (Hausner *et al.,* 1991; Reiter *et al.*, 1990). *In vitro* transcription experiments using cell free extracts of methanogenic archaeon *Methanococcus thermolithotropicus* have shown that the function of aTFB (archaeal transcription factor B) could be replaced by eucaryal TATA binding proteins (TBP), thus identifying **aTFB** as an archaeal TATA binding protein (Gohl *et al.,* 1995). The degree of sequence similarity between the transcription factors of archaea and eukarya is quiet high suggesting that they could be performing analogous functions in archaea and eukarya respectively (Thomm, 1996).

A thermostable type II restriction endonuclease has been isolated from *Sulfolobus acidocaldarius* whose recognition sequence was found to be GGCC (Prangisvilli *et al,* 1985). In the hyperthermophilic archaeon *Sulfolobus shibatae* that grows between 75-85°C, a 60 kDa heat shock protein was found to be induced on exposure of the organism to heat shock at 85-90°C (Trent *et al,* 1990). This heat shock protein helps the organism

survive at lethal temperatures (Trent *et al,* 1994). This protein shared functional features with bacterial chaperonins (Trent *et al,* 1991; Phipps *et al,* 1993) viz; ATPase activity, recognition and binding of unfolded proteins and stimulation of protein folding. Surprisingly, on further studies these proteins showed no sequence similarity with the bacterial chaperonins. Instead, they showed remarkable identity to a group of non-heat shock proteins in eukaryotes known as TCP1s (Trent *et al.,* 1990; Kagawa *et al.,* 1995).

There is very little knowledge about the ribonucleases from the archaeal kingdom. Apart from non-specific degradation, RNases also bring about a variety of specific reactions that are required for RNA processing and turnover. Three proteins p1, p2 and p3 endowed with RNase activity have been purified and sequenced from *Sulfolobus solfataricus* (Fusi *et al.,* 1993). These proteins showed high sequence similarity to the 7 kDa group of DNA binding proteins isolated in *Sulfolobus* strains (Choli *et al.,* 1988a; Choli *et al.,* 1988b). However, the C terminal of p3 was found to be closely related to some eubacterial RNases (Fusi *et al.,* 1995a). The gene encoding p2 was synthesized and expressed in *Escherichia coli.* The recombinant protein produced was found to be indistinguishable in any of its catalytic properties from the *Sulfolobus solfataricus* native enzyme (Fusi *et al.,* 1995b). Further, NMR and photo CIDNP spectroscopies of the recombinant ribonuclease p2 showed that Trp23 and Phe31 residues may be involved in RNA binding (Consonni *et al,* 1995). The proteins p1, p2 and p3 show a narrow substrate specificity suggesting that they could be involved in RNA processing (Fusi *et al.,* 1995a). These RNases of the archaeal kingdom are unique as they do not contain any histidine residues in contrast to the RNases from other organisms. Yet, another thermostable ribonuclease of 9 kDa with DNA binding properties known as SaRD was isolated from *Sulfolobus acidocaldarius* (Kulms *et al.,* 1995).

RNaseP, a ribonucleoprotein enzyme that cleaves the precursor sequences from the 5'ends of pre-tRNAs has been characterized from two archaeal species namely *Sulfolobus acidocaldarius* and *Haloferax volcanii* (Nieuwlandt *et al.,* 1991; Darr *et al,* 1992; La Grandeur *et al.,* 1993). When compared to the bacterial counterparts these archaeal RNaseP RNAs were not found to be catalytic by themselves (Brown and Haas 1996; Haas *et al,* 1996). The genes encoding RNaseP RNA from eight more archaeal

species namely *Halococcus morrhuae*, *Natronobacterium gregoryi*, *Halobacterium cutirubrum*, *Halobacterium trapanicum*, *Methanobacterium thermoautotrophicum* strain AH, *M. thermoautotrophicum* strain Marburg, *Methanothermus fervidus* and *Thermococcus celer* strain AL-1 were cloned and a secondary structure model for archaeal RNaseP RNA was developed. The archaeal RNaseP were found to be similar in both primary and secondary structure to bacterial RNaseP RNAs, but not catalytically proficient all by themselves (Haas *et ai,* 1996).

An RNA endowed with endonuclease activity that catalyses the excision and maturation of 16S rRNA from pre-rRNA has been characterised from the hyperthermoacidophile *Sulfolobus acidocaldarius.* It showed sequence similarity to the eukaryotic U3 small nucleolar RNA thus implicating that U3 mediated processing of pre rRNA was not specific to eukaryotes (Potter *et al.*, 1995).

1.3 Histone like proteins from prokaryotes :

Chromosomal DNA is normally more than a thousand times longer than the cell in which it resides. Cells have specialized proteins that compact the DNA and make it more organized, thus making it accessible to proteins that regulate DNA activities. One level of compaction is brought about by highly basic, small abundant proteins called histones. The histones assemble the DNA into bead like structures called nucleosomes (Kornberg, 1974). Each nucleosome is composed of two copies each of the four core histones H2A, H2B, H3 and H4 forming an octamer around which the DNA is wrapped (McGhee and Felsenfeld, 1980; Igo-Kemenes *et al.*, 1982).

Like the eukaryotes, prokaryotes too compact their DNA within the cell. But the mechanism of packaging is still not very clear. Till this day, there is no clear evidence for a nucleosome like structure in bacteria. The intracellular DNA in bacteria is found to be in a condensed state called the nucleoid (Stonington and Pettijohn, 1971; Worcel and Burgi, 1972; 1974). The stabilization of the nucleoid is brought by a few basic DNA binding proteins, RNA and also the membrane (Pettijohn, 1982; 1988).

Prokaryotic cells lack histones, but they do contain a variety of small DNA binding proteins that compact DNA. These proteins are referred to as **histone like proteins** or "Chromatin associated proteins" (Ussery *et al,* 1994). Several groups purified proteins based on histone like properties from prokaryotes. Four proteins HU, H-NS, HLP1 and H have been identified as prokaryotic histone like proteins.(Drilica and Rouvierre-Yaniv, 1987). In addition two other proteins, the integration host factor (IHF) and factor for inversion stimulation (FIS) have been identified as potentially important proteins in DNA organisation. Among the histone like proteins identified from bacteria, HU protein or DBP II of *Escherichia coli* has been thoroughly characterized. The HU proteins are found to be highly conserved in different prokaryotes, eubacteria, blue green algae and also plant chloroplast (Drilica and Rouvierre-Yaniv, 1987). The *Escherichia coli* HU protein is a small basic protein (9 kDa) that was found to be associated with the bacterial nucleoid (Rouvierre-Yaniv and Kjelgaard, 1979). HU protein exists as a hetero-dimer composed of two subunits a and β that share almost 70% amino acid sequence homology. HU has been referred to variously by different groups such as H2 (Cukier Kahn, 1972), HD (Spassky *et al,* 1984) BH2 (Varshavsky *et al.*, 1977) or NS (Suryanarayana and Subramanian, 1978). Further studies on HU protein have shown that its prominent function is the wrapping of DNA into nucleosome like particles. Electron microscopy of these particles revealed that they contain on an average 8-10 dimers of HU and 275 bp of DNA (Rouvierre-Yaniv *et al,* 1979; Broyles and Pettijohn, 1986). It was found to have stimulatory effect on the initiation of DNA replication *in vitro* at the *Escherichia coli* replication origin (Dixon and Kornberg, 1984). It has also been shown to stimulate transcription of λ DNA (Rouvierre-Yaniv and Gross, 1975). A wide variety of site specific DNA-protein interactions either require HU or are stimulated by it. Among these are bacteriophage Mu transcription (Craigie *et al,* 1985; Lavoie and Chaconas, 1990) initiation of DNA replication (Dixon and Kornberg, 1988; Bramhill and Kornberg, 1988; Hwang and Kornberg, 1992) gene inversion (Johnson *et al,* 1986) and modulation of binding specificity of regulatory proteins, such as CRP (Flashner and Gralla, 1988), IHF (Bonnefoy and Rouvierre-Yaniv, 1992) and Lex A repressor. A

protein similar to HU has been purified from *Bacillus globigii* whose binding to DNA is non-specific and cooperative (Imber *et al.*, 1987).

IHF was originally identified as a factor required for bacteriophage lambda site specific recombination (Nash and Robertson, 1981). It is a 20 kDa heterodimer composed of a (11 kDa) and β (9 kDa) subunit. The subunits share 30% homology with each other and also with the members of HU family. But unlike HU, IHF binds to a specific DNA sequence (Leong *et al.*, 1985, Gardner and Nash, 1986).

H-NS protein (also known as H1a) was identified as an abundant protein of the *Escherichia coli* nucleoid which binds strongly to DNA. It is a neutral protein with no sequence homology to either HU or IHF (Pon *et al*, 1988). Unlike HU, H-NS does not wrap DNA *in vitro* or change the linking number of circular DNA. Yet, H-NS binding compacts DNA significantly as seen by large increase in sedimentation velocity of the DNA-protein complex. It binds DNA non-specifically with a preference for curved DNA sequence (Yamada *et al.*, 1990; Bracco *et al*, 1989). H-NS brings about transcriptional inhibition by binding to the promoters which contain curved DNA sequences (Owen-Hughes *et al.*, 1992; Ueguchi *et al*, 1993).

Recently H-NS was shown to be involved in the post transcriptional regulation of the rpoS gene (Takefumi *et al.*, 1995). Very little is known about the other histone like proteins. HLP-1 is 17 kDa protein coded by the Fir A gene (Varshavsky *et al.*, 1977; Lathe *et al*, 1980; Schaffer and Zillig, 1973). The product of Fir A gene was identified by a mutation that suppressed a rifampicin resistant mutation of RNA polymerase. It has been suggested that the 17 kDa HLP 1 could be a part of the scaffold structure and might also be responsible for maintaining the independent supercoiled domains observed *in vivo* (Sinden and Pettijohn, 1981).

TF1 is a histone like protein encoded by the *Bacillus subtilis* bacteriophage SPO1 ((Johnson and Geiduschek, 1972). TF1 is a 22 kDa homodimer which binds and bends DNA. It shows preference for DNA that contains hydroxy methyl uracil instead of thymine (Greene *et al.*, 1986b). TF1 recognizes specific sites on SPO1 chromosome and binds to these sites forming nested complexes on either side through protein-protein

interaction (Greene *et ah,* 1986a). Thus, TF1 organises the SPO1 DNA into a chromatin like structure.

1.4 Histone like proteins of Archaea :

The first histone like protein identified in Archaea is HTa from *Thermoplasma acidophilum* (Searcy, 1975; Stein and Searcy, 1978). HTa is a low molecular weight basic protein that closely resembles the eukaryotic histones. It is acid soluble with a strong net positive charge and is composed of 89 amino acids. This protein strongly stabilizes DNA against thermal denaturation. It was found to show significant homology to eukaryotic histones H2A, H3 and to *E. coli* HU protein (Searcy and Delange, 1980b). HTa was found to bind tightly to DNA forming nucleosome like nucleoprotein complexes. Thus protecting the DNA from nuclease digestion. The protected fragment size of DNA was about 40 bp (Searcy and Stein, 1980a; Searcy, 1986). Later, it was shown by immunochemistry that this protein is localized in the intracellular DNA (Bohrmann *et ah,* 1990).

MC1 (previously Hmb) was identified as the most abundant histone like protein present in various species of *Methanosarcinacea* (Chartier *et ah,* 1985). It is a 93 aminoacid polypeptide with a marked hydrophilic character (Chartier *et ah,* 1989). MC1 preferentially binds to double stranded DNA in a non-cooperative manner (Culard *et ah,* 1993) and can protect DNA against thermal denaturation (Chartier *et ah,* 1988). Electron microscopy of MC1 DNA complexes have revealed that MC1 binding does not induce a DNA wrapping around the protein like the core of histones (Toulme *et ah,* 1995). However, significant changes in DNA structure were observed on MC1 binding like formation of DNase I hypersensitive sites, sharp DNA bending and compaction of relaxed circular DNA (Laine *et ah,* 1991; Teyssier *et ah,* 1994; Toulme *et ah,* 1995). These changes suggest that supercoiling may alter MC1 binding. It was found to preferentially bind negatively supercoiled DNA inducing a drastic DNA conformational change leading to increasing electrophoretic mobility of DNA (Teyssier *et ah,* 1996). It has been

speculated that *in vivo* MC1 preferentially binds to DNA loops and assists specific proteins in building higher order structures via sharp bending.

Green *et al* (1983) reported isolation of two chromosomal basic DNA binding proteins from the organism *Sulfolobus acidocaldarius*. The two proteins were HSa (Mr. 14000) and NHSa (Mr. 36000). Together these proteins protected about 5% of the DNA against the nuclease digestion but could not afford protection against thermal denaturation.

Grote *et al* (1986) purified a set of small basic and abundant DNA binding proteins from *Sulfolobus acidocaldarius* with molecular weights 7 kDa, 8 kDa and 10 kDa. In the first group five proteins (7a-7e) have been identified, while in the second and third groups two proteins each were present (8a, 8b and 10a, 10b). The 7 kDa proteins show microheterogeneity in their sequences and are probably evolved through gene amplification (Choli *et al.,* 1988b). Electron microscopic studies of the protein DNA complexes of 7, 8 and 10 kDa proteins showed that they bind both ssDNA and ds DNA to form compact structures (Lurz *et al.,* 1986). Similar kinds of proteins from *Sulfolobus solfataricus* (Kimura *et al.*, 1984; Choli *et al.,* 1988a) have also been identified.

The 7 kDa group in *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus* have been characterised in more detail. The 7 kDa group has a characteristic N-terminal sequence of alternating lysine residues in the order of X-K-X-K-X-K-X-K (Choli *et al.,* 1988). The structure of the 7 kDa protein from *Sulfolobus solfataricus* Sso 7d was determined using NMR spectroscopy (Baumann *et al.*, 1995a). Sso 7d binds strongly to double stranded DNA and increases the melting temperature of DNA by 39°C upon binding. Thus, the biological function of this protein is to protect the genetic material in *Sulfolobus solfataricus.* Recently, the solution structure of 7d protein from *Sulfolobus acidocaldarius* Sac 7d has been reported (Edmondson *et al,* 1995). The genes for two Sac 7 DNA binding proteins Sac 7d and Sac 7e from the extreme thermophilic archaeon *Sulfolobus acidocaldarius* have been cloned into *Escherichia coli* and sequenced (Mc Afee *et al,* 1995). The thermodynamics of the binding of Sac 7d protein to double strand DNA has been characterised using spectroscopic signals from both the protein and DNA (McAfee *et al,* 1996).

Recently, a 7 kDa protein was identified from *Sulfolobus solfataricus* that exhibited high affinity for homologous DNA and was able to stabilize it against the thermal denaturation. The protein was able to protect DNA from DNase I digestion in a dose dependent manner (Faraone and Farina, 1995). The aminoacid composition of this protein showed a high content of lysine and glutamic acid residues. The partial aminoacid sequence of the protein suggests that it could belong to the 7 kDa protein family studied in *Sulfolobus acidocaldarius* (Kimura *et al.*, 1984; Choli *et ed.,* 1988a, 1988b). It has been shown that this protein of *Sulfolobus solfataricus* can be ADP-ribosylated *in vitro* . The ADP-ribosylation reaction was found to be NAD dependent. Ribosylation was brought about enzymatically via ADP ribosylating enzymes and also by chemical attachment of free ADP ribose (Faraone *et al.,* 1995). The ADP ribosylating enzymes in *S. solfataricus* were detected immunologically using antibodies raised against eukaryotic mono (ADP ribose) transferase and poly (ADP ribose) polymerase (Faraone *et al.,* 1996).

Another DNA binding protein Hmf was isolated from the archaeal hyperthermophile *Methanothermus fervidus* which was found to compact double stranded DNA into nucleosome-like structures NLSs (Sandman *et al.*, 1990). HMf is a mixture of two very similar polypeptides Hmf 1 (hmf A) and Hmf 2 (hmf B). These polypeptides Hmf A and Hmf B (molecular masses of 7468 and 7667 kDa respectively) were 84% identical in their amino acid sequence and were closely related to eukaryal nucleosome core histones at primary sequence level. Thus, this suggests that the archaeal and eukaryal histones must have evolved from a common ancestor and may be related structurally and functionally.

It was recently observed that the ratio of Hmf 1 and Hmf 2 could vary substantially. The composition of Hmf preparations is growth phase dependent and the homodimers of Hmf A and Hmf B do have different DNA binding properties consistent with forming NLSs in active and inactive regions of the genome (Sandman *et al.,* 1994). The DNA in the nucleosome like structures formed by Hmf binding is constrained in positive supercoils rather than negative supercoils as seen in eukaryotic nucleosomes. Hmf binding increases the helical periodicity of DNA molecules by approximately 11 bp/

turn thus resulting in positive supercoils. Electron microscopic studies of Hmf-DNA complexes have shown that around a core of four Hmf molecules 90-150 bp of DNA was wrapped in 1.5 positive toroidal supercoils (Musgrave *et al.*, 1991). Recently, the three dimensional structure of Hmf B dimer was determined by nuclear magnetic resonance methods and was found to be very similar to the dimer subunits within the histone core octamer of chicken nucleosome. The structure thus confirmed that this archaeal protein Hmf B is a histone (Starich *et al.*, 1996).

Very recently, it has been shown that the mesophillic methanogen *Methanobacterium formicum* JF1 contains three archaeal histones HFoA1, HFoA2 and HFoB. The encoding genes have been cloned and sequenced. The Hfo histones are 75-82 % identical to the Hmf sequences. The Hfo proteins too bind and compact DNA into nucleosome like structures identical to those formed by Hmf proteins (Darcy *et al.*, 1995). Another histone-like protein HANI has been identified from the archaeon *Thermococcus* SpAN1. The gene for this protein han1A has been cloned and sequenced. The protein showed homology with archaeal histone like proteins of "Hmf family" and also eukaryotic histone H4 (Ronimus and Musgrave, 1996).

Yet, another 9 kDa thermostable protein was isolated from the extremophile archaeon *Sulfolobus acidocaldarius* which showed ribonuclease activity as well as DNA binding properties. It was designated as SaRD (Kulms *et al.*, 1995). It showed amino acid sequence similarities to different RNases as well as DNA binding proteins from thermophilic archaea.

Reddy and Suryanarayana (1988; 1989) isolated nucleoid for the first time from the archaeon *Sulfolobus acidocaldarius* and purified four low molecular weight, basic, DNA binding proteins namely HSNP-A (12 kDa), HSNP-C (9 kDa), HSNP-C' (8 kDa) and DBNP-B (11 kDa). Three of these proteins HSNP-A, C and C' showed strong helix stabilizing properties and protected DNA against thermal denaturation whereas the other protein DBNP-B failed to show such an effect. DBNP-B, now referred to as Renaturase binds strongly to single and double stranded DNA. The protein aggregates nucleic acids and promotes renaturation of complementary single stranded DNA (Sreenivas, PhD thesis; 1994). It has been reported recently that HSNP-A, HSNP-C and HSNP-C from

Sulfolobus acidocaldarius are present exclusively on the ribosome free genomic DNA of the cell suggesting that they could be responsible for DNA compaction and genome organization (Bohrmann *et al.,* 1994). HSNP-C' was shown to be immunologically related to Sac 7d protein and condenses DNA into compact structure (Celestina and Suryanarayana, 1995).

1.5 High Mobility Group proteins :

Apart from histones, the other group of chromosomal proteins that are involved in assembly of nucleosomes are the HMG proteins. They are less abundant than histones and demonstrate some specificity of binding to various DNA conformations. Though these proteins have been studied extensively, their cellular role still remains undefined.

Thus HMG proteins have been defined as chromosomal proteins that are extractable from chromatin in 0.35 M NaCl, soluble in 2-5 % perchloric acid with molecular weight lower than 30,000 and contain high content of charged amino acids (Johns EW, 1982; Bustin *et al.,* 1990). The HMG proteins can be grouped into three distinct families- the HMG 1/-2 family, the HMG 14/-17 family and the HMG-I family.

1.5.1 HMG 1/-2 family:

It comprises of proteins that have molecular weights of approximately 25,000 and is the most abundant of HMG family. The HMG1 and HMG2 bind to both double and single stranded DNA with a preference for the latter (Isackson *et al.,* 1979, Bonne *et al.,* 1982). They can also distinguish different single stranded conformations and show a preference for interactions with cruciform DNA (Hamada and Bustin, 1985; Bustin and Soares, 1985; Bianchi *et al.*, 1989). There are reports which suggest that this group of proteins may play a role in chomosomal replication (Bonne *et al.*, 1982, Duguet *et al.*, 1978, Alexandrova *et al.*, 1984). Antibodies to HMG1/2 were found to inhibit DNA synthesis (Alexandrova *et al.*, 1984). There are also reports showing that these proteins

can stimulate DNA polymerases (Duguet and DeRecondo, 1978; Alexandrova and Beltchev, 1988). They may also function in some aspects of transcription (Tremethick and Molloy, 1988; Watt and Molloy, 1988).

1.5.2 HMG 14/17 family :

It consists of proteins with a molecular weight of 10,000-12,000 kDa and are found to have higher affinity for nucleosomes rather than DNA (Sandeen *et al.*, 1980, Yau *et al.,* 1983). Each nucleosome has two putative binding sites for either HMG 14 or HMG 17 where these proteins bind and affect histone DNA interactions (Shick *et al,* 1985). Several experimental evidences suggest that HMG 14 and HMG 17 may modulate the chromatin structure of transcriptionally active genes (Weisbrod *et al,* 1980, Weisbrod, 1982 a; 1982 b). Reconstitution studies too indicate that these proteins preferentially bind to nucleosomes enriched in active sequences (Sandeen *et al.,* 1980).

1.5.3 HMG-I family :

It is comprised of the protein HMG-I (also known as a protein) and its isoform HMG-Y. These two proteins resemble the HMG 14/17 proteins in their amino acid composition. Rapidly growing, transformed, or malignant cells and neoplastic tissue are enriched in these proteins (Vartiainen *et al.,* 1988; Johnson *et al.,* 1988). These proteins could have a possible role in nucleosome phasing or condensation of AT rich DNA into nucleosomes (Strauss and Varshavsky, 1984).

The **HMG box** is a novel type of DNA binding domain which is present in the high mobility group of proteins HMG1 and HMG2. The **HMG box** has a set of highly conserved aromatic and basic residues. The HMG box motif s are unique with respect to the othe: DNA binding motifs identified so far as they have the ability to recognize four-way junctions (Bianchi *et al.*, 1992., Ferrari *et al.,* 1992). Several **HMG box** containing proteins have been identified such as the nucleolar transcription factor UBF (Jantzen *et*

al, 1990), the lymphoid transcription factors TCF-1 and LEF-1 s(Van de Wetering *et al,* 1991; Travis *et al.,* 1991; Waterman *et al.,* 1991), the fungal mating type genes mat-Mc and MATAl(Kelly *et al.,* 1988; Staben and Yanofsky, 1990). and the mammalian sex determining factor SRY (Sinclair *et al,* 1990) and the mitochondrial transcription factor mt TF1 (Parisi and Clayton., 1991).

Thus the **HMG family** members are found in animals, plants and yeast. By aligning the HMG box sequences of all known members phylogenetic trees were constructed in order to understand their evolution. This kind of analysis gave rise to two distinct families - one with a single sequence specific HMG box and the other consisting of relatively non specific DNA binding proteins with multiple HMG boxes (Laudet *et al,* 1993).

HMG like proteins have been isolated from lower eukaryotes like *Saccharomyces cerevisae, Neurospora crassa, Dictyostelium discoideum, Aspergillus nidulans, Tetrahymena pyriformis* and *Tetrehymena thermophila.* The complete aminoacid sequence of HMG like proteins from *T. thermophila* (Roth *et al.,* 1989) and *T. pyriformis* (Hayashi *et al,* 1989) have been determined. The non histone chromosomal protein from *S. cerevisae* has been isolated and its amino terminal sequence shows 40% identity to the HMG1 of calf thymus (Kolodrubetz and Burgum, 1990).

SCOPE AND OBJECTIVES OF THE PRESENT INVESTIGATION

Sulfolobus acidocaldarius a thermoacidophilic sulfur dependent crenarcheote is unique in its survival at extreme conditions of temperature (75-80°C) and acidic pH of 3.0. The genetic material in this organism needs an effective compaction mechanism along with thermal stability. The reported G + C content of DNA and the intracellular salt concentration are very low, thus reinforcing the need for intracellular factor or components that confer stability to the DNA in the organism.

The organisation of DNA has been extensively studied and the 'nucleosome' was derived as the basic unit of organisation. The structural organisation of intracellular DNA in prokaryotes is still not well understood. Hence, in an attempt to understand the thermal stability of intracellular DNA and its organisation, four low molecular weight DNA binding proteins namely HSNP-A, HSNP-C' and HSNP-C (HSNP-helix stabilizing nucleoid associated protein) were found to stabilize DNA against thermal denaturation. The fourth protein DBNP-B showed no such helix stabilizing property. HSNP-C is the most abundant of the three nucleoid associated helix stabilizing proteins implicating the involvement in structural organization of genetic material in the organism. Further work has supported this aspect showing its localization on the ribosome free domains of genomic DNA by electron microscopic studies (Bohrmann *et al.*, 1994).

HSNP-C is very similar to 7 kDa group of DNA binding proteins isolated from Sulfolobus species (Choli *et al*; 1988a, 1988b; Grote *et ai*, 1986). The 7 kDa group comprises of five proteins (7a, 7b, 7c, 7d and 7e) with microheterogeneity in the aminoacid sequence. Hence in an attempt to understand the role of these helix stabilising isoforms with respect to DNA compaction and condensation, three of the abundant isoforms corresponding to 7c, 7d and 7e (HSNP-C'*a*, HSNP-C and HSNP-C'*b*) have been purified characterised with respect to their DNA protein interactions. We have employed a variety of techniques like fluorescence titrations, gel retardation assays, affinity chromatography on nucleic acid matrices, nuclease protection assay, DNA

aggregation and reanaturation assays to study the nucleic acid binding properties of HSNP-C' and its isoforms.

