CHAPTER 2
Organisms used in the present study:

*Sulfolobus acidocaldarius* strain DSM 639 was obtained from Deutche Sammlung Von Mikroorganisemen, Gottigen, Germany. *Escherichia coli* JM109 containing pUC19 plasmid was obtained from the laboratory of Dr. A.R.Subramanian, MaxPlanck Institute for Molecular Genetics, Berlin, Germany.

**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, 0.1% casein acid hydrolysate, 0.1% glucose, 0.02% sodium chloride, 0.13% ammonium sulphate, 0.03% pottasium dihydrogen phosphate, 0.025% magnesium sulphate, 0.07% calcium chloride and the pH was adjusted to 2.8 with 1M sulfuric acid (Kikuchi and Asai, 1984). The growth of the cells was arrested by neutralising the culture with 1M Tris base solution. The cells were harvested by centrifugation at 6,000 x g for 15 minutes. The cell pellets were finally suspended in 10 mM Tris-Cl (pH 7.6), 50 mM KCl, 10 mM magnesium acetate and 7 mM β-mercaptoethanol and centrifuged at 6,000 x g for 20 minutes. The cell pellets obtained were stored at -80°C until further use.

*E. coli* was grown in enriched medium at 37°C with good aeration (Minks et al, 1978).

**Isolation of M13 ss DNA and RF DNA:**

M13 ss DNA and RF DNA were isolated according to Messing, (1983). *Escherichia coli* JM109 was grown at 37°C on a 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 A600 of 0.3. The cells
were then infected with M13 mp7 phage particles with an MOI of 10. Incubation was continued at 37°C for another 6 to 8 hours. The titer was usually $10^{11}$ to $2 \times 10^{12}$ 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 6000 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 then suspended in 0.3 volumes of 10 mM Tris-Cl (pH 7.6) and 0.1 mM Na$_2$-EDTA. The resuspended phage solution was suspended again in 6% PEG 6000 and 0.5 M NaCl and left at 4°C for 60 minutes and the pellet was resuspended in 0.15 volumes of 10 mM Tris-Cl (pH 7.6) and 0.1 mM Na$_2$-EDTA. The M13 ss DNA 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 a small volume of 10 mM Tris-Cl (pH 7.6) and 0.1 mM Na$_2$-EDTA.

M13 RF DNA from the cell pellet was isolated by the procedure of Sambrook et al, (1989). The cell pellet containing RF was suspended in 50 mM glucose, 10 mM Na$_2$-EDTA and 25 mM Tris-Cl (pH 8.0). To this 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. Potassium phosphate (4 ml) was added to the above mixture and the suspension was mixed gently by swirling. The suspension was kept on ice for 10 minutes and centrifuged at 12,000 x g for 15 minutes. The clear supernatant was taken out carefully and incubated with RNase A (20 μg /ml) at 37°C for 30 minutes. The DNA was extracted with equal volume of buffered phenol followed by buffered phenol-chloroform. The aqueous phase was collected, mixed with one tenth volume of 3M sodium acetate (pH 4.8) and 2.5 volumes of ethanol. The DNA was collected by centrifugation and washed once with 70% ethanol and dissolved in a small volume of 10 mM Tris-Cl (pH 7.6) and 0.1 mM Na$_2$-EDTA. The RF DNA was linearised by incubating DNA with Eco R$\lambda$ followed by phenol-chloroform extraction and precipitation with ethanol.
Isolation of pUC19 supercoiled DNA:

pUC19 supercoiled DNA was isolated as described by Wang and Rossman, (1994). LB plates containing 25 µg /ml ampicillin were streaked with E. coli JM109 containing pUC19 plasmid and the culture was grown for 24 hours at 37°C. A single colony was picked from these plates and inoculated into 25 ml LB medium, supplemented with 1% glucose and 25 µg /ml ampicillin. The culture was grown with vigorous shaking at 37°C until late logarthmic phase (A600 = 0.3). 250 ml prewarmed LB medium containing ampicillin, glucose was inoculated with 2.5 ml of the above culture and grown at 37°C for 16 hours. The bacterial cells were harvested by centrifugation at 5,000 x g for 10 minutes at 4°C. The bacterial pellet obtained was resuspended in 10 ml lysis buffer (50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM Na2-EDTA and 5 mg /ml lysozyme). This cell suspension was incubated at RT for 5 minutes and 20 ml of freshly prepared alkaline solution (0.2N NaOH, 1% SDS) was added, mixed and placed on ice for 10 minutes. 12.5 ml of saturated ammonium acetate solution was added, mixed well by swirling and placed on ice for 10 minutes. It was centrifuged at 12,000 x g for 10 minutes at 4°C and to the supernatant obtained 0.7 volumes of ice-cold isopropanol was added and placed on ice for 20 minutes. The DNA precipitate was collected by centrifugation at 12,000 x g for 15 minutes at 4°C. The DNA pellet was redissolved in 6 ml of acidic extraction solution (0.75 M NaCl, 10 mM Na2-EDTA, 0.3M Na acetate (pH 4.2)). The DNA solution was left on ice for 15 minutes, an equal volume of water-saturated phenol was added and continuously inverted for 2 minutes. The upper aqueous phase was separated by centrifugation at 12,000 x g for 10 minutes at 4°C. The organic phase was reextracted with an equal volume of acidic extraction solution. 3.5 ml of reverse extraction buffer (1.5 M Trizma base, 5 mM Na2-EDTA) and 3.5 ml of chloroform were added to the organic phase and this mixture was continuously inverted for 5 minutes and centrifuged at 12,000 x g for 10 minutes. The upper aqueous phase obtained was carefully transferred to another tube and the DNA in the aqueous phase was precipitated by adding 0.1 volumes of 3M sodium acetate (pH 4.2) and 0.7 volumes of ice-cold isopropanol. The DNA pellet obtained was washed twice with 70% ethanol and dissolved in TE buffer (10 mM Tris-Cl (pH 7.6) and 0.1 mM Na2-EDTA).
Isolation of pBR322 supercoiled DNA:

*E. coli* DH5a cells containing pBR322 were grown and supercoiled DNA was extracted according to Wang and Rossman, (1994) as described above.

pBR322 and pUC19 supercoiled DNA were linearised by incubating with *Hind* III followed by phenol-chloroform extraction and precipitation with ethanol.

