3. Materials and methods

Bacterial strains

*E. coli* DH5α  
F-1, *rec A1 end A1 hsd R17* (rK-, mK+)

D(lac ZYA-arg F) UI69 (f80lac ZdM15)

*sup E44 thi-1 gyr A96 rel A1* (BRL, 1986).

**XL1-Blr** MRF'  
D (mcrA) 183 D (mcrCB- hsdSMR- mrr) 173 end A1

*sup E44 thi-1 rec A1 gyr A96 rel A1 lac [ F' proAB

lacI9ZDM15] Su' (Stratagene).

The bacterial strains were stored either lyophilised or in glycerol at -70°C.

Plasmids and cloning vectors

PUC18 was from New England Biolabs, Inc.; pBluescript SK-, λZAP II vector was obtained from Stratagene, Inc.

Bacterial growth media, antibiotics and common reagents

1. Ampicillin:  
100µg/ml solution in sterile double distilled water.

2. LB (Luria Bertani) medium:  
1% bactotryptone, 1% sodium chloride, 0.5% bacto- yeast extract; pH adjusted to 7.0 with 0.1N NaOH.

3. IPTG:  
1M stock of isopropyl thio-β- D- galactoside in sterile double distilled water.

4. X-gal:  
5mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside in dimethyl formamide.

5. SOB:  
2% bactotryptone, 0.5% bacto-yeast extract, 200µl 5M sodium chloride, 150µl 1M potassium chloride, 1ml 100mM magnesium sulphate in a
total volume of 100ml.

6. SOC: 200μl 1M glucose in 100ml SOB.

7. TE: 10mM Tris-HCl (pH 7.5-8.0), 1mM EDTA.

8. RF I: 1.2% rubidium chloride, 1% magnesium chloride, 1.5% w/v glycerol, 0.15% calcium chloride, 3ml of 1M potassium acetate (pH 7.5); pH was adjusted to 5.8 with 0.2M acetic acid and volume was made upto 100ml.

9. RF II: 2ml of 0.5M MOPS [3-(N-morpholino) propane sulphonic acid] pH 6.8, 1.1% calcium chloride, 0.12% rubidium chloride, 15% w/v glycerol; pH was made upto 6.8 with NaOH and volume was made upto 100ml.

10. Denaturing solution: 0.5M NaOH, 1.5M sodium chloride.

11. Neutralising solution: 1M Tris-HCl (pH 8.0); 1.5M sodium chloride.

12. 1 x SSC: 8.765g of sodium chloride, 4.41g sodium citrate in double distilled water. pH was adjusted to 7.0 with NaOH and volume made upto one litre.

13. SM: 0.58% sodium chloride, 0.2% magnesium sulphate, 50mM Tris-HCl (pH 7.5) and 0.01mg gelatin in 100ml double distilled water.

**Animals and handling procedures**

Snakes Rat snakes male and females were caught from the wild during the breeding season (late summer and monsoon). Animals were sacrificed by cervical dislocation.
Mice   Balb/c 4-6 week old female mice were used for raising antibodies against BBP. The blood was drawn retroorbitally.

Ovaries and other tissues were collected from freshly dissected rat snakes. Small ovaries, in early stages of development, were collected (as appears in the figure on the next page). The samples were instantly frozen by dipping in liquid nitrogen and subsequently stored in -70°C.

**Chemicals**

All the chemicals used in this study were obtained from commercial sources as listed below:

Agarose NA, β-mercaptoethanol, BSA (Fraction V), caesium chloride, calcium chloride, DTT, ethidium bromide, formamide, glycerol, guanidium thiocyanate, HEPES, lysozyme, magnesium chloride, MOPS, N-lauryl sarcosyl, PEG 8000, proteinase K, SDS, RNase, TEMED, NBT, BCIP and Tris base were purchased from Sigma chemical company. Acrylamide, bis-acrylamide and sodium acetate were purchased from BDH. Sephadex G-50 and high range and medium range protein molecular weight markers were obtained from Pharmacia. Nitrocellulose filter discs of 0.45µ and 0.22µ pore sizes were purchased from Millipore. DNase, Hybond-N membranes and T4 DNA ligase were obtained from Amersham. Nick translation kits and random priming kits were obtained from BARC. Restriction enzymes were bought from New England Biolabs, Pharmacia, BRL, Bangalore Genei. Sequencing kits were purchased from Promega. Calf intestinal phosphatase, IPTG, X-gal, lambda Hind III markers were bought from Boehringer Mannheim.