The present study shows some important properties of the protein like aggregation of DNA, which increases the effective concentration of DNA facilitating reactions like renaturation of complementary strand pairing. Apart from its involvement in DNA organisation, the study also reflects its multifunctional potential. Nucleotide binding i.e. GTP binding and the associated GTPase activity, ribonuclease activity are the manifestations of the multifunctions of HSNP-C and its isoforms.

CHAPTER 2

MATERIALS AND METHODS

2.1 Organisms used in the present study :

Sulfolobus acidocaldarius strain DSM 639 was obtained from Deutche Sammulung Von, Mikroorganismen, Gottignen, Germany. *Escherichia coli* A19 was obtained from the laboratory of Dr.A.R.Subramanian, Max planck Institute for Molecular Genetics, Berlin, Germany.

2.1.1 Growth of the organisms :

Sulfolobus acidocaldarius was grown at 75°C for 40-48 hrs with vigorous aeration in a medium containing 0.1% yeast extract, 0.1% bactotryptone, 0.5% casamino acids, 0.1%, glucose, 0.2% sodium chloride, 0.13% ammonium sulphate, 0.03% potassium dihydrogen phosphate, 25 mM magnesium sulphate, 0.07% calcium chloride and pH was adjusted to 2.8 with 1 M sulfuric acid (Kikuchi and Asai, 1984). The growth of the cells was arrested at mid logarithmic phase by neutralizing the culture with 1 M Tris base. The cells were harvested thereafter by centrifugation at 7,000 RPM (in a GS-3 rotor of Sorvall RC5C centrifuge) for 15 minutes. The cell pellets were finally suspended in 10 mM Tris-HCl (pH 7.6), 50 mM KCl, 10 mM magnesium acetate and 7 mM mercaptoethanol ($TKM_{10}Me_7$), centrifuged at 8,000 RPM for 30 minutes and the final pellets were stored frozen at -80°C untill further use. *E. coli* was grown in enriched medium at 37"C with good aeration (Minks *et al.*, 1978).

2.2 Purification of HSNP-C and its other forms :

2.2. 1 Isolation of post-ribosomal supernatant (S-100) :

Cells harvested at mid logarithmic phase were ground with double the weight of alumina at 4°C till a soft and sticky paste was obtained. Alternatively, the cells were

lysed by the addition of Triton X 100 and NP-40 to the cell suspension to a final concentration of 0.1% and 0.5% respectively. The cell lysate was extracted with buffer (3 ml per gram of cells) containing 20 mM sodium phosphate (pH 7.0), 50 mM NaCl, 10 mM magnesium acetate, 7 mM mercaptoethanol and 2 µg DNase I (RNase free) per gram of cells. The crude cell extract was centrifuged at 30,000 X g for 30 minutes at 4°C to remove the debris. The supernatant (S-30) obtained was centrifuged at 1,00,000 X g for $4^1/_2$ hours to pellet the ribosomes. The upper 2/3 rd of the supernatant was collected carefully (S-100) and was used as the starting material for purification of HSNP-C and its isoforms.

2.2.2 Isolation of acid soluble proteins :

Acid soluble proteins from the post ribosomal supernatant (S-100) were extracted with one tenth volume of 2.75 M sulfuric acid, added drop wise with slow stirring at 4°C. The acid treated sample was left for slow stirring at 4°C for 4 hours. The precipitated proteins were removed by centrifugation. The clear supernatant obtained was dialysed against buffer containing 20 mM sodium phosphate (pH 7.0), 40 mM NaCl, 1 mM Na_2-EDTA and 7 mM mercaptoethanol for 20 hours. It was further clarified by centrifugation to remove any proteins precipitated during dialysis. This acid extract is enriched with four basic proteins HSNP-A, DBNP-B, HSNP-C and HSNP-C. HSNP-C and its isoforms were further purified by ion exchange chromatography.

2.2.3 Purification of HSNP-C and its forms from **S-100** acid **extract :**

HSNP-C and its isoforms were purified according to Grote *et al* (1986) and Reddy and Suryanarayana (1988; 1989) with some modifications. The dialysed acid extract was applied on to a CM-cellulose column (2 X 12 cm) previously equilibrated with the column buffer (20 mM sodium phosphate, 40 mM NaCl, 1 mM Na_2-EDTA, 7 mM mercaptoethanol). The bound proteins were eluted with a linear gradient of 0.04 M - 0.4 M NaCl containing column buffer. Fractions of 5 ml were collected at a flow rate of 30 ml/hr and aliquots from alternate fractions (20 µl) were analysed by SDS-PAGE. The

protein eluted as three peaks depending on the basicity of these proteins. The first peak contained **HSNP-C'*a*** which co-eluted with another helix stabilising protein HSNP-A The second peak contained pure **HSNP-C'** and the third peak contained **HSNP-C'*b*** which co-eluted with another helix stabilising protein HSNP-C. These three forms namely **HSNP-C'a, HSNP-C** and **HSNP-C'*b*** correspond to proteins 7c, 7d and 7e respectively as described by Grote *et ai,* 1986. The fractions corresponding to each peak were pooled separately for further purification.

The peak I pool was dialysed to bring down the salt concentration to 100 mM NaCl. The proteins in the peak I pool were separated by affinity chromatography on double stranded DNA cellulose column. The dialysed protein solution was applied onto a double stranded DNA cellulose column of 5 ml capacity equilibrated with column buffer containing 100 mM NaCl. The bound proteins were eluted with 250 mM NaCl containing column buffer. Fractions of 1 ml were collected with a flow rate of 10 ml/hour. The alternate fractions were analysed by 15% SDS-PAGE. The fractions containing pure **HSNP-C'a** were pooled and dialysed against 10 mM Tris (pH 7.6).

The peak II pool was dialysed to bring down the salt concentration to 40 mM NaCl and then rechromatographed on a smaller column of CM-cellulose. The column was eluted with buffer containing 500 mM NaCl. The peak fractions containing pure **HSNP-C** were pooled and dialysed against 10 mM Tris (pH 7.6).

The peak III pool was dialysed against dilute acid (0.1 M H_2SO_4) for 12 hours, redialysed against column buffer and then rechromatographed on a smaller column of CM-cellulose. The protein was eluted with column buffer containing 0.5 M NaCl. The peak fractions containing pure **HSNP-C'*b*** were pooled and dialysed against 10 mM Tris (pH 7.6).

2.2.4 Concentration of the protein :

Further concentration of the purified proteins namely HSNP-C'a, HSNP-C and HSNP-C'*b* were achieved by lyophilisation. The lyophilised samples were dissolved in

small volumes of 10mM Tris-HCl pH 7.6 and dialysed against the same. All the protein samples were stored at -20°C until use.

2.2.5 Protein estimation :

Protein concentration was determined by the Folin reagent (Lowry *et al*., 1951) using bovine serum albumin as a standard.

2.3.1 Antibodies to HSNP-C :

Antiserum to purified HSNP-C was raised in rabbit as described by Stoffler and Wittmann, (1971). About 200 μg of protein was emulsified in Freund's complete adjuvant and injected subcutaneously into rabbit at multiple sites. Booster injections each of 50 |ig of protein in Freund's incomplete adjuvant were given subcutaneously at 3rd, 4th, 5th and 6th weeks. Rabbits were bled after third booster injection through the pinna vein. Antiserum was collected after centrifugation of the clotted blood.

2.3.2 Isolation of IgG :

The IgG from the antiserum to HSNP-C was purified by adsorption to protein A (Sambrook and Maniatis, 1990). Antibodies bind to protein A mainly by hydrophobic interactions and can be disrupted at low pH.

A one ml column of protein A-sepharose was equilibrated with 0.1 M Tris-HCl (pH 8.0). The antiserum (1 ml) was adjusted to 0.1 M Tris-HCl (pH 8.0) and loaded onto the column. The bound IgG was eluted with five bed volumes of 100 mM glycine (pH 3.0). Fractions of 500 μl were collected in 1.5 ml Eppendorf tubes containing 50 μl of 1 M Tris (pH 8.0). The fractions containing IgG were identified by measuring their absorbance at 280 nm. The fractions that showed high absorbance (OD_{280} of 1.3) were pooled, dispensed into aliquots and stored at -20°C until use. The residual proteins from the column were removed by washing the column sequentially with 10 volumes of 3 M

urea, 1 M LiCl and 100 mM glycine (pH 2.5). The pH of the column was readjusted to 8.0 by washing it with 10 volumes of 1M Tris (pH 8.0). The column was stored at 4°C in 100 mM Tris-HCl (pH 8.0) containing 0.02% sodium azide.

2.4.1 SDS-Polyacrylamide gel electrophoresis :

Polyacrylamide gel electrophoresis was performed in 15% gel slabs using the procedure of Laemmli (1970). The ratio of acrylamide to bisacrylamide in the gel was 15:0.04. The 15% resolving gel was in 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS. The stacking gel was 5% polyacrylamide in 0.125 M Tris-HCl pH 6.8 and 0.1% SDS. Protein samples were treated with 0.1% SDS and 1% 2-mercaptoethanol at 65°C for 15 minutes or boiled at 100°C for 2 minutes. The electrophoresis was run at 120 volts for 6-7 hours. The gels were removed when the bromophenol blue dye reached the bottom of the gel. The gels were washed with 7.5% acetic acid for 30 minutes and stained with 0.1% coomassie brilliant blue R-250 in 50% methanol and 7.5% acetic acid for 1 hour. The gels were destained with a solution containing 5% methanol and 7.5% acetic acid.

2.4.2 Silver staining :

The polyacrylamide gels were silver stained according to Blum and Gross (1987), with slight modifications. The gels were first fixed for 6 hrs in 10% acetic acid, 20% methanol containing 0.5 ml formaldehyde per liter solution. The gels were then washed once with 50% ethanol for 20 minutes followed by 30% ethanol for 20 minutes. Gels were then treated with sodium thiosulphate (0.2 gm per litre) for 1 minute and rinsed thrice with water (20 sec each). The gels were then impregnated with silver nitrate (0.5 gm per litre) containing 0.75 ml formaldehyde per litre, for one hour. Excess silver nitrate was washed off with two 30 seconds washes with water. Gels were then developed with a solution containing sodium carbonate (30 gm), sodium thiosulphate (4 mg) and (0.5 ml) formaldehyde per litre. Colour development was stopped by washing the gel with water followed by soaking the gel in 10% acetic acid and 10% methanol.

2.5 Western Blotting :

Western blotting was done according to Towbin *et al.*, (1979) with minor modifications. Proteins were separated on 15% SDS-PAGE (Laemmli, 1970) and electrophoretically transferred on to a nitrocellulose membrane (0.45 μm pore size) using the transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS and 20% v/v methanol, pH 8.3). After transfer, the blots were air dried and soaked in 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% NP-40, 3% BSA (blocking buffer) for 2 hours. The blots were then incubated with diluted anti HSNP-C' IgG (1:1000 - 1:5000) in the above buffer. After 2 hours, the blots were washed 3 times for 10 minutes each in the above blocking buffer and incubated with secondary antibody (anti rabbit IgG from goat) coupled to alkaline phosphatase at a dilution of 1 : 5000 in blocking buffer. The blots were washed thoroughly in alkaline buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$). The colour development was done in freshly prepared solution of nitroblue tetrazolium (66 μl from 50 mg/ml stock) and 5-bromo 4-chloro 3-indolyl phosphate (33 μl from 50 mg/ml) in 10 ml of alkaline buffer.

2.6 Aminoterminal sequencing of HSNP-C :

The aminoterminal of HSNP-C was sequenced on Applied Biosystems 477A sequencer and analysed on a 120A HPLC analyser at Molecular Core Facility, HMC, Hershey, PA (U.S.A). About 20 picomoles of the protein was blotted onto a PVDF membrane for sequencing. The sequencing was kindly financed by Dr. K. Sreenivas, post doctoral fellow, Hershey.

2.7 Protein protein interactions of HSNP-C :

2.7.1 Formaldehyde crosslinking of HSNP-C (self aggregation):

HSNP-C in 10 mM Triethanolamine-HCl (pH 7.6) and 50 mM sodium chloride was reacted with 1% formaldehyde (pH 7.6) at 23°C (Jackson, 1978). At different time intervals, small aliquots were taken out and the crosslinking was quenched by the addition of TCA to 10% final concentration. The TCA precipitates were collected by centrifugation, washed with acetone and dissolved in SDS gel loading buffer. The crosslinked products were analyzed by electrophoresis on 15% SDS-polyacrylamide gels (Laemmli, 1970).

2.7.2 Interaction of HSNP-C with other proteins :

2.7.2.1 Coupling of HSNP-C'to epoxy activated sepharose :

One gram of epoxy activated sepharose 6B was pre-swollen in distilled water on a sintered glass funnel. It was then washed with coupling solution (0.2 M $NaHCO_3$ pH 9.5 containing 0.5 M NaCl). HSNP-C (4 mg) was dialysed against coupling solution . This was then mixed with the moist cake of epoxy sepharose in a mini beaker and left for shaking on a shaker water bath for 16 hours at 4°C. The excess ligand was washed off using coupling solution followed by distilled water, 0.1 M bicarbonate buffer pH 8.0 and finally 0.1 M sodium acetate pH 4.0. The excess reactive groups were blocked with 1 M ethanolamine overnight. The excess ethanolamine was washed off by washing, with 1 M bicarbonate buffer pH 8.8 containing 0.5 M NaCl.

2.7.2.2 Isolation of protein complexes that bind to HSNP-C':

The resin epoxy sepharose HSNP-C was packed into a small column of 3 ml capacity and equilibrated with column buffer containing 10 mM Tris-HCl (pH 7.6), 40 mM NaCl and 1 mM EDTA. The sample i.e. S-100 or post ribosomal supernatant was dialyzed against the column buffer and then loaded onto the epoxy sepharose HSNP-C column at a slow flow rate of 4 ml/hr. The column was left overnight at 4°C and then washed thoroughly with 20 bed volumes of column buffer. The bound proteins were eluted with buffer containing 250 mM NaCl and followed by buffer containing 500 mM NaCl. The alternate fractions were electrophoresed on 15% SDS-PAGE to analyse the proteins that bound to HSNP-C. The gel was stained with coomassie blue.

2.7.3 Interaction of HSNP-C with other proteins analysed by gel filtration : (Sephacryl S-200 chromatography):

In this experiment the post ribosomal supernatant (S-100) was subjected to gel filtration chromatography on Sephacryl S-200 and the fractions obtained were analysed by SDS-PAGE to study the aggregation of HSNP-C and its interaction with other proteins. Preswollen Sephacryl S-200 was deaerated and then packed onto long thin column (100 ml) and equilibrated with 20 mM Tris-HCl (pH 7.6), 200 mM KC1, 1 mM EDTA. S-100 (post ribosomal supernatant) was dialysed against the same buffer and then loaded onto the S-200 column. Fractions of 1 ml were collected from the column at a flow rate of 4 ml/hour. Alternate fractions were analysed for presence of protein by SDS-PAGE and immunoblotting.

2.8 Agarose gel electrophoresis :

The DNA in gel mobility shift and renaturation assays were separated and analyzed by electrophoresis on 0.6% - 1.0% agarose gels. The electrophoresis was carried out in either low ionic strength TAE buffer (Lohman *et al.*, 1986) for gel retardation assays or in 40 mM Tris acetate (pH 7.8) and 1 mM Na_2-EDTA buffer. After electrophoresis, the gels were stained in 0.5 μg/ml ethidium bromide in electrophoresis buffer and photographed after UV illumination.

2.9 Gel mobility shift assay :

Gel mobility shift analysis of HSNP-C nucleic acid complexes was carried out exactly as described by Lohman, *et al* (1986). The reaction was carried out in 30 μl reaction volume in 10 mM Tris-HCl (pH 7.0), 0.1 mM Na_2-EDTA buffer containing 25 mM NaCl. HSNP-C nucleic acid complexes were formed at different protein to nucleic acid ratio and electrophoresed on 0.7% agarose gel in 20 mM Tris-HCl (pH 7.8), 0.4 mM sodium acetate, 0.2 mM Na_2EDTA. The gels were then stained in 0.5 μg/ml ethidium bromide in electrophoresis buffer and photographed under UV illumination.

2.10 Isolation of DNA from *Sulfolobus acidocaldarius :*

DNA from *Sulfolobus acidocaldarius* was isolated by a slight modification of the procedure described by Hempstead, (1990). The cell culture (1 litre) was neutralized with 1 M Tris base and the cells were then collected by centrifugation. The cell pellet was washed twice with buffer containing 50 mM Tris-HCl (pH 7.6) and 10 mM Na_2-EDTA ($T_{50}E_{10}$) and suspended in 1 ml of the same buffer and stored at -80^{0}C for 1 hour. The frozen cell pellet was rapidly thawed in a water bath at 60^{o}C and 1 ml of 1% SDS in $T_{50}E_{10}$ and 11 ul of proteinase K (10 mg per ml in 0.1 % SDS, T_{10} (pH 8.0) E_1) were

added. The mixture was incubated at 60°C for 15 min. RNA was digested by the addition of 11 µl of RNase A (5 mg per ml in 0.1 M sodium acetate, pH 4.8) and incubated at 37°C for 1 hour. SDS was precipitated by the addition of 100 µl of 5 M potassium acetate (untitrated) followed by incubation on ice for 30 minutes. The potassium-SDS precipitate was removed by centrifugation at 12,000 X g for 10 minutes. The supernatant was mixed with equal volume of chloroform : isoamyl alcohol (24 : 1 v/v) for 15 minutes at room temperature and the phases were separated by centrifugation. This extraction was repeated twice. The aqueous phase was precipitated by addition of 1/10 volume of sodium acetate (pH 4.8) and 2.5 volumes of 95% ethanol. The DNA was spooled on a glass rod, washed by immersion in 70% ethanol and briefly air dried to remove excess ethanol and dissolved in buffer containing 10 mM Tris-HCl (pH 7.6) and 1 mM Na_2-EDTA.

2.11 Isolation of ^{3}H labelled DNA from *Escherichia coli :*

E. coli DNA was labelled with (^{3}H Methyl) thymidine as described by Mahler, (1967), and the DNA was isolated according to Marmur (1961). The specific activity of DNA was 5,000 cpm per µg.

2.12 Isolation of M13 ssDNA and RF DNA :

M13 ssDNA and RF DNA were isolated according to Messing (1983). *Escherichia coli* JM 109 was grown at 37°C on 2% agar plate containing M9 medium with glucose as a carbon source. A single colony was picked and inoculated to 2x YT medium and allowed to grow to an A_{600} of 0.3. The cells were then infected with M13 mp 7 phage particles with an MOI of 10. Incubation was continued at 37°C for another 6 to 8 hours. The phage titer was usually 10" to 2 X 10^{12} per ml.

Infected cells were collected by centrifuging the culture at 6,000 X g for 10 minutes at 4°C. The supernatant containing M13 phage particles was centrifuged again to remove any bacterial cells. The supernatant was made 0.5 M in NaCl and 6% in PEG

respectively and incubated at 4°C for 60 minutes. The turbid solution was centrifuged at 10,000 X g for 15 minutes and the supernatant was removed carefully without disturbing the M13 phage pellet. The pellet was suspended in 0.3 volumes of 10 mM Tris-HCI (pH 7.6) and 0.1 mM Na_2-EDTA and the phage was reprecipitated with 6% PEG 6,000 and 0.5 M NaCl as mentioned above. The phage precipitate was collected by centrifugation at 10,000 X g for 15 minutes and the pellet was resuspended in 0.15 volumes of buffer containing 10 mM Tris-HCI (pH 7.6) and 0.1 mM Na_2-EDTA. The M13 ssDNA from the collected phage particles was isolated by extracting the phage solution once with buffered phenol followed by buffered phenol : chloroform. The M13 ssDNA was concentrated by precipitation with ethanol and the DNA was dissolved in small volume of buffer containing 10 mM Tris-HCI (pH 7.6) and 0.1 mM Na_2-EDTA.

M13 RF DNA from the infected cell pellet was isolated by the procedure of Sambrook and Maniatis (1989). The cell pellet containing RF was suspended in 50 mM glucose, 10 mM EDTA and 25 mM Tris-HCI (pH 8.0). To the cell suspension, 0.5 ml of freshly made 20 mg/ml lysozyme in the above buffer was added, mixed and incubated on ice for 10 minutes. Then, 5 ml of 0.2 M NaOH and 1% SDS were added, mixed gently and incubated on ice for 10 minutes. 5 M potassium acetate (4 ml) was added to the above mixture and the suspension was mixed by swirling. The suspension was left on ice for 10 minutes. The white pellet was removed by centrifugation at 12,000 X g for 15 minutes. The clear supernatant was taken out carefully without picking up the precipitate and incubated with DNase free RNase A (20 µg per ml final concentration) at 37°C for 30 minutes. The DNA was then extracted with buffered phenol followed by buffered phenol : chloroform. The aqueous phase was collected, mixed with 1/1 Oth volume of 3 M sodium acetate (pH 4.8) and 2.5 volumes of ethanol. The DNA was collected by centrifugation, washed once with 70% ethanol and dissolved in a small volume of 10 mM Tris-HCI (pH 7.6) and 0.1 mM Na_2-EDTA. The RF DNA was linearised by incubating DNA with Eco RI followed by phenol-chloroform extraction and precipitation with ethanol.

2.13 Preparation of **ssDNA** and **dsDNA** cellulose :

ss DNA and dsDNA cellulose were prepared according to the method described by Alberts and Herrick (1971).

2.13.1 Activation of cellulose :

The cellulose was washed several times with boiling ethanol to remove pyridine. It was then quickly washed at room temperature successively with 0.1 M NaOH, 1 mM Na_2-EDTA and 10 mM HC1 solutions respectively. It was later washed with water to neutrality and used for preparation of DNA cellulose.

2.13.2 Coupling of DNA to cellulose :

To make DNA cellulose, a solution of DNA at 1-3 mg/ml in 10 mM Tris-HCl (pH 7.4), 1 mM Na_2-EDTA (Tris-EDTA) is transferred to a glass beaker. Clean, activated and dry cellulose was added with occasional stirring with the help of a flat bottomed glass rod until the paste thickens (~ 1 g cellulose per 3 ml). The lumpy mixture was spread in a petriplate and left at room temperature covered with a gauze until dry. The thoroughly dried cellulose powder was suspended in 20 volumes of 10 mM Tris(pH 7.4) and 1 mM Na_2-EDTA and left at 4°C for a day. After a quick wash to remove the free DNA, the DNA cellulose is stored as a frozen slurry in 10 mM Tris (pH 7.4), 1 mM Na_2-EDTA containing 0.15 M NaCl. To check the efficiency of coupling an aliquot of the DNA cellulose is taken in an Eppendorf tubes made upto 500 µl with buffer containing 0.15 M NaCl and heated at 100°C for 20 minutes. The A_{260} of the released DNA is measured.

To make ssDNA cellulose the DNA is denatured at 100°C in 10 mM KH_2PO_4, 1 mM Na_2-EDTA. After rapid cooling, the DNA is made upto the desired concentration (3 mg/ml) with 10 mM Tris-HCl (pH 7.4) 1 mM Na_2-EDTA and processed as above.

2.14 DNA cellulose chromatography :

In the present work DNA cellulose chromatography (ds/ss DNA) was used to assess the strength of binding of the purified proteins - HSNP-C'*a*, HSNP-C' and HSNP-*C'b* to **DNA.** ds DNA and ss DNA cellulose were packed into different columns and equillibrated with buffer containing 20 mM Tris-HCl (pH 7.6), 40 mM NaCl, 1 mM EDTA and 7 mM 2-mercaptoethanol. 50 μg of each of the purified proteins -HSNP-C'*a*, HSNP-C and HSNP-C'*b* were loaded separately onto each of these columns at a flow rate of 2 ml/hr. The columns were washed with 40 mM NaCl containing buffer and eluted with a step-wise gradient of 100-500 mM NaCl. Fractions of 0.2 ml were collected and analysed for the presence of protein by fluorescence intensity measurements and also by SDS-PAGE.

2.15 Fluorescence Titrations :

Fluorescence measurements of all protein samples were carried out in a FP-777 Jasco spectrofluorimeter. The measurements were performed in 10 mM Tris-HCl (pH 7.0) and 20-50 mM NaCl at 25°C. Excitation and emission band widths were 5 nm and 10 nm respectively. After each addition of nucleic acid to protein, the contents were mixed gently and allowed to stand for 30-40 seconds. The fluorescence emission intensities were recorded after the fluorescence signal was stabilized. Average of the three readings, within an interval of 60 seconds were taken for each titration point. The fluorescence units (arbitrary units) were corrected for dilution. We have checked for inner filter effects (if any) under our experimental conditions which were found to be negligible.

2.15.1 Reverse Titrations :

Aliquots of nucleic acid or other quenchers were added to a fixed concentration of HSNP-C (10 μg) in 1 ml of reaction buffer [10 mM Tris-HCl (pH 7.0); 50 mM sodium

chloride] and the decrease in fluorescence intensity was measured. The initial protein fluorescence was taken to be 100% and all other measurements were made with reference to the initial fluorescence of the protein. Titrations were also performed with different mononucleotides.

2.16 GTPase assay :

HSNP-C' was checked for polynucleotide dependent GTPase activity following the procedure of Ames and Dubin (1960) and Finger and Richardson (1981). Increasing amounts of HSNP-C was incubated with 10 μg per ml nucleic acid (ssDNA or dsDNA) in 40 mM Tris-HCl (pH 7.0), 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM Na_2-EDTA, 0.1 mM DTT and 1 mM GTP at 37°C for 15 minutes. The reaction was stopped by the addition of 1.1% ascorbic acid and 0.28% ammonium molybdate in 0.67 N sulfuric acid and the mixture was incubated at 45°C for 20 minutes. Readings were taken at 820 nm. An absorbance of ~ 0.8 corresponds to release of 50 n moles of Pi as a result of hydrolysis of GTP.

2.17 DNA Aggregation assays :

These experiments were performed as described by Krasnow and Cozzarelli (1982) with slight modifications. The reactions were carried out in 40 μl reaction volume in 10 mM Tris-HCl (pH 7.6), 1 mM DTT or 10 mM sodium acetate (pH 5.0), 1 mM DTT in the presence or absence of $MgCl_2$. The reactions were performed with both non labelled and ^3H labelled DNA.