Preparation of ss and ds DNA-cellulose:

ss DNA and ds DNA-cellulose were prepared according to the procedure described by Alberts and Herrick, (1971). The cellulose was activated by washing several times with boiling ethanol to remove pyridine, then quickly washed at room temperature successively with 0.1M NaOH, 1 mM Na$_2$-EDTA and 10 mM HCl solutions respectively. It was later washed with water to neutrality, dried and used for coupling of DNA.

For ds DNA-cellulose, a solution of 1-3 mg/ml DNA in 10 mM Tris-Cl (pH 7.4), 1 mM Na$_2$-EDTA (Tris-EDTA) is taken in a glass beaker. Activated dried cellulose was added with occasional stirring with a flat bottomed glass rod until the paste thickens (~ 1g cellulose/3 ml). This lumpy mixture was dried by spreading on a petriplate and left at room temperature covered with a gauze. The thoroughly dried cellulose powder was suspended in 20 volumes of Tris-EDTA and left overnight at 4°C. After a quick wash to remove the free DNA, the DNA-cellulose is stored as a frozen slurry in Tris-EDTA buffer containing 0.15 M NaCl. The efficiency of coupling is checked by measuring A$_{260}$ of supernatant obtained after centrifugation of an aliquote of the DNA-cellulose suspension incubated at 100°C for 20 minutes.

For ss DNA-cellulose the DNA is denatured at 100°C in 10 mM potassium phosphate and 1 mM Na$_2$-EDTA. After rapid cooling the DNA is made upto 3 mg/ml concentration with Tris-EDTA buffer and coupled to activated cellulose as described above.
Preparation of ss DNA by heat denaturation:

ds DNA of known concentration was taken and heated in a boiling water bath for 3 minutes. It was then immediately chilled in ice-cold water for 5 minutes, again boiled at 100°C for 5 minutes and chilled for 10 minutes in ice-cold water. The concentration of the resulting ss DNA is almost the same as that of ds DNA.

Preparation of ss DNA by alkaline denaturation:

To the ds DNA, 0.1 M NaOH was added and incubated on ice for 20 minutes and precipitated with 2.5 volumes of ethanol. The DNA precipitate was collected by centrifugation, washed with 70% ethanol and dissolved in TE buffer (10 mM Tris-Cl (pH 7.4) and 1 mM Na$_2$-EDTA).

Isolation of Nucleoid:

Nucleoid from *S. acidocaldarius* was isolated by a procedure described by Reddy and Suryanarayana, (1988). Cells (2 gm) harvested in midlogarithmic phase were suspended in 4 ml of 10 mM Tris-Cl (pH 7.6) containing 1% NP-40, 2 mM spermidine-HCl, 10 mM Na$_2$-EDTA and incubated at 10°C for 30 minutes. The lysate was centrifuged at 10,000 x g for 10 minutes. The cleared viscous supernatant was layered on a 30% sucrose cushion in 10 mM Tris-Cl (pH 7.6), 3 mM magnesium chloride and centrifuged at 10,000 x g for an hour. The concentrated nucleoid pellet obtained was dissolved in 3 ml of 20 mM Tris-Cl (pH 7.6), 150 mM KCl, 1mM Na$_2$-EDTA and 6 mM β-mercaptoethanol and the $A_{260}$ was measured.

Micrococcal nuclease digestion of nucleoid:

This was performed following the procedure of Owen-Hughes and Workman, (1996). Nucleoid (1 μg) fraction was incubated with 0.1 units of micrococcal nuclease in 20 mM Tris-Cl (pH 8.8), 50 mM NaCl, 50 mM MgCl$_2$ and 1 mM CaCl$_2$ at 37°C for different time intervals. The reaction was stopped by adding SDS and EDTA to 1% and 25 mM final concentrations respectively. The
reaction products were analysed on a 1.4% agarose gel. The optimum conditions for MNase digestion were determined by incubating the nucleoid with increasing concentrations of MNase for different time intervals.

**Sephacryl S-1000 column chromatography of *Sulfolobus acidocaldarius* nucleoid:**

The nucleoid isolated was gently sonicated and centrifuged at 3,000 x g for 10 minutes to remove the contaminating cell debris. This cleared and sheared nucleoid was chromatographed on a sephacryl S-1000 gel filtration column. A sephacryl S-1000 column of 100 ml bed volume was packed and equilibrated with 20 mM Tris-Cl (pH 7.6), 150 mM KCl, 1mM Na$_2$-EDTA and 6 mM β-mercaptoethanol (nucleoid buffer). The sheared nucleoid was loaded on the S-1000 column and eluted with two bed volumes of the nucleoid buffer. Fractions (2 ml) were collected at a flow rate of 25 ml/hr and the absorbance was measured at 260 nm. Fractions (50 μl of each) were analysed on a 15% SDS-polyacrylamide gel. DNA in the fractions was analysed on a 0.8% agarose gel. The DNA concentration in the fractions was measured by ethidium bromide fluorescence assay.

