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'

Crude Cell Extract

- Centrifugation: 30,000g 30 min 4°C
- Supernatant (S-30)
  - Centrifugation: 1,000,000g 4 hrs
  - Ribosomal Pellet
  - Post Ribosomal Supernatant (S 100)

- Acid extraction
  - Centrifugation: 10,000g 10 min
  - Acid Precipitated Proteins
  - Acid Soluble Proteins

- CM Cellulose Chromatography
  - Binding: 40 mM NaCl Buffer
  - Elution: Linear Gradient of 0.04-0.4 M NaCl Buffer
  - Peak I HSNP-C' & HSNP-A
  - Peak II HSNP-C'

- ds DNA Cellulose Chromatography
  - Initial Fractions HSNP-A, HSNP-C' a
  - Binding: 100 mM NaCl Buffer
  - Elution: 250 mM NaCl Buffer
  - Pure HSNP-C' a

- Concentration on small CM Cellulose Column

- Pure HSNP-C'

- Peak III HSNP-C' & Trace Amounts of HSNP-C

- Dialysis Against 0.1 N HCl
  - Rechromatography on CM Cellulose
  - Pure HSNP-C' b
**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.
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 HCl (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-chromatographed on a smaller CM cellulose column and the fractions were analysed by SDS-PAGE. Pure protein HSNP-C' was eluted as a sharp peak (Fig. 3.1F). The peak fractions containing the protein HSNP-C 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.
**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: 10µg of HSNP-C'a; lane 3: 10µg of HSNP-C; lane 4: 10µg of HSNP-C'b.
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 *S. 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'.
SAMPLE: HSNP-C

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**REPETITIVE YIELD ANALYSIS:**

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**Average AA Repetitive Yield:** 86.00 X

**Combined AA Repetitive Yield:** 85.55 X 0.598

**Theoretical Initial Yield:** 89.92 pmol (74.94 X)
Fig. 3.2 B: Comparison 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 7d and *Sulfolobus solfataricus* namely Sso 7d.

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<th>Sac 7d</th>
<th>Sac 7e</th>
<th>Sso 7d</th>
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Fig. 3.2 C: Comparison of the amino terminal sequence of HSNP-C with that high mobility group proteins.

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<th>HMG-14</th>
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Fig. 3.2 D: Comparison of the amino terminal sequence of HSNP-C with that of the ribonucleases from *Sulfolobus* species.

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<th>p3 ENDORIBONUCLEASE (S. solfataricus)</th>
<th>SaRD PROTEIN (S. acidocaldarius)</th>
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</table>

NOTE: The NH₂ terminal sequences of Sac 7a, Sac 7b, Sac 7d, Sac 7e proteins are from Kimura et al. (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 *S. 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. Crosslinking 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 crosslinking at pH 8.3 (Fig. 3.3.1) produced a series of crosslinked 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 crosslinking 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 A280 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.
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 electrophoretic 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. 3.3.2 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.
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. 3.3.3B: 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.
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', 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, 3.2 μg, 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.
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 of M13 RF DNA and HSNP-C:

An experiment was performed to see the effect of salt (300 mM KCl) 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 KCl 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 fg, 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 ssDNA (control); lane 2-4 : M13 ssDNA incubated with 1.6 μg each of HSNP-C'α, HSNP-C' and HSNP-C h respectively.
Fig. 3.4.1.1: Gel mobility shift analysis of HSNP C M13 dsDNA complexes in the presence of 150 mM and 300 mM KCl. 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, KCl 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.
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.
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.
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 (\(u\), moles of ligand bound per mole nucleotide). In the case of HSNP-C, the ratio of \(Q_{\text{obs}}/Q_{\text{max}}\) was taken to be equal to the ratio of bound ligand/total ligand, where \(Q_{\text{max}}\) was obtained by the low salt titration curve. The calculation of \(u\) and L_f was according to Bujalowski and Lohman (1987). \(Q_{\text{max}}\) values of 85% for HSNP-C'\(b\) and 72% for HSNP-Ca and HSNP-C were obtained respectively. Scatchard plots of \(u\) 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'\(b\) were \(8.5 \times 10^6 \text{M}^{-1}\); \(8.5 \times 10^6 \text{M}^{-1}\); 1.3 \(\times 10^7 \text{M}^{-1}\).