2.17.1 Aggregation assay (radioactive method):

The reaction mixtures containing constant amount of ^3H *E. coli* DNA (native or denatured) were incubated at 37°C with increasing amounts of protein. After 30 minutes, the reaction mixtures were centrifuged for three minutes at 14,000 X g . The top

20 µl were removed carefully and added to 100 µl of 0.1% SDS in water. To the bottom 20 ul, 100 µl of 0.1% SDS was added. Radioactivity in the top and bottom fractions was measured in a liquid scintillation counter. Aggregation was defined as decrease in radioactivity in top 20 ul relative to an unsedimented control mixture.

2.17.2 Aggregation assay (non-radioactive method):

In the case of aggregation assays where non labelled DNA was used, the assay was carried out exactly as described above. This experiment was done to follow the protein distribution with DNA upon aggregation. After centrifugation, the total supernatant was removed carefully without touching the walls of the Eppendorf centrifuge tube. The pellet was dissolved in 10 µl of 5 X SDS gel loading buffer. Both the samples were boiled at 65°C for 15 minutes and analyzed by 15% SDS-PAGE.

2.17.3 Aggregation assay (spectrophotometric method):

DNA aggregation was also studied by the extent of light scattering monitored by measuring the increase in absorbance at 320 nm spectrophotometrically. Increasing amounts of HSNP-C were added to a fixed amount of DNA (5 ug) in 1 ml of reaction buffer containing 10 mM sodium acetate, 1 mM Na_2EDTA. The formation of DNA-protein aggregates were assayed by measuring the increase in absorbance at 320 nm. The assay was performed at two different temperatures viz.; 37°C and 65°C.

2.18 Renaturation assay :

DNA renaturation assays were carried out according to Sung *et al* (1992) with slight modifications. The reaction was carried out in 20 ul small volume in 10 mM sodium acetate (pH 5.0), 12 mM $MgCl_2$, 1 mM DTT with 400 ng heat denatured *X* DNA

and increasing amounts of HSNP-C'. The reaction mixtures without DNA were preincubated at 37°C for 5 minutes. The reaction was then started by the addition of denatured DNA and incubation was continued for 15 minutes at 37°C. The reaction was then quenched by the addition of SDS and EDTA to final concentrations of 1 % and 25 mM respectively. The renaturation products were analyzed by electrophoresis on 0.8% agarose gels in 40 mM Tris-acetate (pH 7.8) and 2 mM Na_2-EDTA buffer. The agarose gels were then stained in ethidium bromide solution and photographed under UV illumination.

The dependence of renaturation on pH was tested at different pH conditions (pH 5.0, 5.5, 6.0 and 7.6). The requirement for Mg^{+2} for renaturation was tested by performing the assay in the absence of magnesium ions. The extent of DNA renaturation by HSNP-C was also followed by subjecting the renaturation products to S1 nuclease digestion.

2.19 Nuclease protection studies :

2.19.1 S1 nuclease protection studies :

Increasing amounts of HSNP-C was incubated with 400 ng of M13 ssDNA (20 µl reaction volume) at 37°C for 15 minutes in 50 mM sodium acetate (pH 5.0), 150 mM sodium chloride and 1 mM zinc chloride. One unit of S1 nuclease was added to the reaction mixture and incubated for one minute at 37°C. The enzyme digestion was quenched and the products were deproteinized by the addition of SDS and EDTA to 1% and 50 mM final concentrations respectively, before loading on to the agarose gel. The electrophoresis was carried out in 40 mM Tris-acetate (pH 7.8) and 2 mM Na_2-EDTA. The gel was stained in ethidium bromide and photographed under UV illumination.

S1 nuclease digestion assay was carried out to see the extent of pairing promoted by HSNP-C. Renaturation assay was done as described in methods section. The reaction was quenched by the addition of SDS to 1% final concentration. S1 nuclease digestion buffer (10X : 500 mM sodium acetate (pH 4.7), 1500 mM NaCl and 10 mM zinc

chloride) was added to the above reaction mixture followed by one unit S1 nuclease and the incubation was continued at 37°C for 5 minutes. The enzyme digestion was quenched by the addition of EDTA and SDS to 50 mM and 1% final concentrations respectively and the reaction products were analyzed immediately on 0.8% agarose gel. The gel was stained with ethidium bromide and photographed under UV illumination.

2.19.2 DNase I digestion studies (analysis by agarose gel) :

Increasing amounts of HSNP-C' were incubated with 400 ng of *X* DNA in 10 mM Tris-HCl (pH 7.6), 50 mM NaCl, 5 mM $MgCl_2$ and 1 mM DTT at 37°C for 15 minutes. DNase I was added to the reaction mixtures at 10 : 1 (DNA : DNase I w/w) ratio and incubated at 37°C for 30 seconds. The digestion was quenched and the samples were deproteinized by the addition of SDS and EDTA to 1% and 25 mM final concentrations respectively. The reaction products were analyzed on 0.8% agarose gel. The gel was stained with ethidium bromide and photographed under UV illumination.

2.19.3 DNase I digestion studies (spectrophotometric method):

The sensitivity of HSNP-C'-dsDNA complexes to DNase I enzyme was also followed spectrophotometrically. The reaction was carried out in a cuvette (600 µl reaction volume) in 10 mM Tris-HCl (pH 7.6), 50 mM NaCl, 5 mM $MgCl_2$ and 1 mM DTT buffer. HSNP-C in increasing amounts was mixed with 5 µg of calf thymus DNA and incubated at 37°C for 15 minutes. DNase I (0.5 µg) was added directly to the reaction mixture in the cuvettes and the kinetics of increase in absorbance at A_{260} nm was recorded. The increase in absorbance was plotted against time.

2.20 RNase activity of different isoforms of HSNP-C:

2.2.1 RNase activity (visualization by PAGE):

RNase activity for HSNP-C and its isoforms was assayed according to Fusi *et al* (1995a) with minor modifications. tRNA (5 μg) was incubated at 60°C for 60 min in the presence of 1.5 μg of HSNP-C or its other forms in 40 mM sodium phosphate buffer (pH 7.0) or 40 mM Na acetate (pH 5.0) or 40 mM Tris-HCl (pH 7.4). The samples were further incubated at 37°C for 30 minutes in the presence of 0.5% SDS and 0.5 (ig proteinase K. The cleavage products were then resolved by PAGE in the presence of 7 M urea using a 15% polyacrylamide gel. After electrophoresis, the gels were silver stained according to Blum and Gross (1987).

2.2.2 RNase activity - (Spectrophotometric assay):

The assay was performed according to Fusi *et al* (1993). Yeast RNA was used as substrate. The reaction mixture was preincubated for 5 minutes at 60°C, followed by addition of HSNP-C' and the incubation was carried over for 30 minutes at the same temperature. The incubation mixture consisted of 0.4 mg/ml RNA and variable 0.1 mg of HSNP-C in 40 mM sodium phosphate (pH 7.8) in a final volume of 250 μl. The reaction was stopped by addition of 250 μl 1.2 M perchloric acid and 22 mM lanthanum nitrate which led to the precipitation of proteins, DNA and undigested RNA. After standing on ice for 20 minutes, the mixture was spun at 10,000 X g for 10 minutes. A 400 μl aliquot of the supernatant was withdrawn and diluted to 2 ml with water. The A_{260} was then determined against a blank sample incubated in the absence of the protein.

2.21 Isolation of HMG protein fraction from rat liver :

The purification was done according to (Christensen and Dixon, 1981) with minor modifications.

2.21.1 Isolation of nuclei :

Nuclei from rat liver were isolated following the procedure described by Thampan (1985). A 10% homogenate of tissue (rat liver) was made in 50 mM Tris pH 7.6, 2 mM $MgCl_2$, 0.2 mM PMSF, 1 mM $CaCl_2$ containing 0.25 M sucrose (all operations were carried out at 4°C) using polytron homogeniser. The homogenate was filtered through 4 layers of cheese cloth to remove any particulate matter. The clarified filtrate was then centrifuged at 800 X g for 15 minutes at 4°C. The pellet which contains the nuclei was washed with the same buffer containing 0.1% Triton X 100 twice to remove any membranous material. For this step a hand homogeniser was used. The suspension obtained was centrifuged at 800 X g for 15 minutes at 4°C. The nuclear pellet was washed once with sucrose buffer devoid of Triton X 100 and was thus used for preparation of HMG protein fraction.

2.21.2 Fractionation of HMG proteins from isolated rat liver nuclei :

The nuclei were extracted three times with 0.35 M NaCl (adjusted to pH 7.0 with 0.1 N NaOH) by sonicating it at half maximal speed for 30 seconds to obtain the 0.35 M NaCl soluble proteins. The 0.35 M NaCl (pH 7.0) extract was then centrifuged at 2000 X g for 15 minutes at 4°C for 1 hour. The supernatant obtained was clarified by passing through a sintered funnel.

2.21.3 Differential precipitation of HMG and LMG group of proteins with Trichloroacetic acid :

The LMG proteins were precipitated from the salt soluble fraction of proteins with TCA to final concentration of 3%. The sample was left at -20°C for 1-2 hrs. It was then centrifuged at 10,000 X g for 15 min at 4°C. TCA was then added to the supernatant to a final concentration of 20%. It was left overnight at -20°C. It was again centrifuged at 10,000 X g to pellet the HMG protein fraction. The pellet was washed twice with acetone and dissolved in a suitable buffer containing 20 mM Tris-HCl (pH 7.6), 0.1 mM PMSF and stored at -20°C until use.

CHAPTER 3

RESULTS

3.1 Purification of HSNP-C' and its isoforms :

The different forms of HSNP-C an 7 kDa DNA binding protein from the archaeon *Sulfolobus acidocaldarius* were purified according to Grote *et al* (1986) and Reddy and Suryanarayana (1989) with a few modifications. The acid extract of the post-ribosomal supernatant was subjected to CM-cellulose chromatography as described in the methods section. The proteins bound to the column were eluted with a linear gradient of 0.04 M - 0.4 M NaCl containing buffer. The fractions were analysed by SDS-PAGE. As can be seen from Fig. 3. 1A the different forms of HSNP-C eluted as three distinct peaks - peak I (lane 4-8), peak-II (lane 9-16) and peak-III (lane 17-21). The forms that eluted in each peak were named as **HSNP-C'*a***, **HSNP-C** and **HSNP-C'*b***. These proteins correspond to 7c, 7d and 7e as described by Grote *et al* (1986) . In the first peak another helix stabilising protein HSNP-A (Reddy and Suryanarayana, 1989) co-eluted with HSNP-C'a. The second peak contained pure HSNP-C. The third peak contained HSNP-C'*b* and trace amounts of another helix stabilising protein HSNP-C. The same fractions were immunoblotted and probed with anti HSNP-C IgG (Fig. 3. 1B and C). The other forms of HSNP-C cross reacted with anti-HSNP-C IgG. The fractions corresponding to each peak were pooled separately and used for further purification.

As mentioned in the methods section the peak I protein pool was dialysed to bring down the salt concentration to 100 mM NaCl and further subjected to affinity chromatography on double stranded DNA cellulose column. The bound proteins were eluted with buffer containing 250 mM NaCl, and the fractions were analysed by SDS-PAGE. The proteins eluted depending on the strength of binding to the matrix. As can be seen from Fig. 3. 1D in the early fractions the other helix stabilizing protein HSNP-A co-eluted. The latter fractions contained pure HSNP-C'a. The fractions containing the pure protein were pooled.

PURIFICATION OF THREE **ISOFORMS** OF **HSNP-C'**

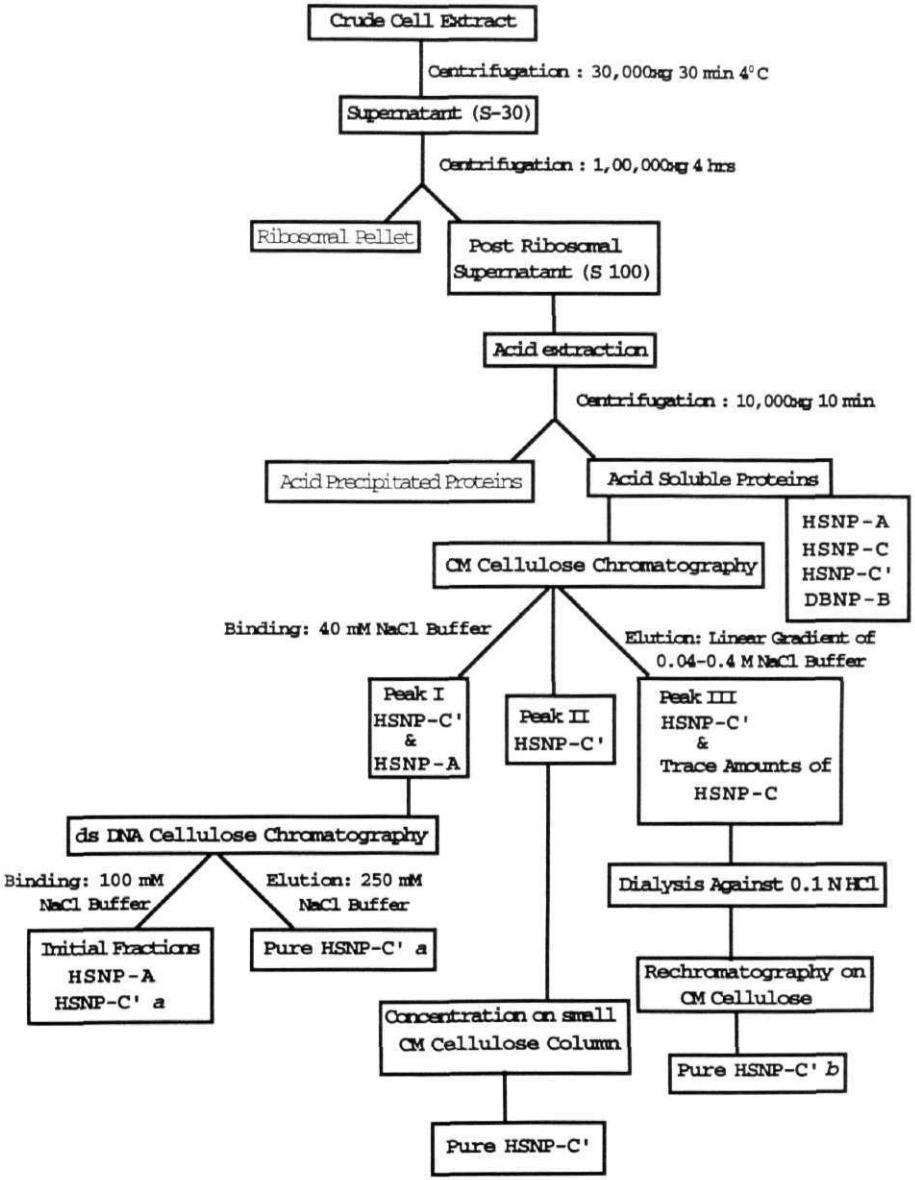
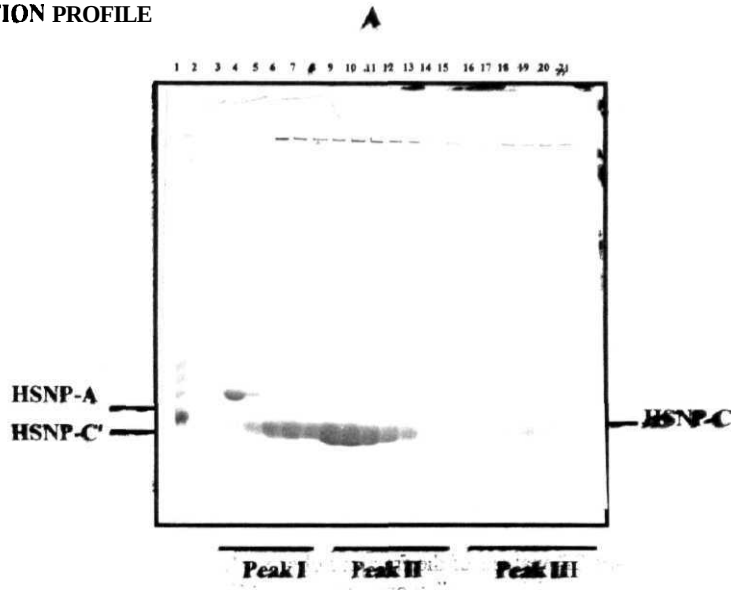


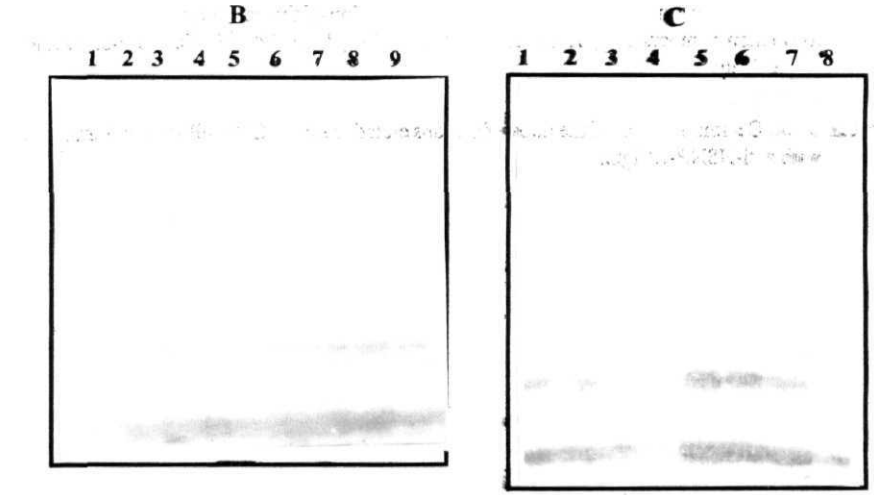
Fig. 3.1 A : SDS-PAGE analysis of the fractions obtained from CM-cellulose chromatography of the acid extract of the post ribosomal supernatant (S-100). Lane 1 : acid extract of the post ribosomal supernatant (S-100), 40 µg protein; lane 2 : flow through of the CM cellulose column, 40 µl; lane 3-21 : 20 µl of every third fraction of the column eluate corresponding to fraction numbers 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, 34, 37, 40, 43, 46, 49 and 52 respectively.

Fig. 3.1 B **and** C : Immunoblot of the above fractions eluted from the CM-cellulose column probed with anti-HSNP-C' IgG.

ELUTION PROFILE



IMMUNOBLOTS



The peak II protein pool was dialysed to bring down the salt concentration to 40 mM NaCl and then rechromatographed on a smaller CM cellulose column to concentrate the protein. The bound protein was eluted with buffer containing 500 mM NaCl and the fractions were analysed by SDS-PAGE. The protein eluted as a sharp peak (Fig. 3. 1E). The peak fractions containing the protein HSNP-C were pooled.

The peak III protein pool was dialysed against 0.1 M HC1 (to remove the trace amounts of HSNP-C, the other helix stabilising protein) centrifuged and the supernatant was re-dialysed against 40 mM NaCl containing buffer. This protein pool was re-chromtographed on a smaller CM cellulose column and the fractions were analysed by SDS-PAGE. Pure protein HSNP-C' fc eluted as a sharp peak (Fig. 3.1F). The peak fractions containing the protein HSNP-C *b* were pooled.

The three purified protein pools were dialysed separately against 10 mM Tris (pH 7.6) lyophilised for further concentration. The lyophilised samples were dissolved in a small volume of 10 mM Tris(pH 7.6), re-dialysed against 10 mM Tris (pH 7.6) and stored at -20°C until use.

The protein concentration was determined according to Lowry *et al* (1951) using BSA as standard. The purity of the concentrated proteins were checked by electrophoresing 10 µg of each protein on SDS-PAGE followed by silver staining (Fig. 3. 1 G). All the three forms of the protein were found to be more than 90% homogeneous.

The yield of the different forms were calculated to be

HSNP-C' *a*	0.35 mg/10 g wet cells
HSNP-C	3 mg/10 g wet cells
HSNP-C'*b*	0.27 mg/10 g wet cells.

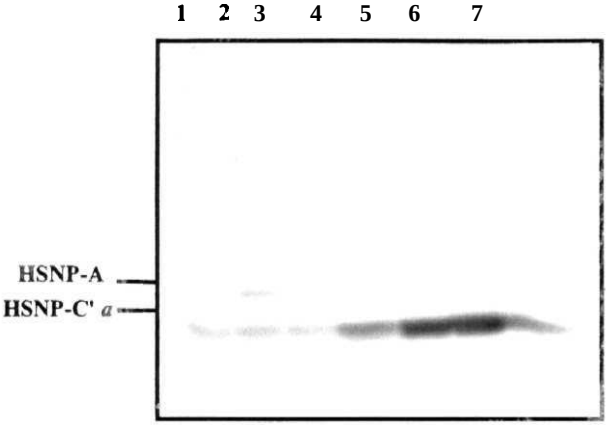
3.2 N terminal sequence of HSNP-C and its similarity to Sac 7d :

The amino terminal of HSNP-C was sequenced on Applied Biosystems 477A sequencer and analysed on a 120A HPLC analyser at Molecular Core Facility, HMC,

Fig. 3.1 **D :** SDS-PAGE analysis of the fractions obtained upon chromatography of peak I fractions on dsDNA cellulose column. Lane 1-7 : fractions 3, 5, 7, 9, 11, 13, 15 respectively

Fig. 3.1 E : SDS-PAGE analysis of the peak fractions obtained upon rechromatography of peak II fractions on a smaller CM-cellulose column. Lane 1: molecular weight markers bovine albumin (66 kDa); ovalbumin (45 kDa); glyceraldehyde dehydrogenase (36 kDa); carbonic anhydrase (29 kDa); trypsinogen (24 kDa); trypsin inhibitor (20 kDa); a lactalbumin (14 kDa); lane 3-7 : fractions 3, 4, 5, 6 and 7 respectively.

D


1 2 3 4 5 6 7
HSNP-A
HSNP-C' a

E

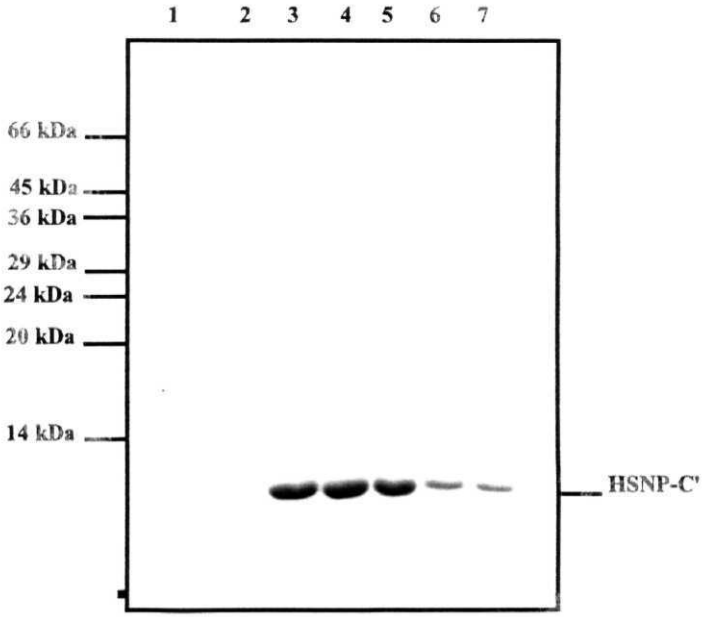
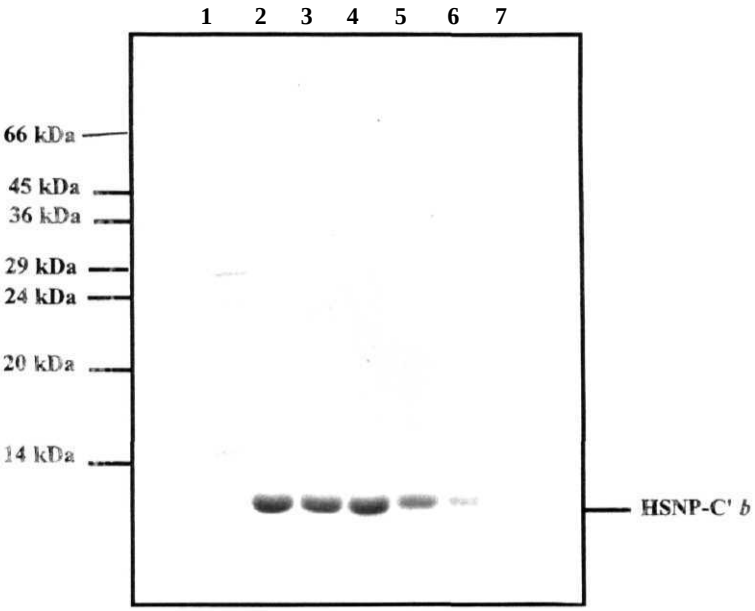

1 2 3 4 5 6 7
66 kDa
45 kDa
36 kDa
29 kDa
24 kDa
20 kDa
14 kDa
HSNP-C'

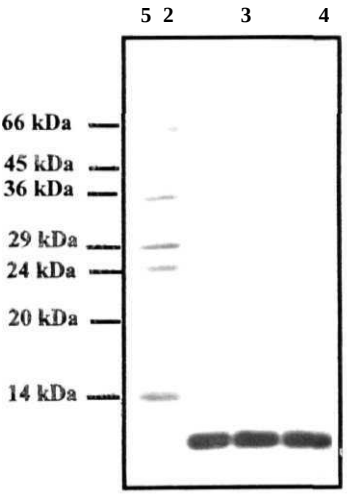
Fig. 3.1 F : **SDS-PAGE** analysis of the peak fractions obtained on rechromatography of peak **III** fraction pool on a smaller **CM-cellulose** column. Lane 1 : molecular weight markers as in Fig. 3.1 E; lane 2-7 : fraction numbers 3, 4, 5, 6, 7 and 8 containing the concentrated **HSNP-C'***b*.

Fig. 3.1 G : SDS-PAGE analysis of all three purified forms of **HSNP-C'**. Lane 1 : molecular weight markers as in Fig. 3.1 E; lane 2 : 10 µg of **HSNP-C'***a*; lane 3 : 10 µg of HSNP-C; lane 4: 10 µg of **HSNP-C'***b*.