Isolation of DNA from the sephacryl S-1000 nucleoid fractions: -

DNA in the sephacryl S-1000 fractions was isolated by phenol-chloroform extraction. Equal volume of buffer-saturated phenol was added to the sephacryl S-1000 fractions and gently mixed for 10 minutes and centrifuged at 5,000 x g for 5 minutes. The upper aqueous phase was removed and the phenolisation step was repeated. Subsequently, the aqueous phase was extracted twice with an equal volume of phenol:chloroform (1:1) by gently mixing for 10 minutes and centrifuging at 5,000 x g for 5 minutes. The upper aqueous phase was collected and extracted twice with chloroform:isoamyl alcohol (24:1) by mixing gently for 10 minutes and centrifuging at 5,000 x g for 5 minutes. The aqueous phase containing DNA was precipitated with 2.5 volumes of ethanol in the presence of one tenth volume of 3M potassium acetate (pH 5.0) and left overnight at -20°C. The DNA pellet collected was washed twice with 70% ethanol and dissolved in a small volume of TE buffer (10 mM Tns-Cl (pH 7.6) and 1 mM Na$_2$-EDTA).
Sephadex G-50 column chromatography of the sephacryl S-1000 peak-II nucleoid proteins:

Sephacryl S-1000 peak-II fractions were pooled, dialysed against 20 mM Tris-Cl (pH 7.6), 50 mM NaCl, 5 mM MgCl₂ and 7 mM β-mercaptoethanol and subjected to DNase I digestion by adding 200 μg DNase I and incubating at 37°C for 1 hr and 65°C for 3 hours. It was then concentrated to 1 ml by lyophilisation, dialysed against column buffer (20 mM Tris-Cl (pH 7.6), 1 mM Na₂-EDTA, 150 mM KCl and 7 mM p-mercaptoethanol) and loaded on a 100 ml sephadex G-50 column preequilibrated with the column buffer. The column was eluted with the same buffer and 4 ml fractions were collected at a flow rate of 5 ml/hr. The absorbance of the fractions was measured at 280 nm and these fractions were analysed on a 15% SDS-polyacrylamide gel (Laemmli, 1970).

DNA-cellulose column chromatography of sephadex G-50 nucleoid fractions:

A ds DNA-cellulose column of 4 ml bed volume was packed and equilibrated with column buffer (20 mM Tris-Cl (pH 7.6), 50 mM KCl, 1 mM Na₂-EDTA and 7 mM p-mercaptoethanol). Sephadex G-50 peak fractions were pooled and diluted to 50 mM KCl in the column buffer without KCl and loaded onto the DNA cellulose column. The column was washed with 5 bed volumes of the column buffer and eluted with a stepwise gradient of 100 mM, 200 mM, 300 mM, 400 mM, 500 mM and 700 mM KCl in column buffer. Fractions (1.5 ml) were collected and the A₂₈₀ was measured. These fractions were analysed by 15% SDS-PAGE.

DNA-cellulose column chromatography of sephacryl S-1000 peak-I nucleoid fractions:

Sephacryl S-1000 peak-I nucleoid fractions were pooled, the KCl concentration was adjusted to 50 mM with 20 mM Tris-Cl (pH 7.6), 1 mM Na₂-EDTA and 7 mM β-mercaptoethanol and subjected to DNase I digestion by treating with 200 μg DNase 1 at 37°C for 1 hr. The DNase I digested nucleoid fraction was dialysed against the column buffer (20 mM Tris-Cl (pH 7.6), 50 mM KCl, 1 mM Na₂-EDTA, 7 mM p-mercaptoethanol) and incubated at 65°C for 3 hrs. It was then loaded on a 5 ml ds DNA-cellulose column preequilibrated with
the column buffer. The column was washed with 5 bed volumes of the column buffer and eluted with a stepwise gradient of 100 mM, 200 mM, 300 mM, 400 mM and 700 mM KCl in the column buffer. 1 ml fractions were collected and the $A_{280}$ was measured. These fractions were analysed by 15% SDS-PAGE.

**DEAE-cellulose column chromatography of sephacryl S-1000 peak-II nucleoid fractions:**

Preswollen DEAE-cellulose was suspended in a buffer containing 20 mM Tris-Cl (pH 7.6), 150 mM KCl, 1 mM Na$_2$-EDTA and 7 mM β-mercaptoethanol (column buffer). The gel was packed into a column of 6 ml bed volume and equilibrated with the column buffer. Sephacryl S-1000 peak-II nucleoid fractions were pooled and loaded onto the DE-52 column, washed with 5 bed volumes of the column buffer and eluted with 1500 mM KCl containing column buffer. 1 ml fractions were collected and the $A_{280}$ was measured. These fractions were analysed on a 15% SDS-PAGE, the DNA concentration was estimated by ethidium bromide fluorescence assay and subjected to DNase I digestion.

DNase I digestion of DEAE-cellulose nucleoid fractions:

DNase I digestion of the nucleoid fractions, obtained from the DEAE-cellulose column was performed by incubating with DNase I at a ratio of 10:1 (DNA:DNase I) in 10 mM Tris-Cl (pH 7.6), 50 mM NaCl, 5 mM MgCl$_2$ and 1 mM DTT at 37°C for 30 seconds. The reaction was terminated by the addition of SDS and EDTA to 1% and 25 mM final concentrations respectively. The reaction products were analysed on a 0.8% agarose gel. The gel was stained with ethidium bromide and photographed under UV illumination.