Nitrocellulose filters were purchased from Schlecher and Schuell. Whatman filter paper (3mm and 1 mm) were from Whatman International Ltd, X-ray films were obtained from Konica corporation. Intensifying screens were purchased from DuPont. All radioactive nucleotides were obtained from BARC. Bacto-agar, Bacto-tryptone and bacto-yeast extract were purchased from Hi Media or Difco laboratories.

All other chemicals were purchased from local manufacturers and were of analytical grade.
3.1 Methods

Sterilisation
All glasswares were sterilized by baking overnight at 250°C. Plastic wares were autoclaved at 15 lb/square inch pressure at 120°C for 20 min. Solutions were prepared in double distilled water or Milli Q water, filtered through 0.45μ nitrocellulose filters and sterilized by autoclaving. Bacterial growth media were sterilized by autoclaving.

Siliconisation
For all manipulations involving minute quantities of nucleic acids the glassware, eppendorfs and Gilson tips were siliconized with 5% solution of dichlorodimethylsilane in chloroform. The siliconized wares were heated at 80°C for 2h, rinsed thoroughly in double distilled water and autoclaved.

Preparation of frozen competent cells
The competent cells were prepared according to Hanahan (1985). A culture of *E. coli* DH5α grown overnight in 1 ml of SOB was inoculated into 100 ml of SOC (1:100 dilution) medium and incubated at 37°C with vigorous shaking till the O.D at 550 nm reached 0.35 (2-3 x 10⁷ cells/ml). The culture was chilled on ice for 15 min and cells were pelleted by centrifugation at 2, 000 rpm for 12-15 min at 4°C. The supernatant was drained thoroughly and the cells resuspended in 0.33 volumes of RFI. After keeping in ice for 15 min the cells were pelleted as before, resuspended in 0.08 volumes of RFII and left at 4°C for further 15 min. Aliquots of the cells (200μl) were distributed into eppendorf tubes, flash frozen in liquid nitrogen and stored at -70°C. Cells kept frozen for 2-3 years could also be efficiently transformed.

Transformation
The frozen competent cells were thawed slowly on ice just before use. Transforming DNA (10 - 100 ng) in a maximum volume of 10μl was mixed with the competent cells and incubated on ice for 40 min. The cells were subjected to heat shock for 90 sec at 42°C followed by rapid chilling on ice. 800μl of SOC was then added to the tubes and incubated at 37°C for 1h with gentle shaking. Transformed cells (50-100 μl) were plated on SOB-agar plates (SOB containing 1.5% bactoagar) containing 100μg/ml of ampicillin, 10mM IPTG and 25μg/ml X-gal. The plates were incubated at 37°C overnight and the recombinant colonies picked up the following day. Bacteria carrying recombinant plasmids of M13 or pUC series form white colonies.
Large scale isolation of supercoiled plasmid DNA

Plasmid DNAs were prepared by alkaline lysis method (Birnboim and Doly, 1979) described by Sambrook et al. (1989) with minor modifications. Bacteria were pelleted from a culture grown overnight in 500ml LB containing appropriate antibiotic, by centrifuging at 5,000 rpm for 10 min and washed in 100ml of ice cold STE (0.1 M sodium chloride, 10mM Tris-HCl pH 8.0, 1mM EDTA). The cells were resuspended in 10ml of solution I (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA) containing 10mg/ml of lysozyme. The contents of the tubes were gently mixed and incubated at room temperature for 10 min. 20ml of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added and the tubes were left on ice for 10 min. 15ml of ice cold 5M potassium acetate pH 4.8, was added to the above. The contents were mixed and chilled on ice for 15 min. The genomic DNA and the bacterial debris were pelleted at 8,000 rpm in a Sorvall SS34 rotor for 30 min at 4°C. The plasmid DNA in the supernatant was precipitated with 0.6 volumes of isopropanol at room temperature for 30 min. The DNA was pelleted at 10,000 rpm for 30 min at room temperature. The pellet was washed in 70% ethanol, dried under vacuum and dissolved in 8ml of TE.

Small scale isolation of plasmid DNA

The same protocol as above was scaled down according to the volume of bacterial culture used for isolation of the plasmid DNA. The DNA was dissolved in TE, treated with RNase A (final concentration 50μg/ml) at 56°C for 1h and the proteins extracted with phenol, phenol: chloroform and chloroform: isoamyl alcohol. The purified plasmid was reprecipitated, washed in 70% ethanol, dried and dissolved in TE.