F


1 2 3 4 5 6 7
66 kDa
45 kDa
36 kDa
29 kDa
24 kDa
20 kDa
14 kDa
HSNP-C' b

G


5 2 3 4
66 kDa
45 kDa
36 kDa
29 kDa
24 kDa
20 kDa
14 kDa

Hershey, PA USA as described in the methods section . It was found to contain a unique sequence of alternating lysine residues namely **val-lys-val-lys-phe-lys-tyr-lys-** at the amino terminal end (3.2A). This feature is a characteristic of the 7 kDa group of proteins from *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus.* It was shown by Choli *et al* (1988) that the sequences for the 7 kDa group of proteins from 5. *acidocaldarius* namely **Sac** 7a, **Sac** 7b, **Sac** 7d and **Sac** 7e at the amino terminal bear high degree of similarity but vary in their lengths at the -COOH terminus and in the degree of monomethylation. On comparison of the N terminal sequence of HSNP-C with the amino terminal sequence of the Sac group of proteins shows that it is 100% identical to Sac 7d (Fig. 3.2B). The sequence alignment was done using BLAST (Basic Local Alignment Search Tool). The purification data shows that HSNP-C is the most abundant of the HSNP-C group of proteins purified just like Sac 7d in the Sac group of DNA binding proteins. Thus, HSNP-C is identical to Sac 7d protein. It is also 100% identical to Sac 7a and Sac 7b at the amino terminus as these proteins differ from each other and Sac 7d only at the carboxy terminus. It shows 93% identity with Sac 7e with differences at residue 1 and 5 where it is Ala and Arg in Sac 7e. It shows 93% similarity in N terminal sequence with that of Sso 7d. It differs at the residue 1 and 2 from Sso 7d. Thus Sso 7d and HSNP-C are similar but not identical at the amino terminal. Kimura *et al* (1984) reported remote resemblance of Sso 7d to eukaryotic DNA binding protein namely the **HMG** group of proteins in having similar but not identical alteration of lysine residues at the N terminus. Comparison of the amino terminus of HSNP-C with the HMG group of proteins is shown in Fig. 3.2 C. Comparison of the amino terminal sequence of HSNP-C with that of the ribonucleases (Fig. 3.2 D) sequenced from *Sulfolobus* species showed 86% identity with p2 endoribonuclease and 86% identity with p3 endoribonuclease of *S. solfataricus* . It also showed 86% identity with SaRD protein, a 9 kDa ribonuclease with DNA binding properties (Kulms *et al.*, 1995).

Fig. 3.2 A： N terminal sequence report of HSNP-C'.

~ fipplied **Biosystems 477A** Protein **Sequence** Report -

SAMPLE : HSNP-C
[initiated 10 Oct 1995 11:53am]

Sample finouirt : 120 pmol

AAcid ft	fincid ID	R.Time (min)	C.Time (min)	Pmol (raw)	Pnol (-bkgd)	Pnol (+lag)	Pnol Ratio	AAcid ID
1	V	19.3Z	19.23	136.07	131.15	137.07	602.07	VAL
Z	K	24.38	Z4.Z0	84.01	79.16	84.05	177.78	LYS
3	V	19.38	19.23	115.52	110.84	118.96	52Z.64	VAL
4	K	?4.38	24.20	42.65	38.88	41.45	87.67	LYS
S	F	Z3.35	23.17	85.37	83.43	92.78	248.54	PHE
G	K	Z4.43	24.20	22.92	20.06	21.43	45.33	LYS
7	Y	15.00	14.80	55.72	53.21	61.69	229.37	TYR
8	K	Z4.4Z	24.20	Z8.18	26.08	Z8.03	59.78	LYS
9	6	8.52	8.48	55.03	45.40	54.47	112.92	GLY
10	E	9.18	9.IB	23.06	1S.6i	Z3.94	71.00	GLU
11	E	9.22	9.18	Z7.13	23.70	Z4.68	73.18	GLU
I?	K	24.32	24.20	11.35	10.30	11.16	Z3.60	LYS
13	E	9.Z7	9.18	17.87	14.43	17.46	51.77	GLU
14	V	19.38	19.Z3	35.72	30.97	40.94	179.83	VAl
15	0	5.83	5.85	16.45	12.72	17.02	41.66	ASP
1b	T	8.10	8.08	37.04	33.46	46.20	118.03	THR
17	S	7.38	7.37	9.77	6.40	8.75	53.00	SER
18								
13	I	Z3.93	23.62	30.23	30.G7	30.67	31.24	ILE
Z0								

REPETITIVE YIELD ANALYSIS:

	Rep.Yield	Variance	
V: 1. 3. 14	89.34 *X*	0.9S9	:VAL
K: 2. 4, 6, 8. 12	83.13 %	0.878	:LYS
E: 10. 11. 13	88.41 I	0.564	:GLU

Average AA Repetitive Yield: 86.00 *X*

Combined AA Repetitive Yield: 8J.55 *X* 0.5ZG

Theoretical Initial Yield: 89.92 pmol (74.94 *X*)

Fig. 3.2 B : Comparision of the amino terminal sequence of HSNP-C' with the 7kDa group of DNA binding proteins from *Sulfolobus acidocaldarius* namely Sac 7a, Sac 7b, Sac 7e and *Sulfolobus solfataricus* namely Sso 7d .

	1 5 10 15
HSNP-C'	: Val-Lys-Val-Lys-Phe-Lys-Tyr-Lys-Gly-Glu-Glu-Lys-Glu-Val-Asp-
Sac 7a	: Val-Lys-Val-Lys-Phe-Lys-Tyr-Lys-Gly-Glu-Glu-Lys-Glu-Val-Asp-
Sac 7b	: Val-Lys-Val-Lys-Phe-Lys-Tyr-Lys-Gly-Glu-Glu-Lys-Glu-Val-Asp-
Sac **7d**	: Val-Lys-Val-Lys-Phe-Lys-Tyr-Lys-Gly-Glu-Glu-Lys-Glu-Val-Asp-
Sac 7e	: **Ala**-Lys-Val-**Arg**-Phe-Lys-Tyr-Lys-Gly-Glu-Glu-Lys-Glu-Val-Asp-
Sso 7d	: **Ala-Thr**-Val-Lys-Phe-Lys-Tyr-Lys-Gly-Glu-Glu-Lys-Glu-Val-Asp-

Fig. 3.2 C : Comparision of the amino terminal sequence of HSNP-C with that high mobility group proteins .

	1 5 10 **15**
HSNP-C'	Val-**Lys**-Val-**Lys**-Phe-**Lys**-Tyr-Lys-Gly-Glu-Glu-**Lys**-Glu-Val-Asp-
HMG-1	Gly-**Lys**-Gly-Asp-Pro-**Lys**-Lys-Pro-Gly-Gly-Lys-Met-Ser-Ser-Tyr-
HMG-2	Gly-**Lys**-Gly-Asp-Pro-Asp-Lys-Pro-Arg-Gly-Lys-**Lys**-Ala-Ala-Tyr-
HMG-14	Pro-**Lys**-Arg-**Lys**-Val-Ser-Ser-Ala-Glu-Gly-Ala-Ala-Lys-Glu-Glu-
HMG-17	Pro-**Lys**-Arg-**Lys**-Ala-Glu-Gly-Asp-Ala-Lys-Gly-Asp-Gly-Ala-Lys-

Fig. 3.2 **D :** Comparision of the amino terminal sequence of HSNP-C with that of the ribonucleases from *Sulfolobus* species.

	1 5 10 15
HSNP-C	Val-Lys-Val-Lys-Phe-Lys-Tyr-Lys-Gly-Glu-Glu-Lys-Glu-Val-Asp-
p2 ENDORIBONUCLEASE (5. *solfataricus*)	**Ala-Thr**-Val-Lys-Phe-Lys-Tyr-Lys-Gly-Glu-Glu-Lys-**Gln**-Val-Asp-
p3 ENDORIBONUCLEASE (*S. solfataricus*)	**Ala-Thr**-Val-Lys-Phe-Lys-Tyr-Lys-Gly-Glu-Glu-Lys-**Gln**-Val-Asp-
SaRD PROTEIN *(S. acidocaldarius)*	**Ala**-Lys-Val-**Arg**-Phe-Lys-Tyr-Lys-Gly-Glu-Glu-Lys-Glu-Val-Asp-

NOTE : The NH_2 terminal sequences of Sac 7a, Sac 7b,Sac 7d, Sac 7e proteins are from Kimura >'tal., (1984) and Choli *et al.*, (1988b); Sso 7d after Choli *et al.*,(1988a); p2 endoribonuclease from *S. solfataricus* after Fusi *et al.*, (1993); p3 endoribonuclease from S. *solfataricus* after Fusi *et al.*, (1995); SaRD protein from 5. *acidocaldarius* after Kulms *et al.*, (1995); HMG -1, HMG-2,HMG-14 and HMG-17 from Walker *et al.*, (1978, 1979).

3.3 Protein-protein interactions of HSNP-C :

3.3.1 Formaldehyde crosslinking (self-aggregation):

Protein-protein crosslinking with zero length cross linker formaldehyde was carried out to study the self aggregation behavior of HSNP-C. Crosslinking was performed at 37°C and 65°C at pH 7.4 and pH 8.3. The products were analysed by 15% SDS-PAGE. Cross linking was also performed in the presence of 300 mM sodium chloride to see if salt has any effect on the aggregation behavior of HSNP-C.

Formaldehyde cross linking at pH 8.3 (Fig. 3.3.1) produced a series of cross linked multimers in the form of a ladder with bands corresponding to dimers, trimers, tetramers, pentamers, hexamers (i.e., 16 kDa, 24 kDa, 32 kDa, 40 kDa, 48 kDa) and also some very high molecular weight aggregates which remained at the interface of the spacer gel and the resolving gel. (lane 1 and 2).

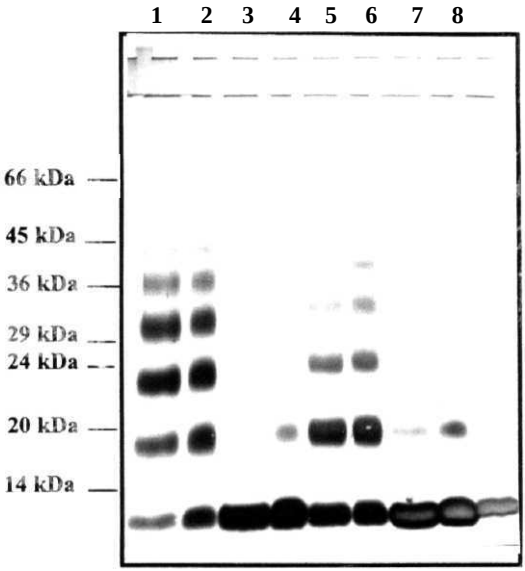
At pH 7.4, only a predominant dimer and a little of high molecular weight aggregate that did not enter the gel (lane 3 and 4) were observed. At pH 8.3 aggregation was higher at 65°C (lane 2) than at 37°C (lane 1). At pH 7.4, the concentration of the dimer increased at 65°C (lane 4). When the cross linking was carried out in the presence of 300 mM NaCl the yield of the aggregates increased at 65°C (lane 5-8).

3.3.2 Interaction of HSNP-C with other proteins :

In this experiment the post ribosomal supernatant (S-100) was subjected to gel filtration chromatography on Sephacryl S 200 matrix. Fractions from no. 34 were analysed on 15% SDS-PAGE as no protein eluted before fraction no. 37 (checked by A_{280} readings). In gel filtration chromatography the larger molecules do not enter the beads and thus are eluted in the early fractions, whereas the smaller molecules enter the beads and get eluted in the latter fractions. Thus, HSNP-C being a low molecular weight (7

Fig. **3.3.1 :** SDS-PAGE analysis of HSNP-C crosslinked with formaldehyde at different temperatures, pH and salt concentration.

Lane 9 : Control HSNP-C; lane 1 : crosslinking at pH 8.3 at 37°C; lane 2 : crosslinking at pH 8.3 at 65 °C; lane 3 : crosslinking at pH 7.2 at 37°C; lane 4 : crosslinking at pH 7.2 at 65 °C; lane 5 : crosslinking at pH 8.3 in the presence of 300 mM NaCl at 37°C; lane 6 : crosslinking at pH 8.3 in the presence of 300 mM NaCl at 65°C; lane 7 : crosslinking at pH 7.2 in the presence of 300 mM NaCl at 37°C; lane 8 : crosslinking at pH 7.2 in the presence of 300 mM NaCl at 65°C.


1 2 3 4 5 6 7 8
66 kDa
45 kDa
36 kDa
29 kDa
24 kDa
20 kDa
14 kDa

kDa) protein would elute in the early fractions only if complexed with some other proteins or if it is aggregating to form higher oligomeric structures. The presence of HSNP-C' was detected by blotting the eluted fractions with anti HSNP-C IgG.

HSNP-C' was seen even in the early fractions corresponding to 43rd ml of elution volume (Fig. 3.3.2 A; lane 5). The protein eluted with different groups of proteins group a - lane 5-8; group b - lane 9-12, group c- lane 13-16, group d-lane 17-19. The above results were confirmed by immunoblotting with anti HSNP-C IgG. As seen in Fig. 3.3.2 B HSNP-C could be detected in lane 5 (i.e. fraction no. 46). HSNP-C was seen eluting in the early fractions could be the aggregated form of the protein or it may also be associated with some other proteins to form high molecular weight aggregates. Immunoblotting also detected the dimeric, trimeric and tetrameric forms of the protein (Fig. 3.3.2 B and C). The monomeric form of the protein eluted from 83-85th fraction as seen both by SDS-PAGE and immunoblotting. Purified protein (HSNP-C) after SDS-PAGE and immunoblotting showed a series of bands similar to formaldehyde crosslinking indicating strong aggregation behaviour of the protein.

3.3.3 Isolation of protein complexes that bound to HSNP-C :

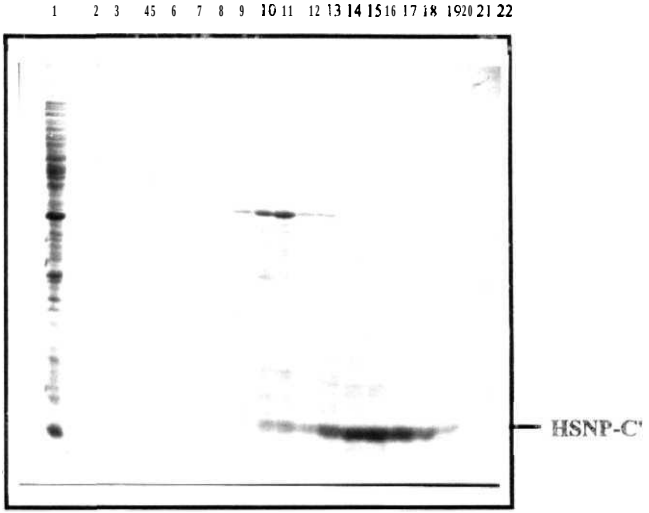
In this experiment the post ribosomal supernatant was passed through a column of HSNP-C coupled to epoxy activated sepharose to see the other proteins interacting with HSNP-C. The coupling of HSNP-C to epoxy activated sepharose was checked by passing anti HSNP-C IgG through the column. The anti HSNP-C IgG bound to the column as can be seen from the electrophorectic analysis of the fractions eluted (Fig. 3.3.3 A, lane 4-7). The column was run exactly in the case of purification of IgG from antiserum on protein A sepharose column (see methods section).

The post-ribosomal supernatant was loaded onto HSNP-C- epoxy sepharose column at very slow flow rates in buffer containing 40 mM NaCl. The column was washed thoroughly with buffer containing 40 mM NaCl and the bound proteins were

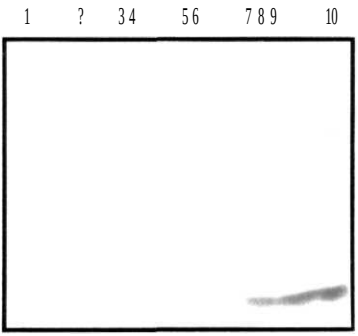
Fig. 33.2 A : SDS-PAGE analysis of the fractions obtained by chromatography of the post-ribosomal supernatant (S-100) through a gel filtration column of Sephacryl S-200. Lane 2 : post-ribosomal supernatant (S-100), 50 µg protein; lane 3-21 : fractions 34, 37, 40, 43, 46, 49, 52, 55, 58, 61, 64, 67, 70, 73, 76, 79, 82, 85 and 88 respectively.

Fig. 3.3.2 B **and** C : Immunoblot of the above fractions probed with anti-HSNP-C' IgG.
(B) Lane 1-10 : fractions 40, 43, 46, 49, 52, 55, 58, 61, 64 and 67 respectively.
(C) Lane 1-7 : fractions 70, 73, 76, 79, 82, 85 and 88 respectively.

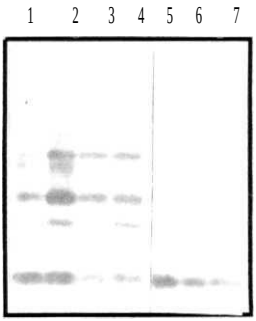
A


1 2 3 45 6 7 8 9 10 11 12 13 14 15 16 17 18 1920 21 22
HSNP-C'

B


1 ? 34 56 789 10

C


1 2 3 4 5 6 7

eluted with a stepwise gradient of 250 mM NaCl and 500 mM NaCl. The fractions were analysed by 15% SDS-PAGE.

Two low molecular proteins and some higher molecular weight proteins (shown by arrow) were eluted at 250 mM NaCl concentration (Fig. 3.3.3 B; lane 8-10). HSNP-C' too was seen eluting with two proteins (mol. wt. 12 kDa and 11 kDa) which are the nucleoid associated proteins **HSNP-A** and **DBNP-B.** No protein was eluted at 500 mM NaCl concentration.

3.4 Nucleic acid binding properties of HSNP-C :

The DNA protein interactions of HSNP-C were studied by three different methods

a) mobility shift assays

b) affinity chromatography on DNA cellulose columns

c) fluorescence titrations

3.4.1 Gel mobility shift assays :

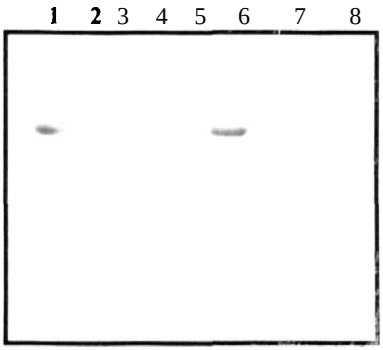
Binding of HSNP-C to different nucleic acids was analysed by gel mobility shift or gel retardation assays on agarose gels. This technique is based on the observation that binding of a protein to DNA leads to decrease in the electrophoretic mobility of DNA in agarose gels. HSNP-C binding to M13 RF DNA, pBR322 RF DNA, supercoiled pUC 19 DNA, M13 linear DNA, 1 kb DNA ladder and M13 ss DNA were shown in Fig. 3.4.1 A, B, C, D and E respectively.

The binding of HSNP-C to the two forms in M13 RF DNA (supercoiled and relaxed forms) resulted in progressive retardation of mobility of DNA with increasing concentrations of the protein (Fig. 3.4.1 A). The supercoiled form of DNA was seen to show immediate retardation at DNA / protein ratio of 1:0.5 itself. At DNA / protein ratio of 1:2 both relaxed and supercoiled forms fused to form a single retarded complex. At very high DNA / protein ratios (lanes 7-9) the protein DNA-complexes were retained in the wells indicating aggregation of DNA. The subsaturated DNA / protein complexes

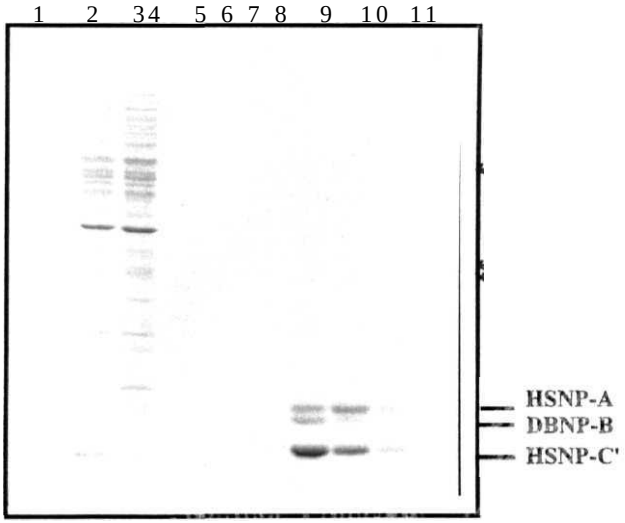
Fig. 3.3.3 A : SDS-PAGE analysis of the fractions obtained on passing anti-HSNP-C IgG through a column of HSNP-C protein coupled to epoxy activated sepharose. Lane 1 : sample applied i.e anti HSNP-C IgG; lane 2-8 : fractions 1, 2, 3, 4, 5, 6 and 7 respectively.

Fig. 3.3.3 B : SDS-PAGE analysis of the fractions obtained upon passing post-ribosomal supernatant (S-100) through a column of HSNP-C protein coupled to epoxy activated sepharose. Lane 1 : Molecular weight markers as in Fig. 3.1 E; lane 2 : S-100 (20 μg protein); lane 4-11 : corresponding to fractions 1, 3, 5, 7, 9, 11, 13 and 15 respectively.

A



B



were seen just below the saturated complexes which entered the gel and stained less with ethidium bromide.

Fig. 3.4.1 B shows the binding of HSNP-C' to the relaxed and negatively supercoiled forms of pBR322 DNA. With increasing concentrations of protein the nucleoprotein complexes showed progressive retardation of mobility. The protein showed co-operative mode of binding as indicated by the gradual formation of retarded complex with some free DNA. There was complete disappearance of the supercoiled form with increasing concentration of protein. Also there was gradual decrease in the intensity of ethidium bromide staining of nucleoprotein complexes as the concentration of protein in the complexes increased.

Fig. 3.4.1D shows the binding of increasing concentrations of HSNP-C to pUC19 supercoiled DNA. The supercoil nucleoprotein complexes showed progressive retardation of mobility with increasing concentration of the protein . At very high DNA / protein ratios w/w (Fig 4.1 D; lanes 9, 10, 11) i.e., 1:64, 1:128 and 1:250 the supercoil DNA- protein complexes were retained in the wells indicating aggregation of DNA. These complexes are representative of saturated complexes. The trailing smears below the wells in lanes 9-11 are subsaturated nucleoprotein complexes.

The binding of HSNP-C to linear DNA was performed using 1 kb DNA ladder from. As can be seen from Fig. 3.4.1C HSNP-C bound to the linear fragments of the 1 kb ladder thus forming retarded nucleoprotein complexes. Fig. 3.4.1E shows the binding of different forms of HSNP-C (i.e. HSNP-C'*a*, HSNP-C and HSNP-C'*b*) to pBR322 DNA. All the three forms bound strongly to DNA thus forming retarded nucleoprotein complexes.

The binding of HSNP-C to single stranded circular DNA was studied using M13 ssDNA (Fig. 3.4.1 F). Retardation in the mobility of M13 ssDNA was seen only from DNA / protein ratios above 1:8. A lot of protein was required to form saturated nucleoprotein complexes (as seen in lane 9 and 10 corresponding to DNA / protein ratios of 1:64 and 1:128) thus indicating its lower affinity for ssDNA.

Fig. 3.4.1 A : Gel mobility shift analysis of HSNP-C'-M13 dsDNA complexes. Lane 1 : 0.4 μg of M13 dsDNA (control); lane 2-4 : M13 dsDNA incubated with 0.1 μg, 0.2 μg, 0.4 μg, 0.8 μg, 1.6 μg, 3.2ug, 6.4 μg and 12.8 μg of HSNP-C' respectively.

Fig. 3.4.1 B : Gel mobility shift analysis of HSNP-C'-pBR322 dsDNA (negatively supercoiled DNA) complexes. Lane 1 : 0.5 μg of pBR322 dsDNA(control); lane 2-10 : pBR322 dsDNA incubated with 0.125 μg, 0.25 μg, 0.5 μg, 1 μg, 2 μg, 4 μg, 8 μg and 16 μg of HSNP-C respectively.

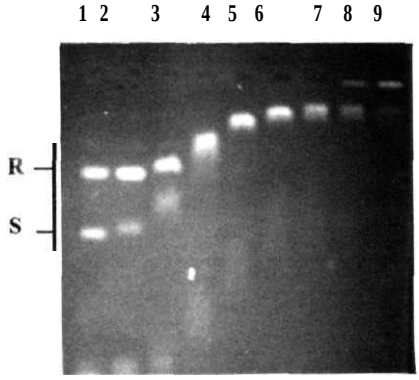
Fig. 3.4.1 C : Gel mobility shift analysis of HSNP-C'-linear DNA (Pharmacia 1 kb DNA ladder)complexes. Lane 1 : Pharmacia 1 kb ladder (10 μg); lane 2-4 : 1 kb ladder incubated with 2.5 μg, 5 μg and 10 μg of HSNP-C respectively.

Fig. 3.4.1 D : Gel mobility shift analysis of HSNP-C'-supercoiled pUC19DNA complexes. Lane 1 : 0.3 μg of supercoiled pUC19DNA(control); lane 2-11 : pUC19DNA incubated with 0.075 μg, 0.15 μg, 0.3 μg, 0.6 μg, 1.2 μg, 2.4 μg, 4.8 μg, 9.6 μg, 19.2 μg and 39.4 μg of HSNP-C respectively.

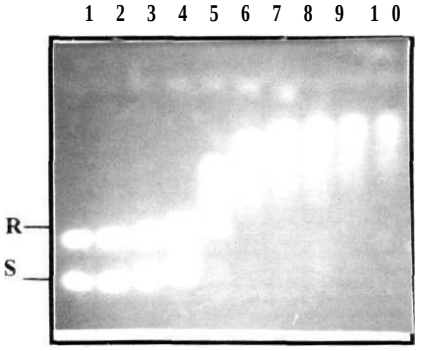
Fig. 3.4.1 E : Gel mobility shift analysis of isoforms of HSNP-C'-pBR322 dsDNA complexes. Lane 1 : 0.500 μg of pBR322 dsDNA (control); lane 2-4 : pBR322 dsDNA incubated with 4 μg each of HSNP-C'*a*, HSNP-C and HSNP-C'*b* respectively.

Note : **R** stands for relaxed form and S stands for supercoiled form.