Isolation of ribosomes and post-ribosomal supernatant fraction (S-100) from *Sulfolobus acidocaldarius*:

This was carried out according to Minks *et al*, (1978) at 0-4°C. Cells harvested at mid logarithmic phase were ground with double the weight of alumina until soft and sticky, extracted with buffer (3ml /gm cells) containing 20 mM Tris-
Cl (pH 7.6), 50 mM KCl, 10 mM magnesium acetate, 7 mM p-mercaptoethanol and 2 μg DNase I (RNase free) per gram cells and centrifuged at 30,000 x g for 30 minutes at 4°C to obtain cell extract (S-30). The S-30 was centrifuged at 1,00,000 x g for 4 hrs (Beckman L8-80, Ti-60 rotor) to pellet the ribosomes. The upper two thirds of the supernatant (S-100) was collected and dialysed immediately against buffer containing 20 mM Tris-Cl (pH 7.6), 50 mM KG, 10 mM magnesium acetate, 7 mM p-mercaptoethanol and 10% glycerol and stored at -80°C. The crude ribosomal pellet was rinsed once with the above buffer and suspended in the same buffer containing 1M ammonium chloride. The ribosomes in 1M ammonium chloride buffer were pelleted by centrifugation at 1,00,000 x g for 4 hrs and the supernatant (ammonium chloride wash) was collected. The ribosomal pellet was suspended in 10 mM Tris-Cl (pH 7.6), 50 mM KCl, 10 mM magnesium acetate, 7 mM p-mercaptoethanol, 10% glycerol and stored frozen at -80°C.

Isolation of acid soluble proteins:

Acid soluble proteins from the concentrated nucleoid or post-ribosomal supernatant (S-100) were extracted with one tenth volume of 2.7 M sulfuric acid, added dropwise to the sample on ice. The acid treated samples were stirred for 4 hours at 4°C and the acid soluble proteins were collected by centrifugation at 15,000 x g for 30 minutes. The clear acid soluble supernatant was dialysed against buffer containing 20 mM Tris-Cl (pH 7.6), 50 mM KCl, 1 mM Na$_2$-EDTA and 7 mM p-mercaptoethanol for 20 hours and clarified at 10,000 x g for 20 minutes.

Purification of DBNP-B:

DBNP-B was purified according to the procedure of Reddy and Suryanarayana. (1989) with some modifications. DBNP-B was purified from the acid extract of nucleoid or S-100 by a single CM-cellulose chromatographic step.

Pre swollen CM-cellulose was suspended in a buffer containing 20 mM Tris-Cl (pH 7.6), 125 mM KCl, 1mM Na$_2$-EDTA and 7 mM p-mercaptoethanol (column buffer). The gel was packed into the column and equilibrated with the above buffer. The acid extracts of post-ribosomal supernatant or nucleoid
containing DBNP-B were dialysed against CM-cellulose column buffer and applied onto the column. The column was washed thoroughly with 250 mM KCl containing column buffer and the proteins were eluted in 500 mM KCl in the column buffer. Fractions (4 ml) were collected at 30 ml/hr flow rate and aliquots (20 μl) from alternate fractions were analysed by 15% SDS-PAGE. In order to remove the salts, the 20 μl aliquots were diluted to 1 ml with water, made to 10 percent in TCA and incubated at 0°C for 2 hours. The samples were centrifuged and the pellets were washed with acetone and dissolved in SDS gel loading buffer.

Concentration of purified DBNP-B:

The fractions containing DBNP-B were pooled and diluted to 125 mM KCl and applied onto a small (2ml bed volume) CM-cellulose column equilibrated with column buffer. After washing the column with 250 mM KCl in the column buffer, the protein was eluted with 800 mM KCl in the column buffer. Protein containing fractions were pooled and immediately dialysed against 10 mM Tris-Cl (pH 7.6) or 10 mM sodium acetate (pH 5.0) and stored frozen until further use at -80°C. The purity of the concentrated protein was checked by electrophoresing 10 μg protein on SDS-polyacrylamide gel followed by silver staining.

Raising antibodies to DBNP-B:

Antibodies to purified DBNP-B were raised as described by Stoffler and Wittman, (1971). About 200 μg of DBNP-B was emulsified with Freund's complete adjuvant and injected subcutaneously into the rabbit at multiple sites. After four weeks, booster doses each of 50 μg of protein in Freund's incomplete adjuvant were given subcutaneously every week till the 6th week. The rabbit was bled after the third booster injection through the pinna vein. Blood collected from the rabbit was first kept at room temperature for 2 hours and then at 4°C for 12-16 hours. It was then centrifuged at 6,000 x g for 15 minutes to remove the clot. The supernatant obtained was recentrifuged at 10,000 x g for 30 minutes. The resulting supernatant was incubated at 56°C for 30 minutes followed by centrifugation at 10,000 x g for 15 minutes. The supernatant obtained (antiserum) was stored frozen in aliquots until further use.
Isolation of IgG from the antiserum:

Antiserum was fractionated by ammonium sulphate precipitation. IgG fraction obtained from 50-60% ammonium sulphate saturation, was dialysed against 70 mM sodium phosphate (pH 6.3) and loaded onto a 5 ml DEAE-cellulose column. The column was eluted with 70 mM sodium phosphate (pH 6.3) and 2 ml fractions were collected. The protein content was analysed by measuring the absorbance at 280 nm. The flowthrough fractions showing a high absorption at 280 nm were pooled and precipitated by adding equal volume of saturated ammonium sulphate solution and centrifuged at 8,000 x g for 40 minutes. The resulting pellet was dissolved in phosphate buffered saline, dialysed against the same buffer and stored frozen in small aliquots at -80°C.