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'\(b\) 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.
Graphs A, B, and C illustrate the percent quenching of HSNP-C'a, HSNP-C', and HSNP-C'b, respectively, as a function of DNA/protein (nucleotide/monomer) ratio at different NaCl concentrations (20 mM, 100 mM, 200 mM).
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' 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 $8.5 \times 10^6 \text{M}^{-1}$.

(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 $8.5 \times 10^6 \text{M}^{-1}$.

(C): Scatchard plot of the data points obtained in fig. 4.3 C 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 $1.3 \times 10^7 \text{M}^{-1}$. 
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; □ □: 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'; □ □: HSNP-C: o-o: HSNP-C'a.
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 fluorescence 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', HSNP-C and HSNP-C' 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' > 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. A, B and C: Back titration of the isoforms of HSNP-C’-DNA complexes with salt. Aliquots of concentrated NaCl were added at $Q_{\text{max}}$ and the increase in fluorescence intensity was measured at each NaCl concentration as described in the materials and methods section. The ratio of the intensity of fluorescence ($F$ in arbitrary units) measured at each addition and the initial fluorescence ($F_0$) was plotted against concentration of NaCl.

(A): HSNP-C’-a-DNA complexes; (B): HSNP-C’-DNA complexes; (C): HSNP-C’-b-DNA complexes.
Fig. 3. 5.1 A: Fluorescence emission spectrum of HSNP-C’α titrated with GTP. HSNP-C’α (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.
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.
Fig. 3. 5.1 C : Fluorescence emission spectrum of HSNP-C'h (1.5 μM) titrated with GTP. HSNP-C'h 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'h (control); spectrum 2-7 : HSNP-C'h titrated with increasing concentration of GTP.
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.

Fig. 3. 1 E: Effect of salt on binding of HSNP-C’ to GTP.

HSNP-C’ (15 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.
Fig. 3.5.2: Quenching of intrinsic fluorescence of HSNP-C with different mononucleotides. o-o: GTP; □ ← : dGTP; A-A : GMP.
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.
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. 
\[\text{o-o : HSNP-C'}a; \Box \Box : HSNP-C; A-A : HSNP-C'b.\]

Fig. 3. 6 B : Quenching of intrinsic fluorescence of the forms of HSNP-C with *E. coli* 23S and 16S rRNA. 
\[\text{o-o : HSNP-C'}a; \Box \Box : HSNP-C; A-A : HSNP-C'b.\]
Fig. 3. 6 C : Reverse titrations of HSNP-C with polynucleotides.
   D-D : poly (U); o-o : poly (A).
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|µl relative to an unsedimented control mixture.

A: Aggregation of native $^3$H labelled *E. coli* DNA by 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.
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 MgCl₂. 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); △-△: 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).
Fig. 3.7. 3 A: SDS-PAGE analysis of native DNA aggregation reaction products.
Lane 1: 2 μg of native *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.

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.
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.
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.
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. **A, B, C, and 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 1 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-HC! pH 7.6, 12 mM MgCl$_2$, and 1 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.

(C): DNA renaturation assay carried out in buffer containing 10 mM sodium acetate pH 6.5, 12 mM MgCl$_2$, and 1 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.

(D): DNA renaturation assay carried out in buffer containing 10 mM sodium acetate pH 6.0, 12 mM MgCl$_2$, and 1 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.
Fig. 3.8. 2: Time course of DNA renaturation promoted by HSNP-C. Lane 1: 0.4 μg of denatured X DNA incubated in the absence of protein (control) deproteinised after 1 min. of incubation time; lane 2: control reaction mixture terminated after 30 min.; lane 3-6: renaturation mixtures in the presence of HSNP-C incubated for 1 min., 5 min., 15 min. and 30 min. before deproteinisation.
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 μg, 0.4 μg, 0.8 μg, 1.6 μg, 3.2 μg and 6.4 μg of HSNP-C respectively.
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 respectively.
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 1 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 heat 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; lane 4, 6, 8, 10, 12: pellet 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; 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.
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 described 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 DNAseI; lane 3-7: DNAseI 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 DNAseI. 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 DNAseI. Increase in A₄₅₀ was continuously recorded at 37°C for 30 minutes. o-o: without HSNP-C'; □-□: with 10 μg of HSNP-C'; A-A: with 20 μg of HSNP-C.
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 μg 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.
degraded tRNA appears as a smear trailing just below the intact tRNA. HSNP-C being a highly basic protein of $p_I$ 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. 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.
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 immunoblotted 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. 3.11A: Isolation of HMG group of proteins from rat liver nuclei. The HMG group of proteins were isolated as described 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).