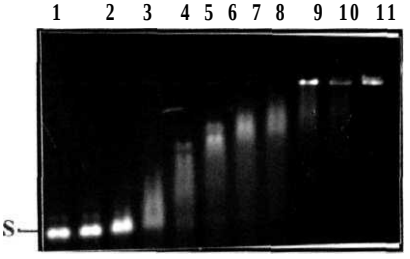
A



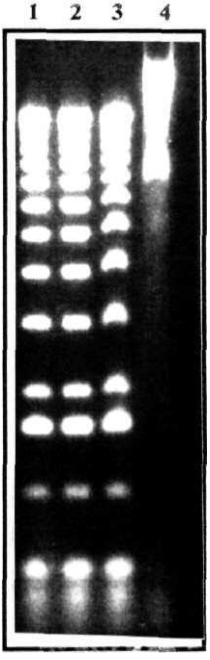
B



i)



C



Σ

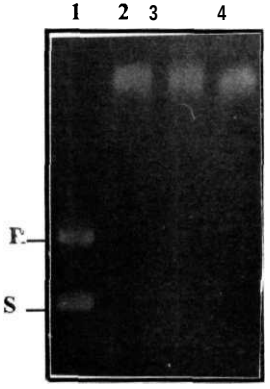


Fig. 3.4.1G shows the binding of different forms of HSNP-C i.e. HSNP-Ca, HSNP-C and HSNP-C' fc to M13 ssDNA. All the three forms bound to M13 ssDNA with mobility of the nucleoprotein complexes to the same extent.

3.4.1.1 *Salt sensitivity of nucleoprotein complexes ofM 13 RF DNA and* ***HSNP-C':***

An experiment was performed to see the effect of salt (300 mM KC1) on the HSNP-C - M13 RF DNA nucleoprotein complexes. DNA-protein complexes were formed by mixing increasing amounts of protein to constant amount of DNA in buffer that lacks salt in two sets. To one set of the nucleoprotein complexes, saturated KC1 was added to a final concentration of 150 mM. To the other set of nucleoprotein complexes, saturated KCl was added to a final concentration of 300 mM . The incubation was continued for another 15 minutes and the products were analysed on an agarose gel. As can be seen from Fig. 3.s4.1.1 the nucleoprotein complexes of M13RF DNA-HSNP-C were reasonably stable in the presence of both 150 mM and 300 mM KCl. However, there is slight decrease in retardation at 300 mM KCl (lane 8-12) when compared to the retardation observed at 150 mM KCl .

3.4.2 Affinity chromatography on DNA cellulose columns :

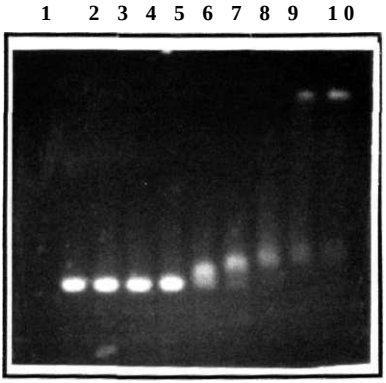
Strength of binding of HSNP-C and its forms HSNP-Ca and HSNP-C'*b* were assessed by affinity chromatography on nucleic acid matrices. These proteins were chromatographed on small columns of ssDNA and dsDNA cellulose. The concentration of salt required to elute the bound protein was determined in each case.

The results show that HSNP-C binds strongest to dsDNA thus requiring approximately 300 mM NaCl (Fig. 3.4.2B) for elution and binds quiet reasonably strongly to ssDNA requiring approximately 250 mM NaCl for elution (Fig. 3.4.2.1B). The protein eluted as two distinct peaks both on dsDNA (0.2 and 0.3 M NaCl) and ssDNA cellulose columns (0.1 and 0.2 M NaCl). The three isoforms of HSNP-C show differences in their elution pattern from dsDNA cellulose column. A major fraction of HSNP-Ca eluted at

Fig. 3.4.1 F: Gel mobility shift analysis of HSNP-C'-M 13 ssDNA complexes. Lane 2 : 0.2 μg of M13 ssDNA (control); lane 3-10 : M13 ssDNA incubated with 0.2 μg, 0.4 μg, 0.8 μg, 1.6 μg, 3.2 ng, 6.4 μg, 12.8 fig, and 25.8 μg of HSNP-C' respectively.

Fig. 3.4.1 G : Gel mobility shift analysis of the isoforms of HSNP-C'-M13 ssDNA complexes. Lane 1 : 0.2 μg of M13 ssDN A (control); lane 2-4 : M13 ssDN A incubated with 1.6 μg each of HSNP-C'*a*,HSNP-C' and HSNP-C *h* respectively.

F



G

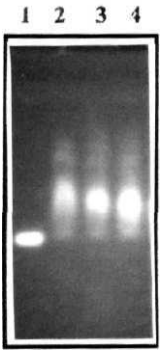
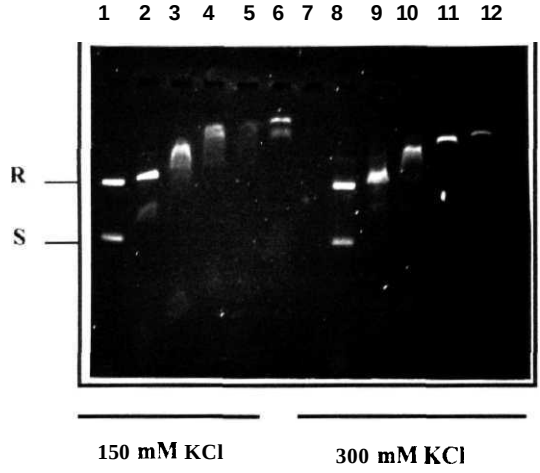


Fig. 3.4.1.1 : Gel mobility shift analysis of HSNP C M13 dsDNA complexes in the presence of 150 mM and 300 mM KO. Lane 1 : 0.3 µg of M13 dsDNA (control) S- supercoiled form, R- relaxed form: lane 2-6 : To the M13 dsDNA-HSNP-C' complexes formed at DNA: protein ratio of 1:0.5, 1:1, 1:2, 1:4 and 1 8, KC1 was added to a final concentration of 150 mM before Loading on the gel; lane 8 : 0.3 µg of M13 dsDNA (control); lane 9-12 : To the M13 dsDNA-HSNP-C' complexes formed at DNA: protein ratio of 1:0.5, 1:1,1:2, 1:4 and 1:8, KCl was added to final concentration of 300 mM before loading on the gel.


1 2 3 4 5 6 7 8 9 10 11 12
R
S
150 mM KCl
300 mM KCl

0.2M NaCl and a smaller fraction at 0.3 M NaCl (Fig. 3.4.2 A). In case of HSNP-C' the fraction eluting at 0.2M NaCl decreased and there was an increase in the fraction eluting at 0.3 M NaCl. There was further decrease in the fraction eluting at 0.2M NaCl in the case of HSNP-C' fc and increase in the fraction eluting at 0.3 M NaCl (Fig. 3.4.2 C).

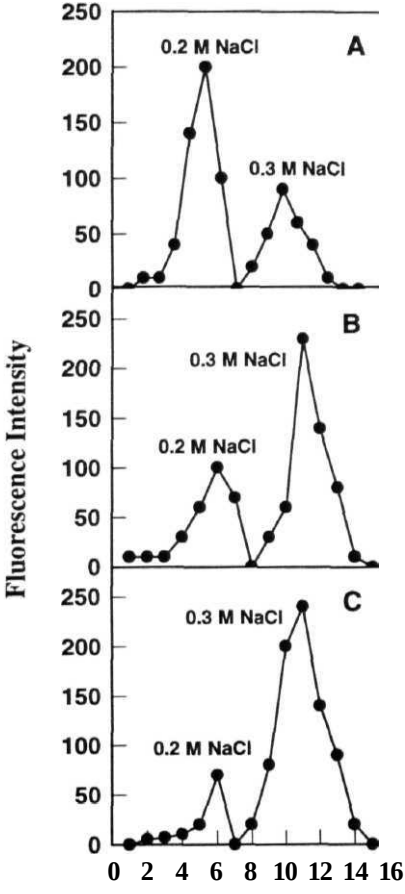
Chromatography on single stranded DNA cellulose column showed that the fraction eluting at 0.1 M NaCl decreased from HSNP-C'*a* to HSNP-C and in HSNP-C *b* there was almost complete disappearance of the 0.1 M NaCl peak (Fig. 3.4.2.1 A, B and C). The fraction eluting at 0.2 M NaCl increased in HSNP-C and HSNP-C'*b* in comparison to HSNP-C'*a*. This experiment suggests that the three isoforms of HSNP-C exist in two fractions which differ in the affinity to DNA cellulose (see discussion)

3.4.3 Fluorescence Titrations :

Protein nucleic acid interactions can be studied by measuring the quenching of intrinsic fluorescence of the protein (due to aromatic amino acids) upon binding of nucleic acid. This property can be used to quantitate binding of proteins to nucleic acids and to obtain thermodynamic binding parameters of the interaction. Such fluorescence titration data are useful in determining (i) the strength of the binding as given by the binding constant, (ii) the binding site size of protein on the nucleic acid lattice and (iii) binding mode of the protein to nucleic acid.

Fluorescence titrations for the study of the interaction of HSNP-C (all the three isoforms) were carried out by reverse titrations (nucleic acid was added to the protein and the resultant decrease in fluorescence intensity was measured). Thermodynamic binding parameters were determined using scatchard formulation according to McGhee and Von Hippel (1974) as described in detail by Kowalczykowski *et al* (1986). The protein was excited at 285 nm and the fluorescence emission intensity was measured at 350 nm. Addition of double stranded DNA (calf thymus) causes quenching of the protein fluorescence. Such titrations were performed at three different salt concentrations (Fig. 3.4.3A, B and C). At low salt (20 mM NaCl) the binding is tight and essentially stoichiometric. At high DNA/ protein ratios saturation in binding was reached as

Fig. 3.4.2 A, B **and** C : Binding of HSNP-C and its isoforms to dsDNA-cellulose.
(A): elution profile of HSNP-C'*a* from dsDNA column; **(B**): elution profile of HSNP-C from dsDNA column; (C): elution profile of HSNP-C'*b* from dsDNA column.

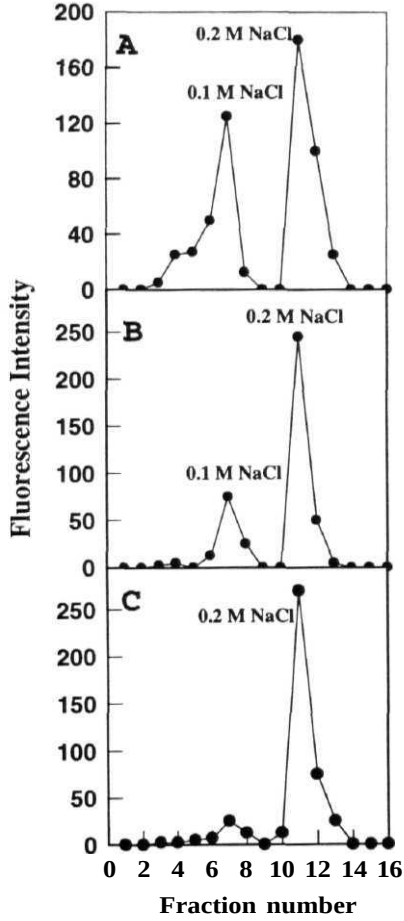


HSNP-C a

HSNP-C'

HSNP-C *b*

Fig. 3.4.2.1 A, B and C : Binding of HSNP-C' and its isoforms to ssDNA-cellulose column. (A): elution profile of HSNP-C *a*; (B): elution profile of HSNP-C; (C): elution **profile** of HSNP-C'*b*.


A
0.2 M NaCl
0.1 M NaCl
B
0.2 M NaCl
0.1 M NaCl
C
0.2 M NaCl
Fluorescence Intensity
Fraction number
HSNP-C' a
HSNP-C'
HSNP-C' b

indicated by the plateau at and above a ratio of 8 nucleotides per protein monomer. The low salt titration curve was used to determine the binding site size, 'n', the average number of nucleotides bound by a protein molecule. The ratio of DNA to protein at the intersection point of the initial and final slope of the titration curve corresponds to the site size. A site size of 4 was obtained for dsDNA for the three isoforms of HSNP-C (HSNP-C'*a*, HSNP-C and HSNP-C'*b*). Titration curves obtained at higher salt concentration (100 mM NaCl and 200 mM NaCl) showed decreased extent of quenching indicating weaker binding at these salt concentrations. The titration curves also do not indicate cooperativity in the binding of the protein to dsDNA (see discussion).

Data were analyzed by the equation 10 of McGhee and Von Hippel (1974). Binding constant (K) was determined from the data points after determining the free protein concentration. (L_f) and the binding density (υ, moles of ligand bound per mole nucleotide). In the case of HSNP-C, the ratio of Q_{obs}/Q_{max} was taken to be equal to the ratio of bound ligand/total ligand, where Q_{max} was obtained by the low salt titration curve. The calculation of υ and L_f was according to Bujalowski and Lohman (1987). Q_{max} values of 85% for HSNP-C'*b* and 72% for HSNP-Ca and HSNP-C were obtained respectively. Scatchard plots of υ versus L_f of the data of binding of HSNP-C to ds DNA at 20 mM NaCl are given in Fig. 3.4.3.1 A, B and C. The intercept on extrapolation of the curve at Y axis gives the intrinsic binding constant 'K' for the non-cooperative binding interaction. The intrinsic binding constants, (K) at 20 mM NaCl for the three isoforms HSNP-Ca, HSNP-C and HSNP-C' fc were 8.5 x 10^6 M^{-1}; 8.5 x 10^6 M^{-1}; 1.3 x 10^7 M^{-1}.

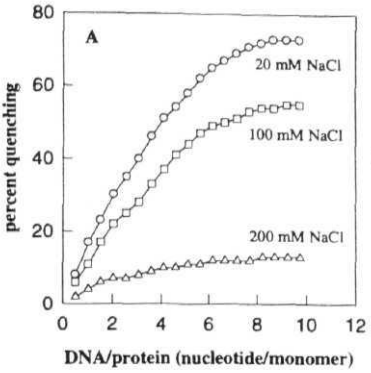
Titrations were also performed with single stranded DNA (with denatured calf thymus DNA and M13 ssDNA). The results are presented in Fig.4.3.2 A and B. The extent of quenching observed was lower than with dsDNA for all the three forms of HSNP-C. Here again HSNP-C' fc was quenched most by single stranded DNA. These results show that HSNP-C'*b* has stronger affinity to both dsDNA and ssDNA than the other two forms of HSNP-C.

Fig. 3.4.3 A, B **and** C : Quenching of intrinsic fluorescence of HSNP-C and its isoforms with ds DNA (calf thymus DNA) at different concentrations of salt. HSNP-C *a* / HSNP-C7 HSNP-C'*b* (1.5 μM) in 1 ml of buffer was titrated with increasing concentration of ds DNA. The fluorescence intensity was measured after each addition.

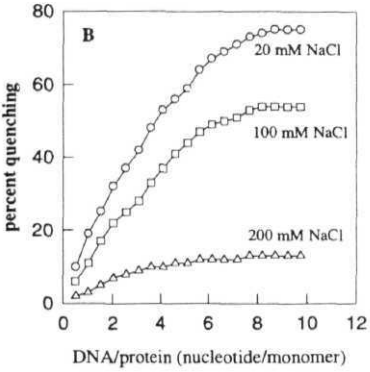
(A): Reverse titration of HSNP-C'*a* with calf thymus DNA at 20, 100 and 200 mM NaCl.

(B): Reverse titration of HSNP-C with calf thymus DNA at 20, 100 and 200 mM NaCl.

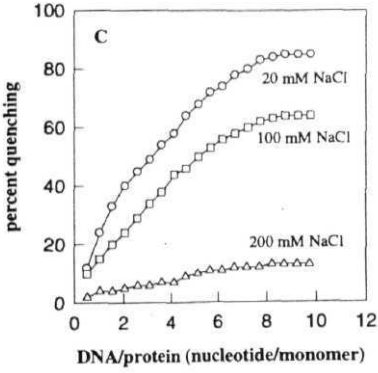
(C): Reverse titration of HSNP-C'*b* with calf thymus DNA at 20, 100 and 200 mM NaCl.



HSNP-C'*a*



HSNP-C'



HSNP-C'*b*

Fig. 3.4.3.1 A, B and C : Scatchard plot of the data obtained in fig. 4.3 A, B and C.

(A): Scatchard plot of the data points obtained in fig. 4.3 A for titrations of HSNP-C'*a* with ds DNA at 20 mM NaCl. The data points were extrapolated on to the y-axis. The intercept on the y-axis gave a value of 8.5 x $10^{6} M^{-1}$.

(B): Scatchard plot of the data points obtained in fig. 4.3 B for titrations of HSNP-C' with ds DNA at 20 mM NaCl. The data points were extrapolated on to the y-axis. The intercept on the y-axis gave a value of 8.5 x $10^{6} M^{-1}$.

(C): Scatchard plot of the data points obtained in fig. 4.3 C for titrations of HSNP-C'*b* with ds DNA at 20 mM NaCl. The data points were extrapolated on to the y-axis. The intercept on the y-axis gave a value of 1.3 x $10^{7} M^{-1}$.

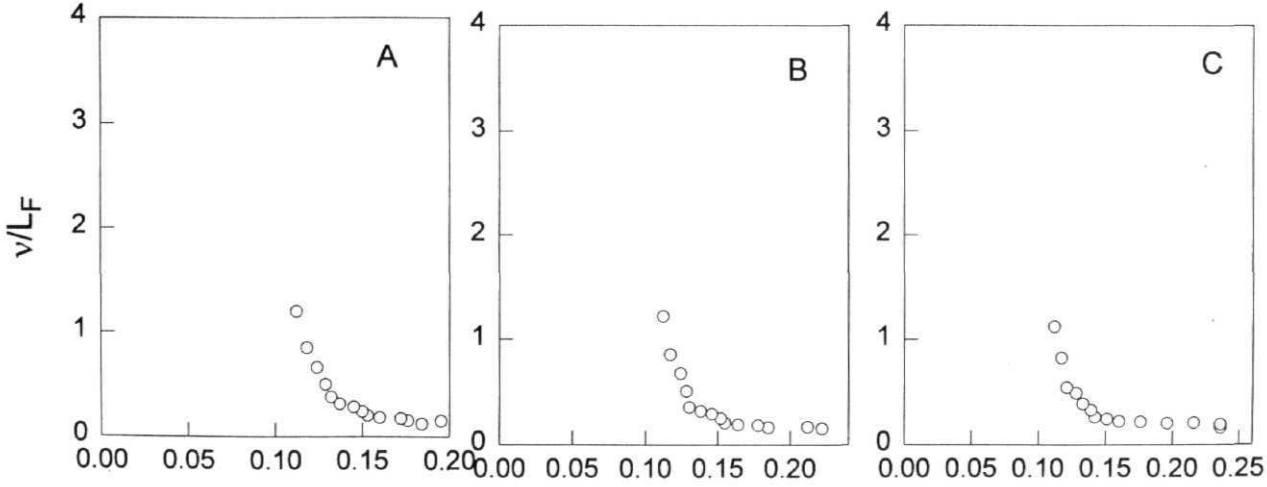
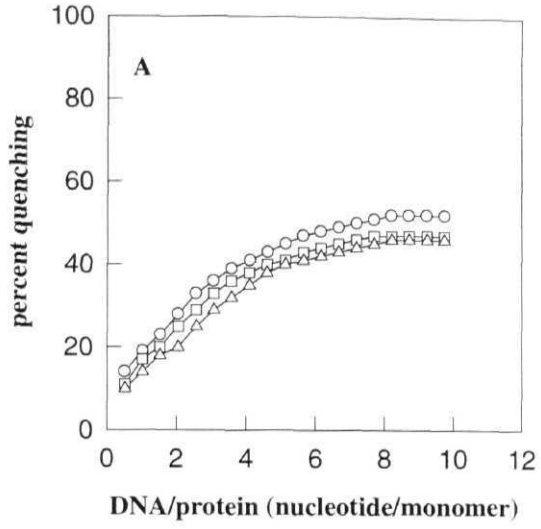
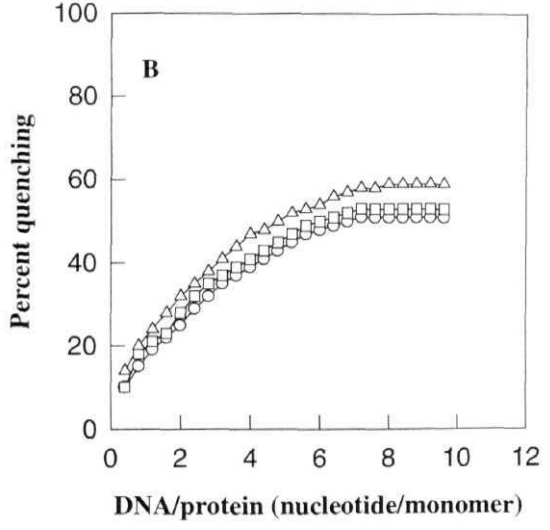

A
B
C
ν/L_F
0
1
2
3
4
0.00
0.05
0.10
0.15
0.20
0.25

Fig. 3.4.3.2 A and B : Reverse **titrations** of HSNP-C and its isoforms with single stranded DNA. Titrations were performed as described in fig. 4.3 in 1 ml of buffer containing 20 mM NaCl. (A) : Reverse titrations **of** HSNP-C and its isoforms with denatured **calf** thymus **DNA. 0-0 :** HSNP-C'*b*: □- □: HSNP-C: A-A HSNP-C'*a*. **(B)** : Reverse titrations of HSNP-C and its isoforms with M 13 ss DNA. A-A : HSNP-C'*b*: □ – □ : HSNP-C: o-o : HSNP-C'*a*.


A
percent quenching
100
80
60
40
20
0
0
2
4
6
8
10
12
DNA/protein (nucleotide/monomer)


B
Percent quenching
100
80
60
40
20
0
0
2
4
6
8
10
12
DNA/protein (nucleotide/monomer)

3.4.4 Salt titrations :

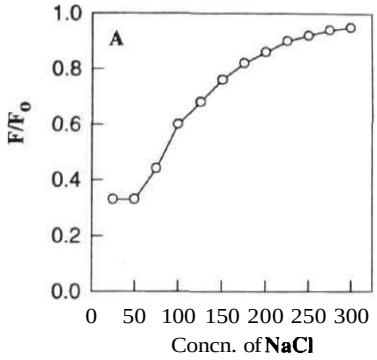
Salt titrations are performed to assess the strength of binding between protein and nucleic acids. To a fixed concentration of HSNP-C' (all three forms) increasing concentration of DNA was added and the increase in quenching was followed till the saturation point (at low salt, 20 mM NaCl). At this stage small volumes of 4 M NaCl were added directly to the nucleoprotein complexes and the increase in flourescence intensity was measured (Fig. 3.4.4 A, B and C). The concentration of the salt where the maximum fluorescence was recovered corresponds to the concentration salt required to dissociate the nucleoprotein complexes. The salt concentration required for recovering 60% of the original fluorescence intensity for HSNP-C'*a*, HSNP-C and HSNP-C'*b* were 100 mM, 120 mM and 170 mM respectively indicating that the strength of binding of the three forms increases in the order HSNP-C'*b* > HSNP-C> HSNP-C'*a*.

3.5 Binding to GTP and the associated GTPase activity :

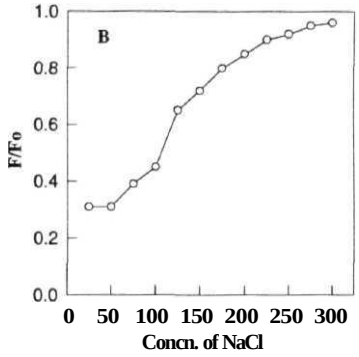
It was earlier found that HSNP-C contains **P-loop motif** and binds to nucleotide and that the binding is independent of the binding to DNA (Celestina, 1996). The binding of nucleotides to the three forms of HSNP-C was determined by fluorescence titrations. Results presented in Fig. 3.5.1 A, B and C show that all the three forms of HSNP-C bind GTP. Using data of experiment in Fig. 3.5.1. A, B and C, percent quenching was plotted against the concentration of GTP (Fig. 3.5.1 D). The extent of quenching of HSNP-C' fc by GTP was appreciably higher than in the case of HSNP-C'a and HSNP-C indicating higher affinity of HSNP-C'*b* to GTP. Titrations performed at higher salt (200 mM NaCl) showed reduced binding to GTP (Fig. 3.5.1E). Fluorescence titrations performed with **GTP,** dGTP and GMP show that the protein binds the three nucleotides with greater affinity to the triphosphate derivative (Fig. 3.5.2). Since HSNP-C bound GTP it was of interest to see if the protein shows GTPase activity. Results presented in Fig. 3.5.3 show that the protein possesses low GTPase activity which is

Fig. 3.4. 4 A, B **and** C : Back titration of the isoforms of HSNP-C'-DNA complexes with salt. Aliquots of concentrated NaCl were added at Q_{max} and the increase in fluorescence intensity was measured at each NaCl concentration as descibed in the materials and methods section. The ratio of the intensity of fluorescence (F in arbittrary units) measured at each addition and the initial fluorescence (F_o) was plotted against concentration of NaCl.

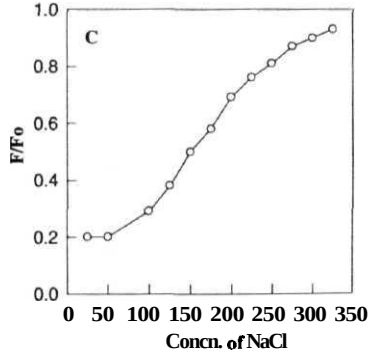
(A) : HSNP-C'*a*-DNA complexes; (B) : HSNP-C'-DNA complexes; (C): HSNP-C'*b*-DNA complexes.



HSNP-C' a



HSNP-C'



HSNP-C' b

Fig. 3. **5. 1** A : Fluorescence emission spectrum of HSNP-C'*a* titrated with GTP. HSNP-C'*a* (1.5 μM) was titrated with increasing concentration of GTP (0.5 to 4 μM) and the fluorescence emission spectrum was recorded after each addition (Excitation at 285 nm). Spectrum 1 : HSNP-C' control; spectrum 2-7 : HSNP-C titrated with increasing concentration of GTP.