Precipitation of DNA

DNA samples were precipitated by addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 0.6 volumes of isopropanol or 2.5 volumes of distilled ethanol. Genomic DNA samples were precipitated at room temperature whereas DNA samples of low molecular weight were precipitated by incubation at -20°C for 2-12h. The precipitated DNA was pelleted by centrifugation at 10,000 rpm for 15 min. The excess salts were removed by washing three times in 70% ethanol. The DNA samples were dried under vacuum and dissolved in appropriate volume of TE (10 mM Tris-HCl pH 7.5, 1mM EDTA).

Purification of plasmid

Plasmids were purified by cesium chloride-ethidium bromide equilibrium density gradient centrifugation. 8g of caesium chloride was added to 8ml of the plasmid in TE and ethidium bromide added to a final concentration of 400μg/ml. The solution was centrifuged at 72,000 rpm in a Beckman VTi80 rotor for 5h at 18°C. The lower band
containing supercoiled plasmid DNA was collected in a syringe fitted with a 21 gauge needle. The ethidium bromide was extracted with water-saturated butanol and the plasmid DNA was dialysed against TE (pH 7.5). All purified plasmids were stored either at -20°C (long term) or at 4°C (short term).

Estimation of nucleic acids
The concentration of nucleic acids present in the samples were estimated by measuring absorbance at 260nm (Sambrook et al., 1989). An O:D. 1 is equivalent to 50μg/ml of double stranded DNA, 40μg/ml of RNA or 20μg/ml of single stranded oligonucleotides.

Restriction enzyme digestion
Restriction enzyme digestion of DNA samples were carried out with 2-5 units/μg of DNA in 25-100μl reaction volume using buffers and incubation conditions recommended by the manufacturers. Reactions were stopped either with EDTA added to a final concentration of 20mM or by incubation at 65°C for 10 min.

End-labelling reaction
End labelling reactions were performed using T4 polynucleotide kinase. 5 units of T4 polynucleotide kinase was added to 50 pmoles of the primer in T4 polynucleotide kinase buffer (50mM Tris-HCl pH 7.6, 10mM magnesium chloride, 5mM DTT, 0.1mM spermidine hydrochloride, 0.1 mM EDTA) containing 30 μCi of γ-32P (specific activity 3,000 Ci/mmol) in a total volume of 10μl. The samples were incubated at 37°C for 45 min. The primer was directly used for sequencing reaction without further purification.

Labelling of DNA molecules for electrophoretic mobility shift assay
DNA probes used in electrophoretic mobility shift assay were prepared only by end filling as nick translated products tend to show altered DNA-binding properties.

In a 50μl reaction, roughly 20μg. of the digestion products were incubated with 0.2mM of dATP/dCTP/ dGTP (4μl of the 1mM stock), 1/10th volume of the Klenow buffer, 30μCi (3μl of 1Ci stock) and 3-5 U of Klenow. The reaction was carried out at 37°C for 45 min after which the products were precipitated, by adding 2 volumes of ethanol. Unincorporated nucleotides were removed by 3 washes of absolute ethanol.

3H Labelling of probes.
3H labelling of probes was performed by the nick translation procedure. In this procedure, equimolar amounts (15 - 20 μM) of 3H labeled deoxynucleoside
triphosphate precursors ($^3$H-dATP, specific activity 70Ci/mMole, $^3$H-dTTP, specific activity 60Ci/mMole) unlabelled dCTP and dGTP, and 1µg DNA were used for each reaction. The total reaction mixture of 50 µl contained $^3$H dATP and $^3$H dTTP, dCTP and dGTP, 10µl of 5 x nick translation buffer, 5µl of enzyme mix. (50pg DNaseI and 2.5U of DNA Pol I in 50mM Tris- HCl pH 7.5, 5mM MgCl$_2$, 50% v/v glycerol and 100µg/µl BSA). The mixture was incubated at 15°C for 90 min. Precipitation of probe and taking of counts was carried out as mentioned above. The specific activity of the probe ranged from 1 -3 x $10^7$ cpm/µg of DNA.

**Measurement of incorporated radioactivity**

Upon completion of the various radiolabelling reactions 1µl of the sample was spotted onto a small piece of nylon membrane, in duplicate and dried with a blow dryer. One of these was washed with cold 10% TriChloro Acetic Acid for 10 min. on ice and later transferred to a small beaker containing 100% ethanol. The membrane was later dried. Both the samples were counted in a Liquid Scintillation Counter, in vials containing the scintillation fluid. The ratio of the TCA washed / TCA unwashed counts was used to estimate the percentage extent of incorporation of the radioactivity and the total radioactivity was estimated by multiplication of the incorporated counts with the appropriate factor.