Gel filtration chromatography of DBNP-B:

Preswollen sephadex G-50 was deaereated and packed into a thin long column of 100 ml bed volume and equilibrated with 20 mM Tris-Cl (pH 7.6), 50 mM KCl, 1 mM Na₂-EDTA and 7 mM β-mercaptoethanol. DBNP-B (200 µg) was loaded on to the column and eluted with the same buffer. Fractions (2 ml) were collected from the column at a flow rate of 5 ml/hr and alternate fractions were analysed for the presence of the protein by both SDS-PAGE and absorbance measurements at 280 nm. Simultaneously protein molecular weight markers viz- bovine serum albumin (66 kDa), ovalbumin (44 kDa) and cytochrome-C (12 kDa) were also chromatographed on the sephadex G-50 column for the determination of the molecular weight of the native state of DBNP-B.

Gel mobility shift assay:

Gel mobility shift analysis of DBNP-B nucleic acid complexes was carried out as described by Lohman et al., (1986). The reaction was carried out in 30 µl reaction volume in 10 mM Tris-Cl (pH 7.6), 0.1 mM Na₂-EDTA and 25 mM NaCl. DBNP-B-nucleic acid complexes were formed at different protein to nucleic acid ratio, incubated for 15 minutes at 37°C and electrophoresed on a 0.8% agarose gel in TAE buffer (40 mM Tris-acetate (pH 7.8) and 1 mM Na₂-EDTA). 3 µl loading dye (50% (v/v) glycerol, 0.05% (w/v) bromophenol blue, 0.05% (w/v)
xylene cyanol) was added to each sample and loaded directly onto the agarose gel. The gels were then stained with 0.5 µg/ml ethidium bromide in TAE buffer and photographed under UV illumination.

Chemical and proteolytic cleavage of DBNP-B:

DBNP-B was cleaved chemically with CNBr and proteolytically with V8 protease, trypsin and chymotrypsin.

Cyanogen bromide cleavage:

DBNP-B was treated with cyanogen bromide according to Giorginis and Subramanian, (1980) with slight modifications. DBNP-B (0.25 mg/ml) in 90% formic acid was treated at room temperature under nitrogen with CNBr (10 mg/ml) in 90% formic acid in a protein:reagent ratio of 1:20 for different time intervals. The products of CNBr cleavage were vacuum dried, dissolved in SDS-gel loading buffer and analysed by 15% SDS-PAGE.

V8 protease cleavage:

V8 protease digestion of DBNP-B was performed according to Cleveland et al., (1977). DBNP-B (1 mg/ml) in 125 mM Tris-Cl (PH 6.8), 10% glycerol and 0.5% SDS, was boiled at 100°C for 2 minutes, cooled and digestion was carried out at 37°C by the addition of protease at a concentration of 10:1 (protein:enzyme) for different time intervals. The reaction was stopped by adding β-mercaptoethanol and SDS to final concentrations of 10% and 2% respectively and boiling the samples for 2 minutes at 100°C. The reaction products after V8 protease cleavage were analysed by 15% SDS-PAGE.

Trypsin and chymotrypsin cleavage:

DBNP-B in 10 mM Triethanolamine (pH 8.0) was reacted with 20:1 protein:enzyme concentration of TPCK-trypsin or chymotrypsin at 37°C for different time intervals. At different time intervals aliquots were taken out and the reaction was quenched by the addition of TCA to 10% final concentration and incubated at 0°C for 30 minutes. The precipitates were collected by
centrifugation, washed with acetone, dissolved in SDS gel loading buffer containing 10 mM DTT and electrophoresed on a 15% SDS-polyacrylamide gel.

Cross linking studies:

DBNP-B crosslinking experiments were performed with three different reagents viz. formaldehyde (HCHO), dimethyl suberimidate (DMS) and difluoro dinitrobenzene (DFDNB).

Formaldehyde crosslinking:

DBNP-B crosslinking with formaldehyde as a crosslinking agent was performed according to Jackson, (1978) with some modifications. DBNP-B in 20 mM Triethanolamine-HCl (pH 7.5) or 10 mM sodium acetate (pH 5.0) was reacted with 200 mM HCHO, pH 7.5 and 5.0 respectively in the presence or absence of 10 mM magnesium chloride at 37°C, 65°C and 80°C for different time intervals. At different time intervals small aliquots were taken out and the crosslinking was quenched by the addition of TCA to 10% final concentration. The precipitates were collected by centrifugation, washed with acetone and dissolved in electrophoresis sample buffer containing 0.1% SDS but devoid of β-mercaptoethanol /DTT and electrophoresed on 12.5% or 15% SDS-polyacrylamide gels. HCHO crosslinking of DBNP-B was also performed at different concentrations of NaCl (50, 100, 150, 200 and 500 mM) for different time intervals at 37°C.

Dimethyl suberimidate crosslinking:

DBNP-B was crosslinked with DMS following the procedure of Thomas and Kornberg, 1975. DBNP-B in 20 mM Triethanolamine (pH 8.5) was reacted with 0.2 mg /ml DMS (in 20 mM Triethanolamine (pH 8.0)) and incubated at 37°C and 65°C in the presence or absence of 10 mM magnesium chloride. At different time intervals, small aliquots were taken out and the crosslinking was stopped by the addition of TCA to 10% final concentration. The precipitates were collected by centrifugation after incubation at 0°C for 30 minutes. The precipitates were washed with acetone and dissolved in electrophoresis sample buffer containing
0.1% SDS and devoid of β-mercaptoethanol / DTT. These samples were analysed by 12.5% SDS-PAGE.