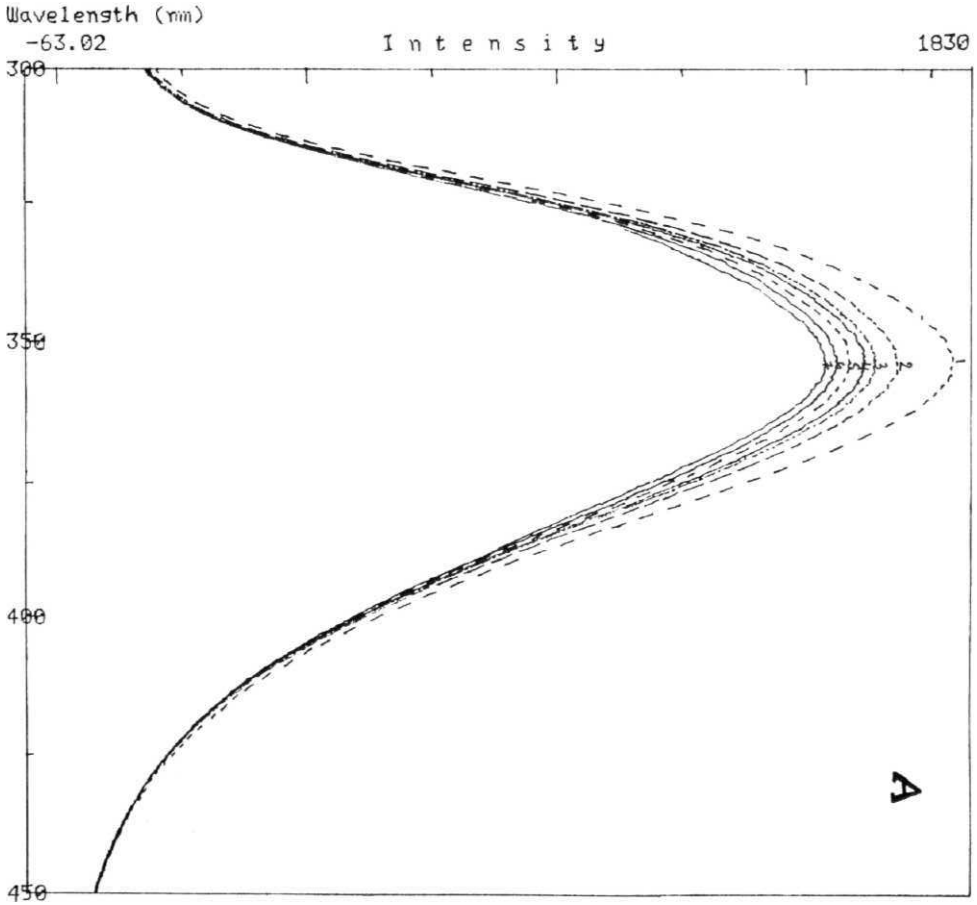

Wavelength (nm)
-63.02
Intensity
1830
300
350
400
450
A

Fig. 3. 5. 1 B : Fluorescence emission spectrum of HSNP-C titrated with GTP. HSNP-C' (1.5 μM) was titrated with increasing concentration of GTP (0.5 to 4 μM) and the fluorescence emission spectrum was recorded after each addition (Excitation at 285 nm). Spectrum 1 : HSNP-C (control); spectrum 2-7 : HSNP-C titrated with increasing concentration of GTP.

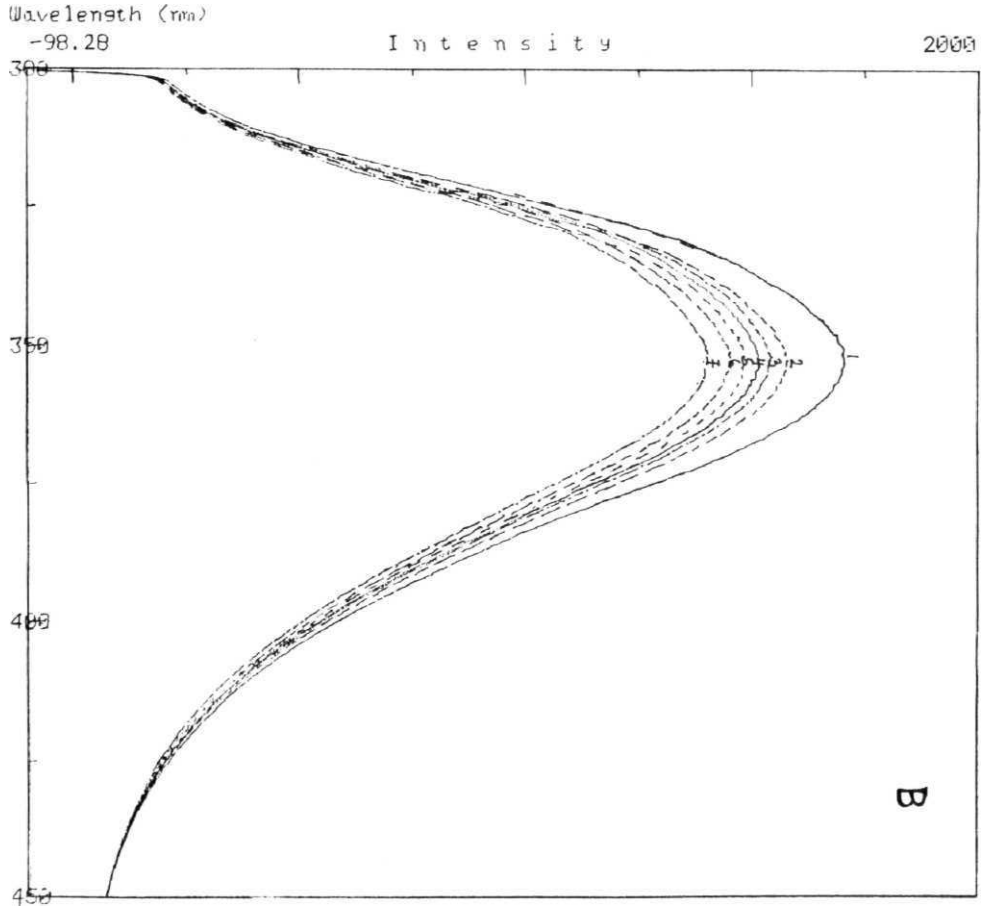

Wavelength (nm)
-98.28
Intensity
2000
300
350
400
450
B

Fig. 3. **5. 1** C : Fluorescence emission spectrum of HSNP-C'*b* (1.5 μM) titrated with GTP. HSNP-*C'b* was titrated with increasing concentration of GTP (0.5 to 4 μM) and the fluorescence emission spectrum was recorded after each addition (Excitation at 285 nm). Spectrum 1 : HSNP-C'*b* (control); spectrum 2-7 : HSNP-C'*b* titrated with increasing concentration of GTP.

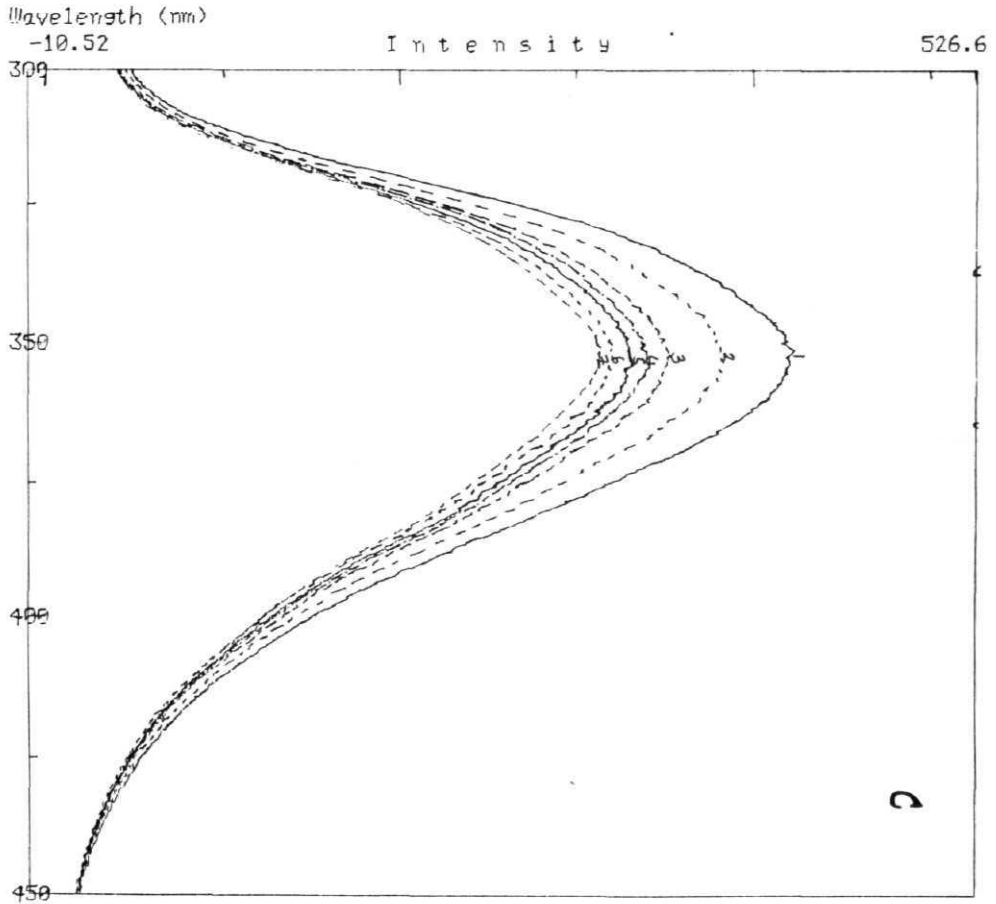

Wavelength (nm)
-10.52
Intensity
526.6
300
350
400
450
1
2
3
4
5
6
7

Fig. 3.5.1 D : Using data from experiment in Fig 5.1 A, B and C. percent quenching **for** HSNP-C and its isoforms was plotted against concentration of GTP.
o-o : HSNP-C'*a*; □ - □ : HSNP-C'; A-A: HSNP-C'*b*.

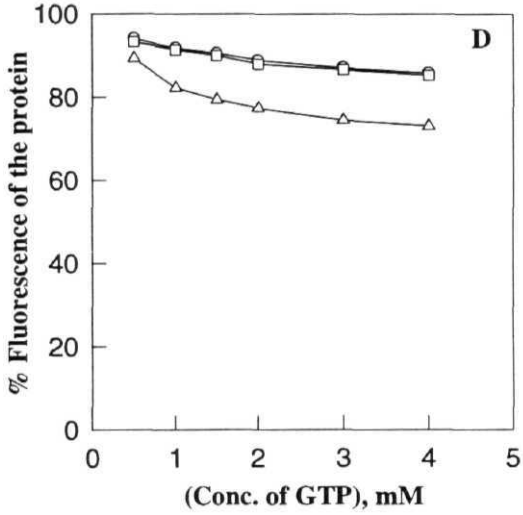

D
% Fluorescence of the protein
100
80
60
40
20
0
0
1
2
3
4
5
(Conc. of GTP), mM

Fig. 3. **5. 1** E : Effect of salt on binding of **HSNP-C'** to GTP.
HSNP-C'(1.5 μM) was titrated with increasing concentration of GTP (0.3 to 6 μM) and the fluorescence intensity was recorded after each addition (Excitation at 285 nm). o–o : titration performed at 20 mM NaCl; □- □: titration performed at 200 mM NaCl.

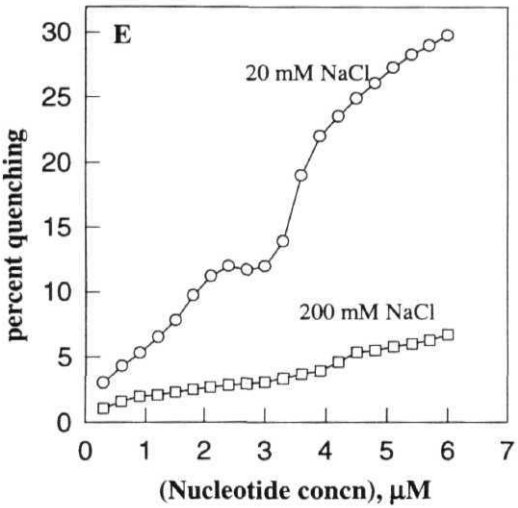

E
20 mM NaCl
200 mM NaCl
percent quenching
(Nucleotide concn), μM
0
5
10
15
20
25
30
0
1
2
3
4
5
6
7

Fig. **3. 5. 2** : Quenching of intrinsic fluorescence of HSNP-C' with different mononucleotides. o-o : GTP; □ –□ : dGTP; A-A : GMP.

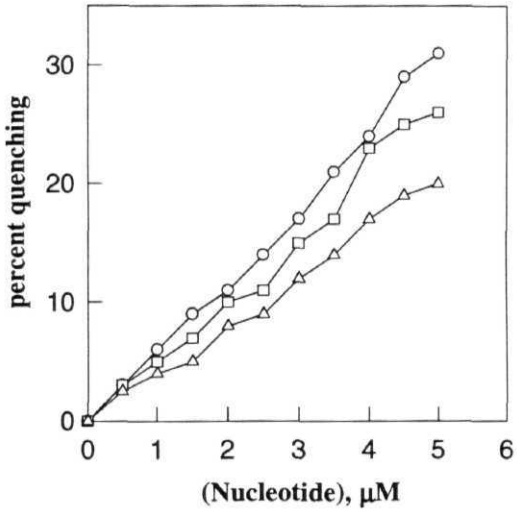
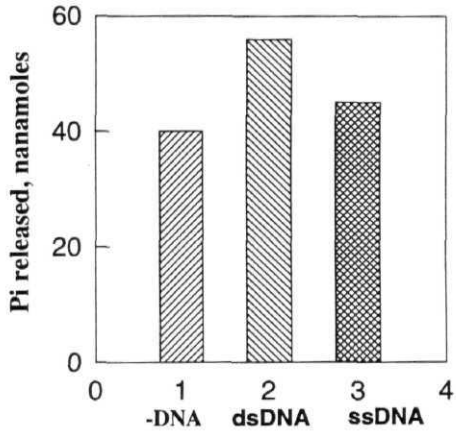

percent quenching
30
20
10
0
0
1
2
3
4
5
6
(Nucleotide), μM

Fig. *3.5.3:* GTPase activity for HSNP-C'.
The assay was performed as described in the methods section in three conditions :
(a) : without DNA; (b): in the presence of ds DNA; (c) : in the presence of ss DNA.


Pi released, nanamoles
60
40
20
0
0
1
2
3
4
-DNA
dsDNA
ssDNA

stimulated marginally by DNA. Low activity observed may be due to suboptimal conditions for the assay.

3.6. Binding to RNA :

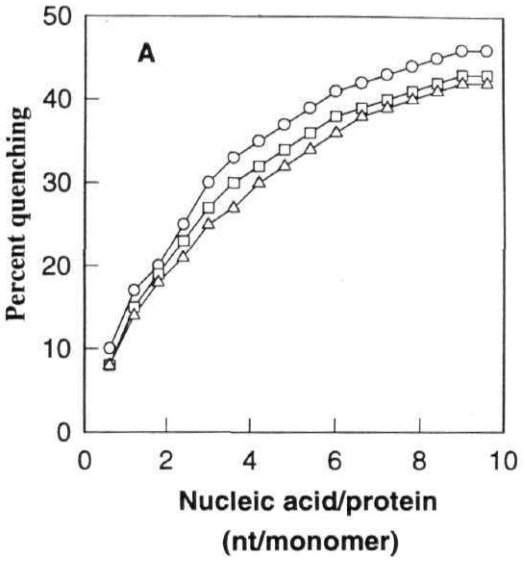
The affinity of HSNP-C to ribonucleic acids was also studied by fluorescence titrations. The three forms of HSNP-C were titrated with tRNA (Fig. 3.6A) rRNA (Fig. 3.6B) and synthetic polyribonucleotides, poly A and poly U (Fig. 3.6C). The fluorescence of the protein was quenched by all the polynucleotides. However, the extent of quenching varied. tRNA and ribosomal RNA which are known to have substantial secondary structure quenched the fluorescence by about 45% whereas synthetic polynucleotides showed lower quenching. Hence, HSNP-C also binds RNA although weakly. The binding to RNA was found earlier to be highly sensitive to salt. Lower than 100 mM salt was sufficient to elute HSNP-C, from columns of poly U and poly A sepharose (Celestina and Suryanarayana, 1995).

3.7 Aggregation of DNA :

It was observed while studying the nucleic acid binding properties of HSNP-C by gel mobility shift assays that at high protein concentration the nucleoprotein complexes failed to enter the gel indicating aggregation of DNA. Therefore the ability of this protein to aggregate nucleic acids was tested by the procedure of Krasnow and Cozzarelli (1982) as described in the methods section. The reaction mixtures containing constant amount of ^{3}H labelled *E. coli* DNA and increasing amounts of HSNP-C were incubated at different pH conditions and centrifuged at 10,000 X g for 10 minutes. The pellet and the supernatant fractions were analysed for DNA. The results presented in Fig. 3. 7.1 A and B show that this protein is able to aggregate both ssDNA and dsDNA. It was found to aggregate DNA at both pH 5.0 and pH 7.4. The aggregation of DNA is dependent on the protein concentration and increases with increase in the concentration of the protein. HSNP-C was able to aggregate 68% of the input dsDNA (Fig. 3.7.1 A) and 65% of the

Fig. 3. 6 A : Quenching of intrinsic fluorescence of the forms of HSNP-C with *E. coli* tRNA. o-o : HSNP-C'*a*; □-□ : HSNP-C; A-A : HSNP-C'*b*.

Fig. 3. 6 B : Quenching of intrinsic fluorescence of the forms of HSNP-C with *E. coli* 23S and 16S rRNA. o–o : HSNP-C'*a*; □-□ : HSNP-C; A-A : HSNP-C'*b*.


A
Percent quenching
50
40
30
20
10
0
0
2
4
6
8
10
Nucleic acid/protein
(nt/monomer)

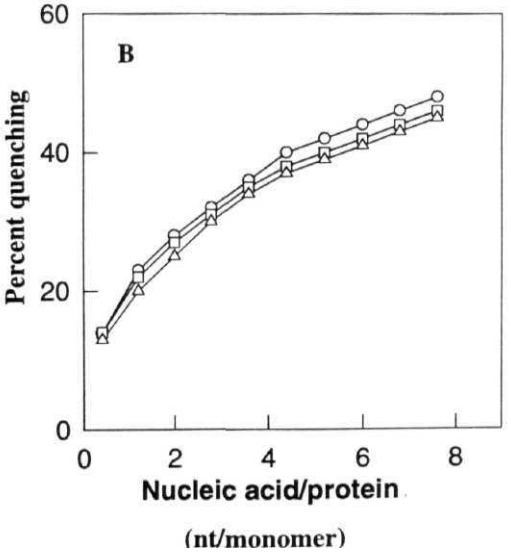

B
Percent quenching
60
40
20
0
0
2
4
6
8
Nucleic acid/protein
(nt/monomer)

Fig. 3. 6 C : Reverse titrations of HSNP-C with polynucleotides.
D-D : poly (U); o-o : poly (A).

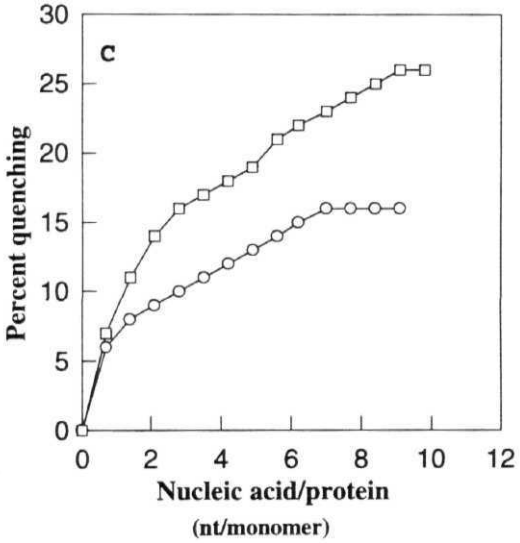
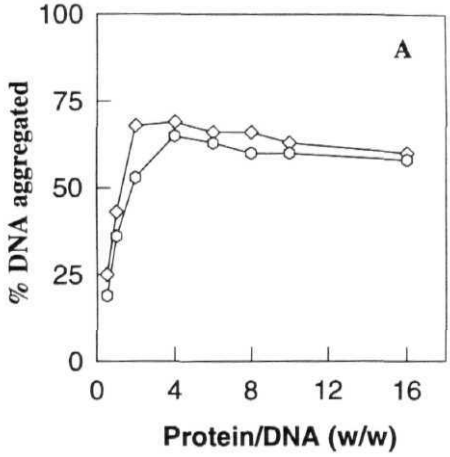
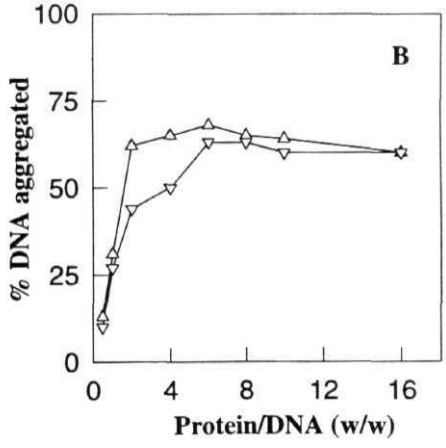

c
Percent quenching
30
25
20
15
10
5
0
0
2
4
6
8
10
12
Nucleic acid/protein
(nt/monomer)

Fig. 3.7.1 A **and** B : Aggregation of ^{3}H labelled *E. coli* DNA (native / denatured) by HSNP-C The assay was performed as described in the methods section. Aggregation is defined as the decrease in the radioactivity of top 20|il relative to an unsedimented control mixture. A : Aggregation of native ^{3}H labelled £. *coli* DNAby HSNP-C; 0-0 : native DNA at pH 5.0; 0-0 : native DNA at pH 7.6. B : Aggregation of denatured ^{3}H labelled *E. coli* DNA by HSNP-C A-A : denatured DNA at pH 5.0; V-V : denatured DNA at pH 7.6.


A
100
75
50
25
0
% DNA aggregated
0
4
8
12
16
Protein/DNA (w/w)


B
100
75
50
25
0
% DNA aggregated
0
4
8
12
16
Protein/DNA (w/w)

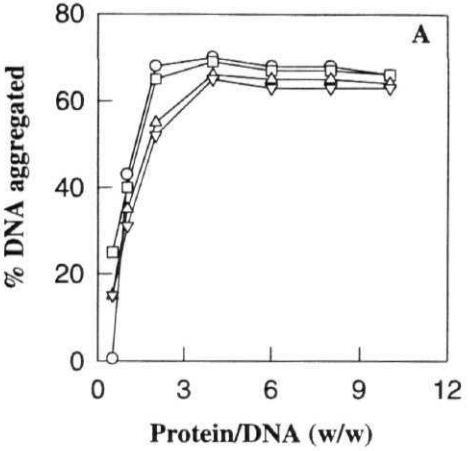
input ssDNA (Fig. 3.7.1 B) . The aggregates formed remained stable with increasing concentration of the protein. Maximum aggregation of native DNA (Fig. 3.7.1 A) was seen from protein to DNA ratios (w/w) of 2.0 (0-0) at pH 5.0. In comparison, aggregation at pH 7.4 required higher protein concentration and maximum aggregation was observed from protein to DNA ratios (w/w) of 4.0 (O–O). A protein / DNA ratio (w/w) of 4.0 corresponds to about 5 nucleotides / protein monomer.

In the case of denatured DNA, aggregation was observed from protein / DNA (w/w) ratios of 4.0 at pH 5.0. (Fig 3.7.1B; A-A). At pH 7.6, the aggregation required higher protein concentration than that required at pH 5.0 i.e. protein to DNA ratios above 6.0 (Fig. 3.7.1B; V-V). Aggregation of DNA by HSNP-C' was also performed in the absence of Mg^{+2} to determine the requirement of Mg^{+2} for aggregation. Results presented in Fig. 3.7.2A show that aggregation of DNA is not dependent on Mg^{+2} as the aggregation pattern was similar to that in the presence of 12 mM $MgCl_2$. Since HSNP-C has affinity for mononucleotides like GTP (as seen in fluorescence titrations with GTP) aggregation assay was performed in the presence of GTP. Results presented in Fig. 3.7.2 B show that GTP had no effect on the aggregation property of HSNP-C as the aggregation pattern observed was similar to that in the absence of GTP.

Aggregation of DNA by HSNP-C was also followed by using non-labelled DNA and the assay was carried out as described in the methods section. Immediately after centrifugation, the total supernatants were removed carefully, mixed with gel loading buffer and electrophoresed. The pellets were dissolved in 40 µl of water, mixed with gel loading buffer and electrophoresed on 15% SDS-PAGE . The gels were silver stained in order to visualise both DNA and HSNP-C(Fig. 3.7.3 A and B). DNA (native / denatured) seen as smear at the top portion of the gel was completely sedimented by HSNP-C as all the DNA was in the pellet (Fig. 3.7.3 A, lane 10 ; Fig. 3.7.3 B, lane 10). The protein also pelleted along with DNA and no traces of either protein or DNA were visible in the supernatant fraction (Fig. 3.7.3 A, lane 11; Fig. 3.7.3 B, lane 11). At higher protein concentration (i.e. between the ratio of 3-6 of protein / DNA) excess or free protein started appearing in the supernatant (Fig. 3.7.3 A and B; lanes 15,17 and 19) but,

Fig. 3.7.2 A : Effect of Mg^{+2} on the aggregation of native and denatured ^{3}H labelled *E. coli* DNA promoted by HSNP-C'. o-o : pH 5.0,12 mM $MgCl_2$ (native DNA); □- □ : pH 5.0 without Mg^{+2} (native DNA); A-A : pH 7.6,12 mM $MgCl_2$ (denatured DNA); V-V : pH 7.6 without Mg^{+2} (denatured DNA).

Fig. 3.7.2 B : Effect of GTP on the aggregation of native and denatured ^{3}H labelled *E. coli* DNA promoted by HSNP-C'. □ – □: pH 5.0, 1 mM GTP (native DNA); Δ–Δ : pH 5.0, without GTP (native DNA); V-V pH 7.6,1 mM GTP (denatured DNA); 0-0 : pH 7.6, without GTP (denatured DNA).


A
% DNA aggregated
80
60
40
20
0
0
3
6
9
12
Protein/DNA (w/w)

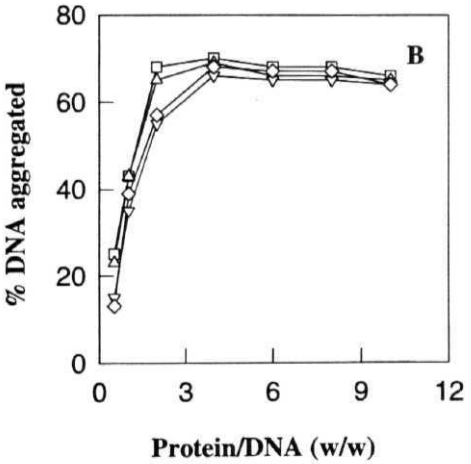

B
% DNA aggregated
80
60
40
20
0
0
3
6
9
12
Protein/DNA (w/w)

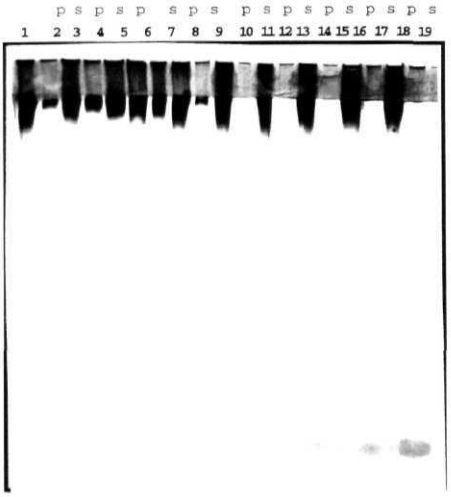
Fig. 3.7. 3 A : SDS-PAGE analysis of **native** DNA aggregation reaction products.
Lane 1 : 2 μg of native 5. *acidocaldarius* DNA without centrifugation (control); lane 2 : pellet (control); lane 3 : supernatant (control); lanes 4, 6, 8, 10, 12. 14, 16, 18 : pellet fractions from reaction mixture incubated with 0.5 μg, 1 μg, 2 μg, 4 μg, 6 fig, 12 fig, 18 μg and 24 μg of HSNP-C' respectively; lane 5, 7, 9, 11, 13, 15, 17, 19 : supernatant fractions from reaction mixture incubated with 0.5 μg, 1 (ig, 2 μg, 4 μg, 6 μg, 12 μg, 18 μg and 24 μg of HSNP-C respectively.

Fig. 3.7. 3 B : SDS-PAGE analysis of **denatured** DNA aggregation reaction products.
Lane 1 : 2 μg of heat denatured *S. acidocaldarius* DNA without centrifugation (control); lane 2 : pellet (control); lane 3 : supernatant (control); lanes 4, 6, 8, 10, 12, 14, 16, 18 : pellet fractions from reaction mixture incubated with 0.5 μg, 1 μg, 2 μg, 4 μg, 6 μg, 12 μg, 18 μg and 24 μg of HSNP-C respectively; lane 5,7,9,11,13,15,17. 19 : supernatant fractions from reaction mixture incubated with 0.5 μg, 1 μg, 2 μg, 4 μg 6 μg, 12 μg, 18 μg and 24 μg of HSNP-C respectively.

A


p s p s p s p s p s p s p s p s p s p s
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

B


p s p s p s p s p s p s p s p s p s p s
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

however no trace of DNA was seen in the supernatants. This again corresponds to about 4-5 nucleotides / monomer protein where the saturation of DNA occurs.

DNA aggregation was also studied by light scattering monitored as increase in absorbance at 320 nm as described in the methods section. DNA aggregation by HSNP-C was performed at 37°C and 65°C. Results presented in (Fig. 3.7.4) show that at protein to DNA ratio (w/w) of greater than 4.0 there was aggregation of DNA as seen by increase in OD at 320 nm. Aggregation was optimal at 37°C. But even at 65°C there was appreciable aggregation.

3.8 DNA renaturation :

Renaturation of complementary strands is a key functional property essential for genetic recombination and repair. Aggregation or condensation of DNA was found to facilitate DNA renaturation (Sikorav and Church, 1991).

Since HSNP-C could aggregate DNA, the ability of the protein to promote renaturation of complementary ssDNA was tested. DNA renaturation reaction was performed with heat denatured λDNA (as described in the methods section). The reaction products were deproteinised with 1% SDS and proteinase K, and then analysed by electrophoresis on 0.8% agarose gels. Heat denaturation of dsDNA generates fragments of complementary ssDNA which can be seen as a smear extending towards the bottom of the gel . Renaturation of this fragmented ssDNA forms a **network of high molecular weight renatured DNA with lots of single stranded gaps** (Fig. 3.8). This renatured DNA fails to enter the agarose gel and remains in the wells of the gel. This assay has been widely used to follow renaturation of complementary ssDNA. (Muller and Stasiak, 1991; Sikorav and Church, 1991 and Sung *et al*., 1992). This assay was thus used to follow DNA renaturation by HSNP-C.

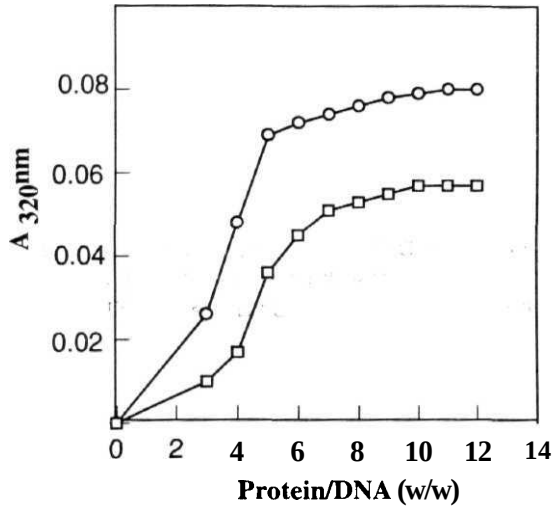

0.08
0.06
0.04
0.02
A 320nm
0
2
4
6
8
10
12
14
Protein/DNA (w/w)

Fig. 3.7. 4 : Aggregation of DNA studied by light scattering method . DNA aggregation promoted by HSNP-C' was studied by measuring the increase in the absorbance at 320 nm. o–o : assay performed at 37° C; □ - □ : assay performed at 65° C.

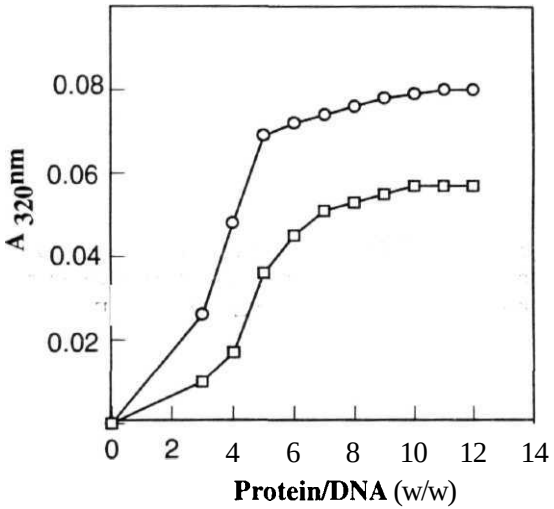
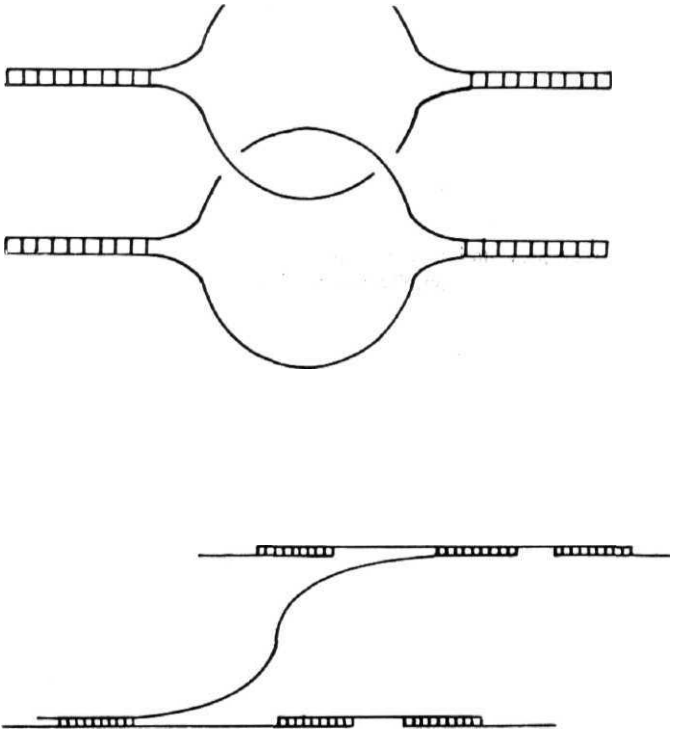

0.08
0.06
0.04
0.02
A 320nm
0
2
4
6
8
10
12
14
Protein/DNA (w/w)

Fig. 3.8 : Schematic representation of network DNA formed by renaturation of randomly sheared DNA molecules (taken from Sikorav and Church, 1992).



3. 8.1 Effect of pH on DNA renaturation by HSNP-C' :

DNA renaturation was carried out under different pH conditions to determine the optimum pH required for renaturation (Fig. 3.8.1). Large amounts of DNA is retained in the wells of the agarose gel after deproteinisation with 1% SDS and proteinase K at pH 5.0. (Fig. 3.8.1 A; lane 2-6) compared to reactions performed at pH 5.5 (Fig. 3.8.1C; lane 2-6), pH 6.0 (Fig. 3.8.1D; lane 2-6) and pH 7.6 (Fig. 3.8.1B; lane 2-6). At pH 5.5 and pH 6.0 (Fig. 3.8.1 C and D) the amount of protein required to bring out renaturation is greater than at pH 5.0. At pH 7.6, there is very little renaturation as can be seen by the very low amounts of DNA is retained in the wells of the gel (Fig. 3.8.1B).

3.8.2 Time course of renaturation :

Renaturation assay was performed at different time intervals to determine the time required for DNA renaturation. Renaturation of DNA by HSNP-C occurs at a very rapid rate as renatured DNA was seen retained in the well within one minute of incubation (Fig. 3.8.2). Renaturation is complete by 15 min. as almost all the DNA appears in the well (lane 5). After 30 minutes of incubation there was no further change in the renatured products (lane 6).

3.8.3 Effect of Mg^{+2} on renaturation :

Renaturation assay was also carried out in the absence of $MgCl_2$ to see if renaturation was dependent on magnesium ions. HSNP-C was found to bring about renaturation even in the absence of magnesium (Fig. 3.8.3). When compared to renaturation in the presence of Mg^{+2} (Fig. 3.8.1 A) there is a slight delay in renaturation initially i.e. at DNA / protein ratios of 1 : 0.5 and 1:1. However, at higher DNA / protein ratios renaturation was complete.

Fig. 3.8. **1 A, B, Cand** D : Renaturation **of** DNA **by** HSNP-C'.

The renaturation assay was carried out as described in the text . Denatured λ DNA was incubated with increasing amounts **of HSNP-C** in **different** pH conditions and **the** reaction products were deproteinised with **a mixture** of 1%SDS and proteinase K and electrophorosed on 0.8% **agarose** gels .

(A): DNA **renaturation** assay carried out in buffer **containing 10** mM sodium acetate **pH** 5.0, 12 mM $MgCl_2$ and) mM **DTT,** Lane 1 : denatured DNA (0.4 μg) incubated in the absence of protein; lane 2-6 : **denatured** DNA **incubated** in the **presence of** 0.2 μg, **0 4** μg, **0.8** μg, 1.6 μg and **3.2** μg of **HSNP-C** respectively .

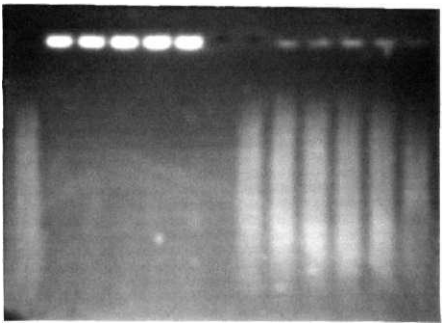
(B): DNA renaturation assay carried out **in** buffer containing 10 mM Tris-HCl **pH** 7.6, 12 mM $MgCl_2$ and 1 mM **DTT. Lane** 1 : denatured DNA (0.4μg)incubated in **the** absence of protein; lane 2-6 : denatured DNA incubated **with** increasing concentration **of protein** as above.

(C): DNA **renaturation** assay carried out **in** buffer containing 10 mM sodium acetate **pH** S.5, 12 mM $MgCl_2$ and 1 **mM** DTT. **Lane** I : denatured DNA (0.4 μg) incubated in the absence of protein; lane 2-6 . denatured DNA incubated with **increasing** concentration of protein as above.

(D): DNA renaturation assay **carried** out in buffer containing **10** mM **sodium acetate** pH **6.0,** 12 mM $MgCl_2$ and i mM **DTT.** Lane 1 ; denatured DNA (0.4 μg) incubated in the absence of protein; lane 2-6 : denatured DNA incubated with **increasing** concentration of protein as above.

A P

1 2 3 4 5 6 1 2 3 4 5 6



C D

1 2 3 4 5 6 1 2 3 4 5 6

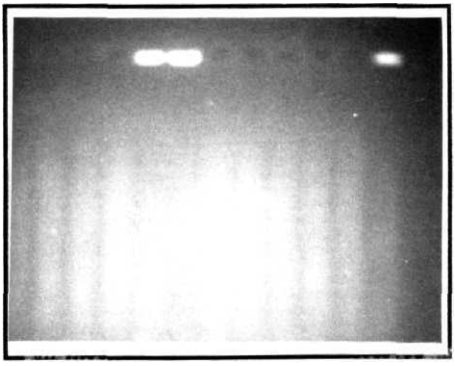


Fig. 3.8. 2 : Time course of DNA renaturation promoted by HSNP-C'. Lane] : 0.4 µg of denatured *X* DNA incubated in the absence of protein **(control) deproteinised** after] min. *of* incubation time; lane *2* : control reaction mixture terminated after 30 min.; tane 3-6 ; renaturation mixtures in the presence of HSNP-C incubated **for** 1 min., 5 min., 15 min. and 30 min. before deproteinisation.

1 2 3 4 5 6

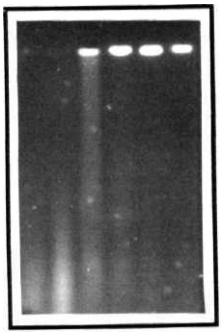
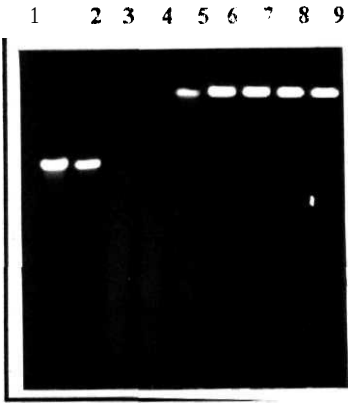


Fig. 3. 8. 3 : Renaturation assay in the absence of magnesium ions. The assay was performed as described in the text. All samples were deproteinized before electrophoresis. Lane 1 : 0.4 μg of native *X* DNA (control); lane 2 : native *X* DNA incubated with 0.4 μg of HSNP-C'; lane 3 : 0.4 μg of heat denatured *X* DNA (control); lane 4-9 : heat denatured *X* DNA incubated with 0.2 |ig, 0.4 μg, 0.8 μg, 1.6 μg, 3.2 μg and 6.4 μg of HSNP-C respectively.


1 2 3 4 5 6 7 8 9

3.8.4 S1 nuclease sensitivity of renatured products :

S1 nuclease digestion of the renatured products was performed to study the sensitivity of renatured DNA to S1 nuclease. S1 nuclease is a single strand specific nuclease. Renaturation assay at different concentrations of HSNP-C' was performed in duplicate and deproteinised with SDS. One set of the reaction products were loaded directly onto the agarose gel (Fig. 3.8.4). The renatured product is seen retained in the wells (Fig. 3.8.4, lane 2-6). Denatured DNA incubated in the absence of protein (control) was not renatured (lane 1). The other set was deproteinised and incubated with S1 nuclease before loading onto the gel. Denatured DNA incubated with S1 nuclease in the absence of HSNP-C was digested into small fragments which migrated to the bottom of the gel (Fig. 3.8.4, lane 6). Although some amounts of the DNA renatured by HSNP-C was digested by S1 nuclease, substantial amounts of DNA was resistant to digestion and remained in the wells of the agarose gel (lane 8-11, Fig. 3.8.4). This resistance to S1 nuclease digestion increases as the concentration of HSNP-C increases, thus indicating the formation of duplex regions in the renatured DNA.

3.8.5 Relation between DNA aggregation and renaturation promoted by HSNP-C :

As aggregation of DNA increases the effective concentration of DNA molecules, it facilitates renaturation. An experiment was carried out to see the relation between DNA aggregation and renaturation in the presence of HSNP-C. The renaturation assay was carried out in the presence of increasing amounts of HSNP-C and the samples were centrifuged at 10,000 X g for 10 minutes. The supernatants and the pellets were deproteinised with 1% SDS and loaded onto a 0.8% agarose gel (Fig. 3.8.5). DNA in the absence of HSNP-C is not sedimented (lane 2) and is seen in the supernatant (lane 3). As a reference, the reaction mixture incubated with DNA alone was loaded onto the gel without centrifugation (lane 1). At DNA / protein ratio of 1:0.5 most of the DNA is seen

Fig. 3.8.4 : Resistance of renatured **DNA** to **S1** nuclease digestion. The assay was performed as **described in the** methods section. Lane 1 : 0.4 **μg** of denatured **λ** DNA (control); lane 2-**5** : renaturation products formed at **DNA** : protein ratios of **1:1, 1:2, 1:4, 1:8** respectively; **lane** 7 : 0.4 **μg** of denatured **λ DNA** digested with **S1** nuclease (control); lane **8-11** : **S1 nuclease treatment** of renaturation products formed at DNA : protein ratios of **1:1**, 1:2, 1 4 and **1:8 respecti vely**.

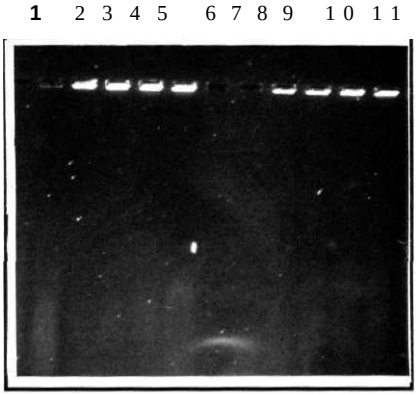
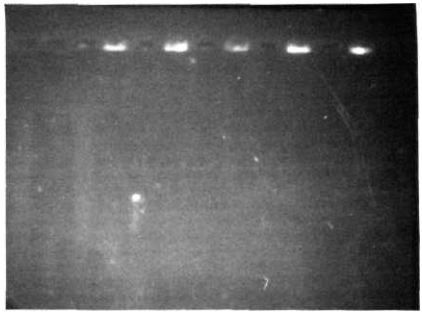

1 2 3 4 5 6 7 8 9 10 11
UNTREATED
S1 TREATED

Fig. 3.8. **5** : Relation between renaturation and aggregation .
DNA renaturation assay with increasing amounts of HSNP-C was carried **out in buffer** containing 10 mM sodium acetate pH 5.0, 12 mM $MgCl_2$ and J mM DTT. The reaction products were centrifuged at 10,000 x g for 10 minutes . The **supernatant and the pellet** fractions were deproteinised with 1% SDS and analysed on 0.8% agarose gel . Lane 1 : 0.4 µg of heal denatured λ DNA (control unsedimented); lane 2 . pellet fraction of **the** reaction mixture (control) incubated in the absence **of HSNP-C;** lane 3 : supernatant fraction of the reaction mixture (control) incubated in the absence **of HSNP-C;** Jane 4, 6, 8, 10, 12 : pellet fractions of **the** reaction mixture incubated with 0.2 µg, *GA* µg, 0.8 µg, 1.6 µg and 3.2 µg **of HSNP-C** respectively; lane 5, 7, 9, 11, 13 : supernatant fractions of the reaction mixture incubated with 0.2 µg, 0.4 µg, 0.8 µg, 1.6 µg and 3.2 µg of HSNP-C respectively .

1 2 3 4 5 6 7 8 9 10 11 12 13



in the pellet fraction (lane 4) and most of it was renatured. With increase in HSNP-C' concentration almost all the DNA was seen in the pellet fractions and was also found to be renatured (lane 6, 8, 10, 12). No traces of denatured DNA were seen in these lanes. Thus from DNA / protein ratios of 1:0.5 (w/w) renaturation was seen and this renatured DNA pelleted upon centrifugation indicating aggregation. The DNA aggregation and renaturation experiments were carried out using only HSNP-C due to the abundant availability of this protein.

3.9 Sensitivity of DNA in nucleoprotein complexes formed by HSNP-C:

HSNP-C binds strongly to dsDNA. The susceptibility of DNA in the nucleoprotein complexes of HSNP-C'-λDNA to nuclease was studied by subjecting them to DNase I enzyme digestion.

DNase I digestion of HSNP-C'-DNA complexes :

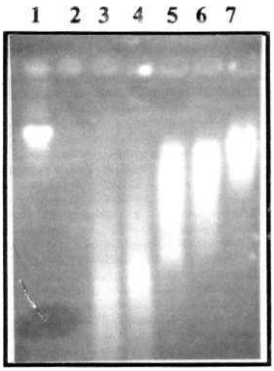
HSNP-C'-λDNA complexes were formed by incubating increasing amounts of HSNP-C with constant amount of DNA. These nucleoprotein complexes were then incubated with constant amount of DNase I (DNase I (w/w) =10). The reaction was stopped, deproteinised with 1% SDS and the reaction products were analysed by electrophoresis on 0.8% agarose gel (Fig 3.9 A) . DNase I digestion of A, DNA generated small fragments which appeared as a smear below the intact DNA (lane 3). The λ DNA in the nucleoprotein complexes was progressively protected from digestion with increasing concentration of the protein (lane 4-7) as seen by the increase in the size of the fragments.

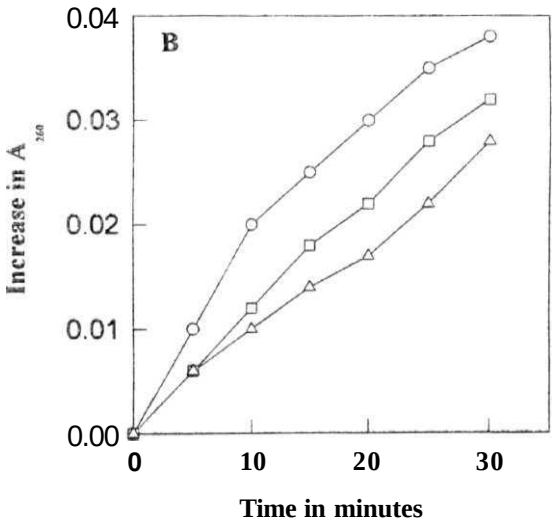
DNase I digestion of dsDNA in the presence and absence of HSNP-C was also studied by spectrophotometry by measuring the increase in hyperchromicity with time of incubation (Fig. 3.9 B); with increase in concentration of HSNP-C the DNA is protected from hydrolysis by DNase I, as is seen by decrease in hyperchromicity. The assay was

Fig. 3.9 A : DNaseI digestion of *X* DNA in the HSNP-C'-DNA complexes. The assay was performed as descibed in the text. Samples were deproteinised before electrophoresis. Lane 1 : 0.4 μg of native *X* DNA (control); lane 2 : λ DNA alone incubated with DNasel; lane 3-7: DNasel digestion of DNA in the HSNP-C'-λDNA complexes formed at DNA : protein ratios of 1:1, 1:2, 1:4 ,1:8 and 1:16 respectively.

Fig. 3.9 B : Effect of HSNP-C' on action of DNasel. The assay was carried out as described in the text . HSNP-C was added to native calf thymus DNA (5 μg) and incubated with 0.5 μg of pancreatic DNasel. Increase in A_{260} was continuously recorded at 37°C for 30 minutes. o–o : without HSNP-C'; □ – □ : with 10|ig of HSNP-C'; A-A: with 20 μg of HSNP-C.

A


1 2 3 4 5 6 7


B
0.04
0.03
0.02
0.01
0.00
Increase in A 260
0
10
20
30
Time in minutes

performed at 1 : 1 and 1: 2 ratios of DNA/protein, as above this ratio aggregation of DNA was seen.

3.10 Ribonuclease activity for HSNP-C' :

Two RNases, p1 and p2 were identified and characterised from *Sulfolobus solfataricus* by Fusi *et al* (1993). Their sequences showed high degree of sequence similarity to the 7 kDa group of DNA binding proteins of *S. solfataricus* (Fusi *et al.*, 1993; 1995). Later, a 9 kDa protein named SaRD was identified from 5. *acidocaldarius* which was found to possess thermostable RNase activity as well as DNA binding properties. The N terminal amino acid sequence (the first 16 residues) of SaRD showed high degree of sequence similarity to the 7 kDa group of DNA binding proteins from *S. solfataricus* and *S. acidocaldarius.* (Kulms *et al.,* 1995). Since the N terminal sequence of HSNP-C showed high sequence similarity to that of p2 and p3 endoribonucleases from *S. solfataricus* and SaRD protein of *S. acidocaldarius* (see Fig. 3.2D), it was of interest for us to investigate if HSNP-C possessed any RNase activity.

The RNase activity was assayed by two different methods :

a) Visualisation of degraded RNA on acrylamide gels by silver staining .

b) Spectrophotometric method by measuring the increase in the absorbance at 260 nm upon degradation of RNA .