Fluorescence titrations of DBNP-B with RNA:

All fluorescence measurements were obtained using JASCO FP-777 spectrofluorimeter. The measurements were performed in 10 mM Tris-Cl (pH 7.6), 1 mM DTT and different NaCl concentrations at room temperature (25°C). The excitation band width was 5 nm and the emission band width was 10 nm. Reverse titrations were performed by adding lattice (RNA) to the ligand (DBNP-B). Small volumes of (2-3 μl) concentrated polynucleotide solution (Poly (A) or poly (U)) were added to DBNP-B (22 μg) in the buffer and the decrease in emission fluorescence intensity was recorded after each addition. After each addition the reactants were mixed gently and left undisturbed for 60 seconds. The fluorescence readings were recorded after another 60 seconds which was the time usually taken for the stabilisation of the fluorescence signal. Usually three readings (at 10 second intervals) were taken for each titration point and the average of these readings were used for the analysis. The fluorescence intensity measured in arbitrary units was corrected for the dilution during titrations. In our experimental conditions the inner filter effect was very insignificant. The excitation was at 274 nm and the emission fluorescence intensity was measured at 304 nm. The initial protein fluorescence was taken to be 100% and all other measurements were made with reference to the initial fluorescence.

The strength of the binding of RNA to the protein was measured by adding increasing concentrations of 4M NaCl to DBNP-B-RNA complexes at saturation in aliquots and the dissociation of the RNA-protein complexes due to increase in ionic strength in the reaction medium was followed by observing the recovery of the protein fluorescence.

Tetranitromethane modification of DBNP-B:

This was performed following the procedure of Riordan and Vallee, (1972). DBNP-B (0.3 mg /ml) in 50 mM Tris-Cl (pH 8.1) and 100 mM KCl was reacted with 5 mM tetranitromethane (in methanol) at 21°C for 30 minutes. The reaction
was terminated by dialysing exhaustively against 100 volumes of 10 mM Tris-Cl (pH 7.5), 30 mM NaCl and 7 mM β-mercaptoethanol for 16 hours. The TNM modified DBNP-B was analysed by 15% SDS-PAGE.

**ss DNA-cellulose chromatography of TNM modified DBNP-B:**

TNM modified DBNP-B was passed through a 1 ml ss DNA-cellulose column equilibrated with 10 mM Tris-Cl (pH 7.6), 125 mM KCl, 1 mM Na₂-EDTA and 7 mM p-mercaptoethanol. The column was washed with 150 mM KG containing above buffer and eluted with a stepwise gradient of 300 mM and 500 mM KCl in 10 mM Tris-Cl (pH 7.6), 1mM Na₂-EDTA and 7 mM p-mercaptoethanol. Fractions (0.2 ml) were collected and analysed for the presence of protein on a 15% SDS-polyacrylamide gel.

**Renaturation assays:**

DNA renaturation assays were carried out according to Sung et al, 1992 with slight modifications. The reaction was carried out in a 20 μl sample volume in 10 mM sodium acetate (pH 5.0), 12 mM magnesium chloride, 1 mM DTT with heat denatured DNA or alkaline denatured DNA and increasing concentrations of DBNP-B. 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 stopped by the addition of SDS and EDTA to final concentrations of 1% and 25 mM respectively. The renaturation products were analysed by electrophoresis on 0.8-1.6% agarose gels or on 7.5% native polyacrylamide gels. Extent of DNA renaturation by DBNP-B was also followed by measuring the resistance of the renaturation products to S1 nuclease digestion. The dependence of renaturation on temperature and pH was followed at different temperatures and pH. Time course was carried out to determine the optimum time for renaturation.

**S1 nuclease digestion of DNA renatured in the presence of DBNP-B:**

S1 nuclease digestion assay was carried out to see the extent of pairing promoted by DBNP-B. Renaturation assay with increasing amounts of DBNP-B and heat denatured X DNA in 10 mM sodium acetate (pH 5.0), 12 mM
magnesium chloride and 1 mM DTT was performed for 30 minutes at 37°C in duplicates. After deproteinisation of the reaction products with 1% SDS, one set of the reaction products was directly loaded on a 0.8% agarose gel while the other set was subjected to S1 nuclease digestion in S1 nuclease digestion buffer (10X: 500 mM sodium acetate (pH 4.7), 1500 mM NaCl and 10 mM zinc chloride) at 37°C for 5 minutes (2 units of S1 nuclease /µg DNA). 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 analysed by electrophoresis on a 0.8% agarose gel. The gel was stained with ethidium bromide and photographed under UV illumination.

DNase I sensitivity of pUC19 supercoiled DNA in the DBNP-B-DNA complexes:

DBNP-B in increasing amounts was incubated with 400 ng of pUC19 supercoiled DNA in 10 mM Tris-Cl (pH 7.6), 50 mM NaCl, 5 mM MgCl2 and 1 mM DTT at 37°C for 45 minutes. DNase I was added to the reaction mixtures to 10:1 ratio (DNA:DNase I) and incubated at 37°C for 1 minute. The digestion was quenched by deproteinising with 1% SDS and 25 mM EDTA. The reaction products were analysed on a 0.8% agarose gel or on a 2.5% acrylamide and 0.5% agarose composite gel.