HSNP-C and its isoforms HSNP-C' *a* and HSNP-C' fc were assayed for RNase activity using *E. coli* tRNA as substrate (Fig. 3.10A). The assay was performed as described in the methods section. 5 μg of *E. coli* tRNA was incubated with 1 μg of protein (HSNP-C'*a*/HSNP-C'/HSNP-C'*b*) in different buffers namely 40 mM sodium acetate pH 5.0, 40 mM sodium phosphate pH 7.0 and 40 mM Tris-HCl pH 7.8 at 56°C for 60 minutes . The samples were further incubated with proteinase K in the presence of 0.5% SDS at 37°C for 30 minutes. The cleavage products were resolved on PAGE in the presence of 7 M urea using a 15% gel.

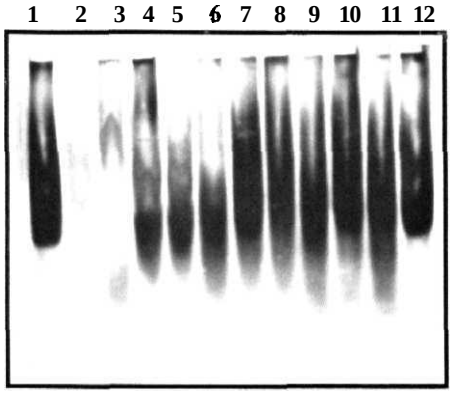
Fig. 3.10 A shows the analysis of degraded products of tRNA formed by the action of HSNP-C'*a*, HSNP-C and HSNP-C'*b* at pH 5.0, pH 7.0 and pH 7.8. The

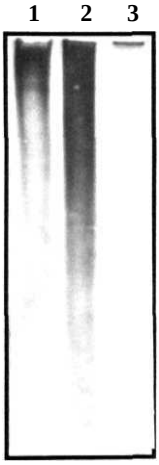
Fig. 3.10 A **and** B : RNase activity of HSNP-C` using *E. **coli*** tRNA and mixture of *E. coli* 23S and 16S rRNA as substrates.

5 µg of ***E. coli*** tRNA or a mixture of *E. coli* 23S and 16S rRNA was incubated with 1 |ig of HSNP-C' ***a*** or HSNP-C or HSNP-C'*b* in 10 µl of different buffers (20 mM sodium acetate pH 5.0, 20 mM sodium phosphate pH 7.0 or 20 mM Tris-HCl pH 7.8) at 56°C for 30 minutes . The samples were deproteinised with a mixture of 0.5% SDS and proteinase K and the degraded RNA was electrophorosed on acrylamide gels and visualised upon silver staining .

(**A**) : RNase activity of HSNP-C and its isoforms with *E.coli* tRNA at pH 5.0, pH 7.0 and pH 7.8. Lane 1 and 12 : tRNA incubated in the absence of protein (control); lane 2 : reaction mixture incubated with HSNP-C alone; lane 3-5 : tRNA incubated with HSNP-C'*a*, HSNP-C and HSNP-C' fr respectively at pH 5.0; lane 6-8 ; tRNA incubated with HSNP-C'*a*, HSNP-C and HSNP-C'*b* respectively at pH 7.0; lane 9-11 : tRNA incubated with HSNP-C'*a*, HSNP-C and HSNP-C' fc respectively at pH 7.8 .

(B) : RNase activity for HSNP-C at pH 5.0 using a mixture of *E. coli* 23S and 16S rRNA as substrate. Lane 1 : rRNA incubated in the absence of protein (control); lane 2 : rRNA incubated in the presence of HSNP-C; lane 3 ; HSNP-C incubated in the absence of rRNA.


1 2 3 4 5 6 7 8 9 10 11 12


1 2 3

degraded tRNA appears as a smear trailing just below the intact tRNA . HSNP-C' being a highly basic protein of pI 10.5 does not enter the gel as the pH of the gel was around 8.3. The slight traces of protein seen in the gel may be due to charge imparted by SDS (lane 2, Fig 3.10 A). HSNP-C'*a* shows maximum activity at all the three pH (Fig. 3.10A, lanes 3,6,9). The activities of HSNP-C and HSNP-C'*b* were low at pH 5.0, but were high at pH 7-8 (lanes 4,5 ; lanes 7,8 ; lanes 10, 11).

Fig. 3.1 OB shows the degradation of a mixture of 23S and 16S rRNA from *E. coli* in the presence of HSNP-C at pH 7.0. The degraded RNA is seen as a long smear extending to the bottom of the gel whereas the control RNA is seen just below the well of the gel. The control protein does not enter the gel in the basic conditions of the gel (Fig. 3.10B, lane 3).

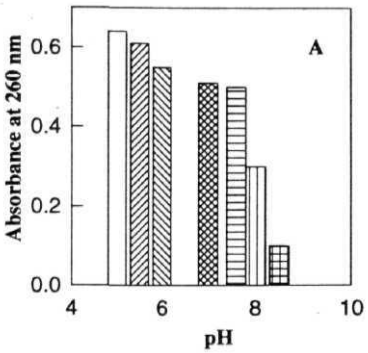
The different forms of HSNP-C were assayed for RNase activity spectrophotometrically using yeast RNA as substrate (according to Fusi *et al.*, 1993) as described in the methods section. RNA (0.4 mg/ml) was incubated with protein (0.1 mg) in different buffers namely 40 mM sodium acetate pH 5.0, pH 5.5, pH 6.0, 40 mM sodium phosphate pH 7.0, 40 mM Tris-HCl pH 7.5, pH 8.0, pH 8.5 and 40 mM sodium carbonate pH 9.5 at 60°C for 60 minutes. The undigested RNA and protein were precipitated with 1.2 M perchloric acid and 22 mM lanthanum chloride and the absorbance of the clear supernatant was measured at 260 nm against a suitable blank. The increase in the absorbance at 260 nm was plotted against pH.

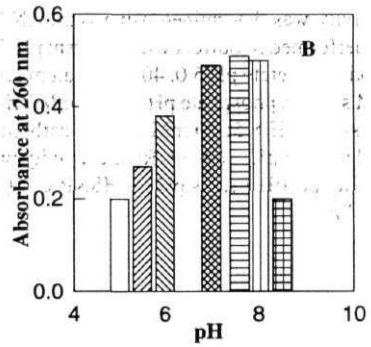
HSNP-C' *a* showed maximal activity at pH 5.0 and substantial activity in the range of pH 5.5 to 7.5. The activity decreased at pH 8.0 and reached zero level at pH 9.5 (Fig. 3.10.1 A). HSNP-C and HSNP-C'*b* showed maximal activity in the pH range of 7.0-8.0 (Fig. 3.10.1 B and C).

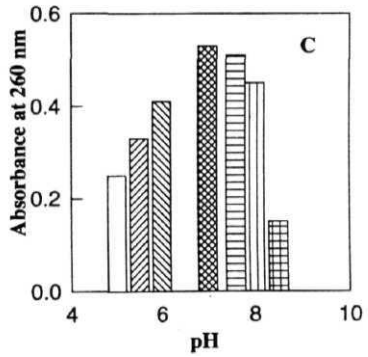
The assay was performed at different temperatures to see the thermostability of the activity. The protein was stable upto 75°C and after which there was a decline in the activity. At 95°C there was complete inactivation of the protein (data not shown).

Fig. 3. 10. 1 A, B **and** C : RNase activity for **HSNP-C'** and its isoforms.

RNase activity was determined using yeast RNA as substrate (Fusi *et al.*, 1993). The assay was performed in buffers with different pH. The composition of the buffers used are : 40 **mM** sodium acetate pH 5.0; 40 **mM** sodium acetate pH 5.5; 40 **mM** sodium acetate pH 6.0; 40 **mM** sodium phosphate pH 7.0; 40 **mM Tris-HCl** pH 7.5; 40 mM **Tris-HCl** pH 8.0; 40 **mM** Tris-HCl pH 8.5; 40 mM sodium carbonate pH 9.5. The absorbance at 260 **nm** was plotted against pH. (A): RNase activity profile versus pH for **HSNP-C'***a*; (**B**): RNase activity profile versus pH for HSNP-C; (C): RNase activity profile versus pH for **HSNP-C'***b*.


A
Absorbance at 260 nm
0.0
0.2
0.4
0.6
4
6
8
10
pH


B
Absorbance at 260 nm
0.0
0.2
0.4
0.6
4
6
8
10
pH


C
Absorbance at 260 nm
0.0
0.2
0.4
0.6
4
6
8
10
pH

3.11 Homology of HSNP-C' to the eukaryotic HMG group of proteins :

The N terminal sequence of HSNP-C has a unique sequence of alternating lysine residues and bears remote resemblance to the HMG proteins in this aspect. This aspect was earlier reported by Kimura *et al* (1984) for the 7 kDa protein Sso 7d from 5. *solfataricus*. This prompted us to investigate if any immunological homology exists between HSNP-C and HMG class of proteins. For this, HMG protein fraction was isolated from rat liver according to Christensen and Dixon (1981) as described in the methods section and immonoblotted and probed with anti HSNP-C IgG.

The procedure for isolation of HMG group of proteins involves the following steps:

a) isolation of 0.35M NaCl soluble proteins from isolated rat liver nuclei

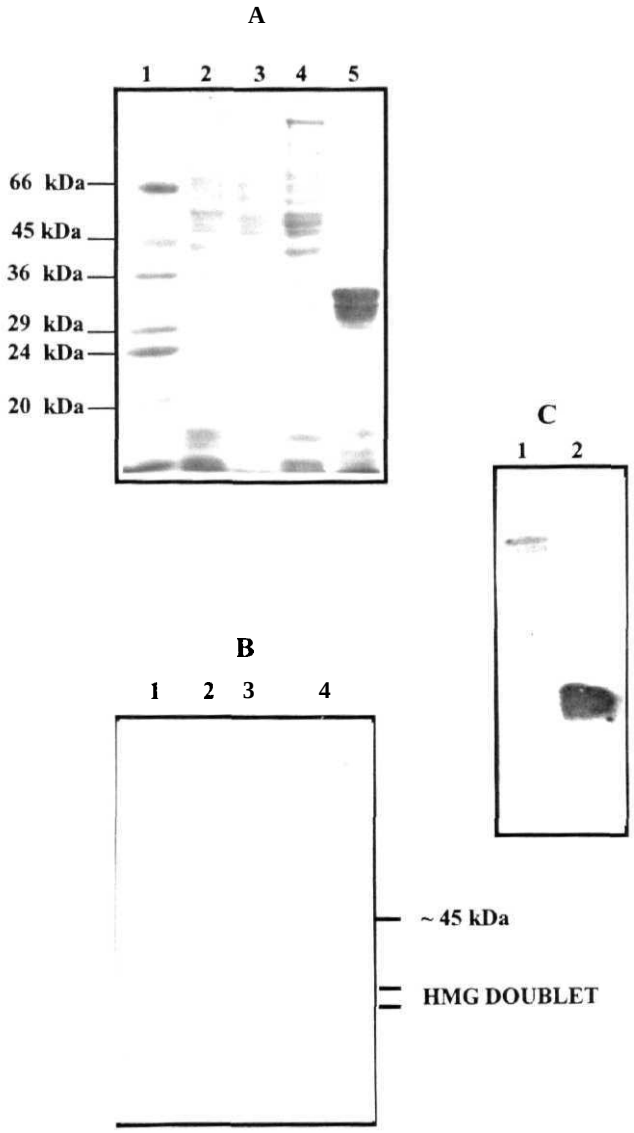
b) Differential precipitation of LMG (low mobility group) proteins and HMG (high mobility group) proteins from the 0.35M NaCl soluble proteins by 3% TCA and 20% TCA respectively.

Fig. 3.11A shows the different steps involved in the isolation of HMG group of proteins. The lane 5 shows the HMG fraction which consists of a distinct doublet of HMG1 and HMG2 of approximately 35 kDa and HMG 14 and 17 around 18 kDa. The proteins from the different steps of extraction of HMG proteins were immunoblotted and probed with anti HSNP-C IgG (Fig.3.11 B). The HMG 1,2 doublet crossreacted distinctly with anti-HSNP-C IgG. Apart from this another protein of approx 45 kDa crossreacted in the nuclear fraction. Fig. 3.11 C shows the strong crossreaction of the HMG fraction with anti HSNP-C'IgG.

Fig. 3.11 A : Isolation of HMG group of proteins from rat liver nuclei . The HMG group of proteins were isolated as decribed in the text . Lane 1 : molecular weight markers, bovine albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde dehydrogenase (36 kDa), carbonic anhydrase (29 kDa); trypsinogen (24 kDa), trypsin inhibitor (20 kDa); lane 2 : rat liver nuclear extract proteins (20 µg protein); lane 3 : proteins precipitated with 0.5 M NaCl pH 7.0 (20 µg protein); lane 4 : proteins precipitated with 3% TCA (LMG group of proteins, 20 µg); lane 5 : proteins precipitated with 20% TCA (HMG group of proteins, 20 µg).

Fig. 3.11 B : Immunoblot of the above samples probed with anti HSNP-C IgG . Details are described in the materials and methods section.

Fig. 3.11 C : Immunoblot showing the cross-reactivity of HMG group of proteins to anti HSNP-C IgG . Lane 1 : HMG fraction from rat liver (20 µg protein); lane 2 : pure HSNP-C the monomeric and dimeric forms (20 µg protein).


A
1 2 3 4 5
66 kDa
45 kDa
36 kDa
29 kDa
24 kDa
20 kDa
C
1 2
B
1 2 3 4
~ 45 kDa
HMG DOUBLET

CHAPTER 4

DISCUSSION

One of the important aspects of genetic material in an organism is its organisation within the cell and its stability. Packaging of eukaryotic DNA has been extensively investigated and the role of histones in the basic organization of the eukaryotic DNA into nucleosomes has been well worked out. In bacteria, it has been assumed that the DNA is organized into a highly condensed nucleoid structure (counter part of chromatin in prokaryotes) with simple free supercoiled domains. The compaction of DNA in bacteria is brought about by histone like proteins (Forterre *et al.*, 1993). However, no definite information is available on the organization of DNA in prokaryotes that include bacteria and archaea.

The archaeal kingdom includes organisms which survive and grow under extreme conditions. The organism of the present investigation *Sulfolobus acidocaldarius* is a sulfur dependent extreme thermoacidophile. The organization DNA in this organism must involve compaction (or condensation) along with thermal stability. The chromosome structure with respect to thermal stability has been widely investigated in order to understand the stability of DNA in these hyperthermophiles (Forterre *et ai*, 1993; Marguet and Forterre, 1994). A chromosome like organization of DNA in these archaeal organisms has implicated the presence of some specific proteins that could be involved in organisation of DNA. Work from our laboratory has identified four DNA binding proteins from the nucleoid of *Sulfolobus acidocaldarius*. Three of these proteins HSNP-A, HSNP-C and HSNP-C' protect DNA against thermal denaturation (Reddy and Suryanarayana, 1988; 1989). These helix stabilizing proteins have shown to be exclusively localized on the nucleoid domains of this organism by immunogold electron microscopy (Bohrmann *et al.*, 1994). The helix stabilising protein HSNP-C, the protein studied in the present investigation has been shown earlier to be one of the important proteins involved in the compaction of the intracellular DNA and in protection of DNA against thermal denaturation (Celestina and Suryanarayana, 1995 and Celestina, 1996). The present work has demonstrated that HSNP-C is analogous to Sac 7d described

earlier (Choli *et al.*, 1988b). It has been reported that 7d protein from *Sulfolobus acidocaldarius* exists in multiple forms 7a, 7b, 7c, 7d and 7e. Of these 7d is the most abundant form. Except for 7c, the sequences for all the other forms of the protein have been determined (Choli *et al.*, 1988a; 1988b). Proteins 7a and 7b differ from 7d in length at the C terminus (7a and 7b of 61 residues and 58 residues respectively) and the extent of monomethylation of lysine residues at the N terminus. Protein 7e has 64 residues as 7d, but 7e has replacement of ala-1 for val-1, arg-4 for lys-4 and met-54 for leu-54. Recently, McAfee *et al.,* (1995) showed that there are only two genes encoding Sac 7 proteins in *Sulfolobus acidocaldarius* corresponding to proteins 7d and 7e. The 7a, 7b, 7c were inferred to be modified forms of 7d.

In order to understand the role of the different forms of these helix stablizing proteins in the organism, the relatively abundant forms 7c, 7d and 7e were purified and characterized with respect to the protein aggregation and the nucleic acid binding properties.

Formaldehyde cross linking of the three proteins was undertaken to understand the aggregation behavior of this protein. The tendency of the protein for self aggregation was noticed during immunoblotting of the purified protein. Protein 7d was earlier shown to aggregate into high molecular weight multimers by crosslinking experiments (Celestina and Suryanarayana, 1995). All the three forms 7c, 7d and 7e formed multimeric aggregates. The yield of multimeric aggregates increased at higher temperature indicating the physiological state of the protein in an oligomeric structure. This protein also associates with other proteins as shown by affinity chromatography. Other nucleoid proteins HSNP-A and DBNP-B were found to bind to HSNP-C'. Further work will be necessary to study the interaction of these protein complexes with DNA.

The protein binds to all forms of DNA as shown by mobility shift assays. The results however, indicate cooperative and stronger binding to supercoiled forms of the DNA. However, fluorescence titrations did not indicate cooperativity. This may be due to the aggregation behavior of the protein. The affinity of the protein to single stranded DNA and single strand polyribonucleotides is much weaker than to double stranded DNA and RNA with substantial secondary structure. Among the three forms studied, HSNP-

C'*b* (corresponding to 7e of Choli *et al.*, 1988 b) showed strongest affinity to dsDNA as indicated by both titrations and binding to DNA cellulose columns. DNA cellulose chromatography of the three forms revealed an interesting heterogeneity in these proteins. All the three forms were eluted in two protein peaks with differing quantity. The fraction eluting in low salt gradually decreased from HSNP-C'a, HSNP-C, and HSNP-C'*b*. The two protein fractions eluting at lower salt concentration may correspond to the methylated fraction of these proteins. Choli *et al* (1988 b) showed that all 7d proteins were methylated to differing extents. From our results on purification of the three 7d proteins and elution on DNA cellulose columns HSNP-C'a and HSNP-C may be a single protein with certain proportion of methylated form. HSNP-C'a and HSNP-C elute in adjacent peaks with possible cross contamination. HSNP-C'a is predominantly the methylated form and it can have some contamination of HSNP-C. The purified HSNP-C may have less of the methylated form. The results of DNA cellulose chromatography support the above conclusion. The extent of methylation reported for 7e protein was less than that of 7d (Choli *et al.*, 1988 b).

Another interesting observation was the ability of the protein to bind nucleotides with strong affinity to GTP. Both HSNP-C and HSNP-C bound GTP with about equal affinity, whereas HSNP-C'*b* (7e) showed stronger affinity. The protein (7d) possesses a sequence Gly-Lys-Thr-Gly-Arg-Gly-Ala-Val-Ser (between residues 37 to 47) which is very similar to the P-loop motif involved in nucleotide binding (Saraste *et al.*, 1990). The binding to GTP by all three forms with weak GTPase activity may indicate some role for GTP in the functioning of this protein. However, present studies do not indicate any definite role of GTP in the functioning of this protein. Additionally the ability of the protein to bind GTP (GTP binding to HSNP-C was earlier shown by us to be independent of the binding to DNA) may also indicate that HSNP-C is a multifunctional protein.

Gel mobility shift assays at high protein to DNA ratios indicated aggregation of DNA by protein and the DNA protein complex failed to enter the agarose gel and remained at the wells. This prompted us to study the aggregation of DNA by protein. We have only tested aggregation and renaturation of DNA by the protein using only

HSNP-C' because of the greater availability of this form. The aggregation caused by the protein reached a plateau and did not change with increase in concentration of protein. This behavior is strikingly different from another DNA binding protein (DBNP-B) from *Sulfolobus acidocaldarius* studied in our laboratory (Sreenivas, 1994). DBNP-B aggregates DNA into crystalline gel at a particular protein / DNA ratio below and above which there was marked reduction in the aggregation.

Aggregation of HSNP-C could be more due to the protein protein aggregation with DNA binding. Earlier we have shown by electron microscopy that the complexes of HSNP-C and dsDNA progressively tend to be more compact with very little of free DNA exposed. The aggregation promoted by DBNP-B required Mg^{+2} whereas that promoted by DBNP-B required Mg^{+2} (optimal at 10 mM Mg^{+2}) . As a consequence of the aggregation, aggregated complementary single stranded DNA could reanneal. The characteristics of DNA aggregation and renaturation by HSNP-C are more like non specific as earlier shown for other nucleic acid binding basic proteins such as ribosomal protein S3 (Bruckner and Cox, 1985), and histones (Cox and Lehman, 1981). The characteristics of DBNP-B promoted aggregation and renaturation are more like recombination proteins (Sreeenivas, 1995).

The associated ribonuclease activity of HSNP-C and its isoforms with tRNA and ribosomal RNA as substrate support the earlier studies of p2 and p3 endoribonucleases (Fusi *et al.*, 1993). However, it is also possible that the purified HSNP-C (although 99% pure by SDS-PAGE) could be contaminated by a RNase activity. Further work is needed to confirm the ribonuclease activity of HSNP-C using recombinant protein. In the absence of any contrary report, the observed and reported RNase activity of HSNP-C may well be another unexplainable function of this protein.

Another important observation presented in this investigation pertains to strong immunological cross activity of anti HSNP-C antibodies to HMG-1 class of proteins of rat liver. The reported remote homology in the sequence of HMG-1/2 and 7d by Kimura *et al.,* (1984). Although, the physiological role of HMG group of protein HMG-1 and HMG-2 is not known several studies have been undertaken on the characteristics and interactions of HMG-1 and HMG-2 proteins (see introduction). Interestingly, Stross *et al*

(1994) reported that HMG box domains of HMG-1 induces DNA looping as shown by electron microscopy. These studies are very similar to those formed by HSNP-C with plasmid DNA at low protein/DNA ratios (Celestina and Suryanarayana, 1995).

Some of the important observations made in the present thesis are heterogeneity in HSNP-C isoforms (due to methylation and differing affinity to DNA), stronger binding of HSNP-C'*b* (7e) to DNA, binding of HSNP-C to nucleotides with GTPase activity, homology to HMG-1/2 and the associated RNase activity. These results suggest that HSNP-C may be multifunctional protein apart from its main role as a structural component of archaeal nucleoid.

CHAPTER 5

SUMMARY

Chapter 1 : Introduction

This chapter begins with an introduction to Archaea - the third biological kingdom, followed by the molecular biological aspects of the archaeal kingdom. In addition to this it also provides a review of the proteins that are histone like from bacterial and archaeal organisms. A brief account of eukaryotic scaffolding proteins namely the HMG group of DNA binding proteins is also given. The first chapter was followed by scope and objectives of the present investigation.

Chapter 2 : Materials and Methods

This chapter provides a detailed description of the methodologies used to carry out the present work. In addition, information about the chemicals and the materials used for the methods used is given. The present investigation was carried out with the help of various techniques such as mobility shift assays, fluorescence titrations, affinity chromatography on nucleic acid matrices, DNA aggregation and renaturation assays.

Chapter 3 : Results

The three abundant isoforms of HSNP-C were purified by CM cellulose chromatography (HSNP-C'*a*, HSNP-C and HSNP-C'*b*). The amino terminal sequencing of HSNP-C (the most abundant of the three isoforms) showed that it is 100% identical to Sac 7d protein. Crosslinking studies with formaldehyde showed that the physiological state of HSNP-C is an oligomeric structure. Gel filtration experiment and affinity chromatography experiments on HSNP-C coupled epoxy sepharose showed the association of HSNP-C with other proteins. The nucleic acid binding properties of HSNP-C and its isoforms were studied by gel mobility shift assays, fluorescence titrations, affinity chromatography on nucleic acid matrices, nuclease protection assay. The protein binds strongly to ds DNA and reasonably strongly to ss DNA. Of the three isoforms, HSNP-C'*b* showed strongest affinity to ds DNA as seen by both affinity

chromatography and fluorescence titrations. The binding constant K for the three isoforms were calculated. Apart from nucleic acid binding, all the three isoforms showed binding to GTP. HSNP-C aggregated both double stranded and single stranded DNA. The aggregates formed remained stable with increase in the concentration of the protein (HSNP-C). Complementary single stranded DNA was renatured by HSNP-C into high molecular weight network DNA. Renaturation promoted by HSNP-C did not require Mg^{+2} or GTP and showed an optimum pH of 5.0. Interestingly, HSNP-C and its isoforms showed ribonuclease activity with tRNA and ribosomal RNA as substrates inspite of strong DNA binding properties. All the three isoforms showed weak binding to RNA. Immunological homology of HSNP-C was observed with eukaryotic DNA binding proteins namely the HMG 1/2 group of proteins.

Chapter 4 : Discussion

Crosslinking of HSNP-C with formaldehyde has shown that this protein exists as multimeric aggregates physiologically. Fluorescence titrations suggest strong binding of HSNP-C to double stranded DNA and weak binding to single stranded DNA. Gel mobility shift assays indicate strong and cooperative binding of the protein to double stranded DNA with greater affinity for the supercoiled form. Of the three isoforms, HSNP-C'*b* showed strongest affinity for double stranded DNA. Chromatography of the three isoforms on DNA cellulose columns revealed an interesting heterogeneity in the isoforms. The salt required to elute each isoform differed depending on the extent of methylation of the forms. Apart from nucleic acid binding, HSNP-C and its isoforms showed nucleotide binding i.e to GTP with a mild GTPase activity. This GTPase activity could be of importance in some other processes in the cell. HSNP-C aggregated both double stranded and single stranded DNA which suggests its role in condensation of DNA in the organism. Aggregation of DNA increases the effective concentration of DNA and facilitates many important reactions like renaturation of complementary single strands. Renaturation and aggregation promoted by HSNP-C was more non specific like that shown by histones (Cox and Lehman, 1981). The associated ribonuclease activity of HSNP-C and its isoforms support the earlier studies of p2 and p3 endoribonucleases

(Fusi *et al*., 1993). The ribonuclease activity and nucleotide binding reflect the multifunctions of the protein HSNP-C apart from DNA binding. Strong immunological homology of HSNP-C'was seen with the eukaryotic scaffolding proteins namely the HMG 1/2 group.

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