DNA aggregation assay:

DNA aggregation by DBNP-B was studied by the extent of light scattering monitored by the increase in absorbance at 320 nm spectrophotometrically. Increasing amounts of DBNP-B in aliquots were added to a fixed amount of λ DNA (2 µg) in 1 ml reaction buffer containing 10 mM sodium acetate (pH 5.0) and 1 mM Na2-EDTA. The formation of DNA-protein aggregates was assayed by measuring the increase in absorbance at 320 nm. The influence of salt and magnesium on DNA aggregation by DBNP-B was tested by performing this assay at different sodium chloride and magnesium chloride concentrations at room temperature (25°C).
**Nicking assay:**

The nicking activity of DBNP-B was tested by incubating DNA containing both supercoiled and relaxed forms or only relaxed form in a reaction volume of 20 µl of 50 mM Tris-Cl (pH 7.5), 50 mM KCl, 0.1 mM Na₂-EDTA, 0.5 mM DTT and 10 mM MgCl₂ with different concentrations of the protein at 37°C and 65°C for different time intervals. The reaction was terminated by the addition of SDS to 1% final concentration and the samples were digested with 2 µl of 0.8 mg/ml proteinase K for 30 minutes at 37°C. Then, 3 µl of loading buffer containing 0.05% bromophenol blue, 0.05% xylene cyanol, 10 mM Tris-Cl (pH 8.0) and 50% glycerol was added and the reaction products were electrophoresed on a 1% agarose gel. The same reaction was performed at different pH and also the influence of magnesium and ATP on the nicking activity of DBNP-B was studied.

**DNA polymerase assay:**

Reaction mixtures (100 µl) contained 40 mM Tris-Cl (pH 7.9), 8 mM MgCl₂, 1 mM DTT, 4 mM ATP, 100 µM each of dATP, dGTP, dCTP, 25 µM dTTP, 1 µCi of [³H] dTTP, 5 µg of activated calf thymus DNA and 0.5 units of *E.coli* DNA polymerase I. After incubation for 20 minutes at 37°C the reaction was terminated by adding 50 µg of denatured calf thymus DNA and 1 ml of ice-cold 10% TCA with 1% sodium pyrophosphate and kept on ice for 30 minutes. Precipitates were collected on whatman GF/C filters, washed thrice with 3 ml each of 10% TCA containing 1% sodium pyrophosphate, washed thrice with ethanol and dried. Acid insoluble radioactivity on the GF/C filters was measured in a Beckman liquid scintillation counter after adding 5ml of toluene scintillation fluid. The effect of DBNP-B on the activity of DNA polymerase I was studied by adding DBNP-B at different DNA:protein concentrations and incubating at 37°C for 15 minutes prior to the addition of DNA polymerase I.

**RNA polymerase assay:**

Reaction mixtures (500 µl) contained 25 mM Tris-Cl (pH 7.9), 10 mM MgCl₂, 1 mM Na₂-EDTA, 0.2 mM each of CTP, GTP, ATP, 0.05 mM UTP, 5 µCi of pH] UTP, 6 µg of activated calf thymus DNA, 1 mM K₂HPO₄ (pH 7.0), 1
mM DTT and 50 μg of bovine serum albumin. The final NaCl concentration was adjusted to 0.15 M for each assay and 2 units of *E. coli* RNA polymerase was added, incubated at 32°C for 30 minutes. The reaction was stopped with 6 ml of 10% TCA containing 1% sodium pyrophosphate after adding 0.25 mg calf liver RNA and kept on ice for 30 minutes. Precipitates were collected on Whatman GF/C filters, washed thrice with ice cold 10% TCA containing 1% sodium pyrophosphate, thrice with ethanol and dried. The acid insoluble radioactivity was measured in a Beckman liquid scintillation counter. The effect of DBNP-B on the activity of RNA polymerase was studied by adding DBNP-B at different DNA:protein concentrations and incubating at 32°C for 15 minutes prior to the addition of RNA polymerase.

**Topoisomerase I assay:**

Wheat germ topoisomerase I was assayed in a 20 μl reaction volume containing 50 mM Tris-Cl (pH 7.5), 50 mM KCl, 10 mM MgCl₂, 0.1 mM Na₂-EDTA, 0.5 mM DTT, 30 μg /ml bovine serum albumin, 0.5 μg pBR322 supercoiled DNA and 2 units of wheat germ topoisomerase I. The reaction mixtures were incubated at 37°C for 1 hour and the reaction was terminated by the addition of SDS and EDTA to 1% and 25 mM final concentrations respectively. The reaction products were digested by adding 2 μl of 0.8 mg /ml proteinase K for 30 minutes at 45°C. Then 3 μl of loading buffer containing 0.05% bromophenol blue, 0.05% xylene cyanol in 10 mM Tris-Cl (pH 8.0) and 50% glycerol was added and the reaction products were analysed on a 1% agarose gel. The effect of DBNP-B on the wheat germ topoisomerase 1 activity was observed by adding DBNP-B at different DNA:protein concentrations and incubating these complexes at 37°C for 15 minutes prior to the addition of topoisomerase I.

**Topoisomerase II assay:**

Rat testis topoisomerase II was purified as described by Galande and Muniyappa, (1996). The Rat testis topoisomerase II relaxation assay was carried out in a 20 μl volume buffer containing 10 mM Tris-Cl (pH 8.0), 50 mM NaCl, 50 mM KG, 5 mM MgCl₂, 0.1 mM Na₂-EDTA, 30 μg /ml bovine serum albumin, 1mM ATP, 400 ng pBR322 supercoiled DNA and 100 nM topoisomerase II. The
reaction mixtures were incubated at 37°C for 30 minutes. The reaction was terminated by the addition of SDS and EDTA to 1% and 25 mM final concentrations respectively. These samples were digested with 2 μl of 0.8 mg/ml proteinase K for 30 minutes at 45°C. Then, 3 μl of loading buffer containing 0.05% bromophenol blue, 0.05% xylene cyanol in 10 mM Tris-Cl (pH 8.0) and 50% glycerol was added and the reaction products were electrophoresed on a 1% agarose gel. The effect of DBNP-B on the relaxation activity of topoisomerase II was studied by adding DBNP-B at different DNA:protein concentrations and incubating at 37°C for 15 minutes prior to the addition of topoisomerase II.

SDS-PAGE:

Protein samples were analysed by electrophoresing on 15% or 12.5% polyacrylamide gels containing SDS as described by Laemmli, (1970). The ratio of acrylamide to bisacrylamide was 30:0.8. Resolving gel consisted of 15% acrylamide, 0.4% N-N' methylene bisacrylamide or 12.5% acrylamide, 0.33% N-N' methylene bisacrylamide in 0.375 M Tris-Cl, 0.1% SDS, pH 8.8. The stacking gel consisted of 6% acrylamide, 0.16% N-N' methylene bisacrylamide in 0.125 M Tris-Cl, 0.001% SDS, pH 6.8. Samples were reconstituted in loading buffer (0.1% SDS, 10 mM DTT, 10% glycerol, 62.5 mM Tris-Cl, 0.05% bromophenol blue, pH 6.8), boiled for 2 mins at 100°C, cooled and loaded. Electrophoresis was carried out at 120V for 6 hrs in electrode buffer (0.05 M Tris-Cl, 0.38 M glycine, 0.1% SDS, pH 8.3).

Staining of polyacrylamide gels:

Coomassie blue staining:

Gels were first washed with 7.5% acetic acid for 30 minutes. The gels were stained with coomassie blue R-250 (0.1% in 50% methanol, 7.5% acetic acid) for 60 minutes at room temperature and destained in 5% methanol, 7.5% acetic acid.
Silver staining:

Gels were stained according to Blum and Gross, (1987). Gels were first fixed in 50% methanol, 12.5% acetic acid overnight, washed with 50% ethanol twice each for 20 minutes and once with 30% ethanol for 20 mins. Gels were then pretreated with Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3} . 5 H\textsubscript{2}O (0.2 g /lit) for 1 min, rinsed with H\textsubscript{2}O for 30 seconds thrice. The gels were impregnated with AgNO\textsubscript{3} (2g /lit) and 0.75 ml 37% HCHO /lit for 20 minutes, and rinsed with water for 30 seconds thrice. Gels were developed with Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3} . 5 H\textsubscript{2}O (4 mg /lit), 0.5 ml 37% HCHO /lit and Na\textsubscript{2}CO\textsubscript{3} (60g /lit) for about 10 minutes. Developing was stopped by washing thrice in H\textsubscript{2}O for 2 minutes and soaking in 50% methanol and 12.5% acetic acid for 10 minutes. The gels were finally washed with 50% methanol.

Western Blotting:

Western blotting was performed according to Towbin et al, (1979) with slight modifications. Proteins were separated on a 15% SDS-Laemmli polyacrylamide gel, and transferred onto nitrocellulose membranes (0.45 μm pore size) electrophoretically for a period of 3-5 hours. The transfer buffer employed contained 25 mM Tris, 192 mM glycine, 0.1% SDS and 20% methanol at pH 8.3. After the completion of the transfer, the blots were air dried briefly and incubated in the blocking buffer (10 mM Tris-Cl (pH 7.6), 150 mM NaCl, 0.1% Tween-20) for 2 hours. The blots were then incubated in the cognate primary antibody (blocking buffer containing 0.1% IgG) for 2 hours. After the incubation, the blots were washed for 1 hour with the blocking buffer containing 0.1% Tween-20 (with three changes of buffer). The blots were finally incubated with the secondary antibody (anti-rabbit IgG from goat coupled to horse radish peroxidase at a dilution of 1:500) for 2 hours. This was followed by thorough washing of the blots for 1 hour with three changes at intervals of 20 minutes. The final washing was carried out in 10 mM Tris-Cl (pH 7.6), 150 mM NaCl. The colour development was done in freshly prepared solution of 0.03% 4-chloro-1-napthol and 0.03% (v/v) H\textsubscript{2}O\textsubscript{2} in 10 mM Tris-Cl (pH 7.6) and 150 mM sodium chloride.
Agarose gel electrophoresis:

DNA was analysed by electrophoresis on 0.8% to 1.6% agarose gels. The electrophoresis was carried out 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 under UV illumination.

Acrylamide- agarose composite gel electrophoresis:

DNase I digested products in the DNA-DBNP-B complexes were separated on composite gels containing 2.5% acrylamide and 0.5% agarose. The ratio of acrylamide to bisacrylamide in the gel was 40:0.4 in TAE buffer (40 mM Tris-acetate (pH 7.8) and 1 mM Na$_2$-EDTA). 1% agarose in TAE was dissolved by boiling, and brought to 50°C, then mixed with equal volume of acrylamide solution and the gel was casted. The electrophoresis running buffer employed was TAE. The gel was stained with 0.5 µg /ml ethidium bromide in TAE buffer and photographed under UV illumination.

Nucleic acid and Protein estimation:

The concentration of DNA in the nucleoid fractions was determined by an ethidium bromide fluorescence assay following the procedure of Morgan et al, (1979). DNA was assayed by exploiting the enhanced fluorescence of ethidium intercalated into duplex regions of DNA. Increasing amounts of calf thymus DNA was added to 1 ml buffer containing 0.5 µg /ml ethidium bromide, 5 mM Tris-Cl (pH 8.0), 0.5 mM Na$_2$-EDTA. The fluorescence emission was measured at 600 nm by exciting at 525 nm with a band width of 20 nm in a JASCO FP-777 spectrofluorimeter. Nucleoid fractions were added to 1 ml buffer and the fluorescence was measured as above. The amount of DNA in the nucleoid fractions was calculated from the calf thymus DNA standard graph.
The concentration of poly (U) and poly (A) were determined spectrophotometrically using molar absorption coefficients of 9800 and 9350 at 258 and 260 nm respectively.

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