**Isolation of total RNA**

Total RNA was prepared from various tissues using the Guanidium iso-thiocyanate method of Chomczynski and Sacchi (1987). 1g of tissue was homogenised in ice-cold GITC (4M guanidium isothiocyanate, 25mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1M β-mercaptoethanol) in a glass homogeniser. 0.1 vol of 2M acetate, pH 4.0, 1 vol. of water saturated distilled phenol and 0.2 vol. of chloroform :: Isoamyl alcohol (48:2 v/v) mixture was added sequentially on ice with thorough mixing after the addition of each reagent. The final suspension was shaken vigorously for 1-2 min incubated on ice for 15min. and centrifuged at 10, 000 rpm for 20min. at 4°C in a Sorvall HB-4 rotor. The aqueous phase containing the RNA was transferred to a fresh tube. The RNA was precipitated with an equal vol. of isopropanol at -20°C for 4-6h. Total RNA was pelleted by centrifugation at 10, 000 rpm for 30min at 4°C. The pellet was dissolved in 3ml. GITC and extracted with an equal volume of phenol and 0.2 volumes of chloroform :: Isoamyl alcohol (48 :: 2 v/v) The RNA was reprecipitated with an equal volume of isopropanol at -20°C for 4-6 h.

The RNA was pelleted as before, washed thrice with 70% ethanol, vaccum dried and dissolved in sterile double distilled water. The quality of the RNA preparation was checked by electrophoresis using a 1% agarose gel in TBE (89mM Tris, 89mM Boric
acid, 2mM EDTA) containing 0.5mg/ml of ethidium bromide. The RNA was quantified by estimating its absorbance at 260 nm using a spectrophotometer.

**Purification of mRNA**

mRNA was purified from the total RNA by affinity chromatography over oligo-dT cellulose. A sterile 1ml plastic syringe was packed with oligo-dT cellulose that washed with 0.1M NaOH and presoaked in water. 10mg of total RNA dissolved in the binding buffer (10mM Tris-HCl pH7.5, 500mM LiCl, 2mM EDTA) was bound onto this matrix by repeated loading. The column was washed with about 10ml of washing buffer (10mM Tris-HCl pH 7.5, 150 mM LiCl, 2mM EDTA) and subsequently eluted with about 3 ml of 2mM EDTA solution. The mRNA was precipitated by ethanol precipitation, and quantitated and checked for quality later.

**cDNA Synthesis**

cDNA was synthesised by the Gubler and Hoffman's procedure (Gubler and Hoffman, 1983) using the Amersham's cDNA synthesis kit as per the manufacturer's instructions.

Essentially the first strand cDNA was synthesised by oligo-dT as well as random priming of 5-10μg of mRNA and using the AMV reverse transcriptase. A typical reaction mix contained:

- 5x 1st strand buffer: 4μl
- NaPPi: 1μl
- Human placental RNase inhibitor: 1μl
- dNTP's: 3μl
- oligo dT random primer: 1μl
- mRNA: 5μl
- water: 2.5μl

The reaction was carried out at 42°C for 45-60min. The enzyme was heat denatured and a 2μl aliquot was removed for quantitation and for gel electrophoresis. The kit uses the *E.coli* RNase to remove the RNA strand in the hybrid mRNA : cDNA product of first strand synthesis and the DNA polymerase for second strand synthesis. To the entire quantity of the first strand reaction 6.5μl of DNA polymerase I and 1 μl of *E.coli* RNaseH was added. The mix was incubated at 22°C for 60min and heat inactivated, following which 1U of T4 DNA polymerase was added and incubation was carried for further 30min to remove any 3' overhangs.
Adaptation of cDNA and size selection
For facilitation in cloning, the cDNA was ligated to blunt ended EcoR1 adaptors at 15°C for 4 h, in a 20μl reaction using 1/10th volume of 10x ligase buffer and 10U of T4 DNA ligase. The unligated adaptors were removed by size exclusion chromatography through Sephacryl S-300 column, provided along with the kit. Fractions containing the cDNA were pooled, precipitated. 25-50ng of the cDNA was ligated with 1μg of EcoR1 predigested and phosphatased λZAP II vector, at 15°C for 4h, in a 10μl reaction under same conditions as during adaptor ligation.

Packaging of ligated products
The ligated cDNA were packaged into phage particles using the packaging extracts obtained commercially. 10μl of the component A and 15μl of component B of the Amersham Packaging kit were added to entire ligated products, diluted to 500μl in SM buffer and stored at 4°C with addition of 20μl of chloroform.

Preparation of plating cells
The XL1 blue cells were grown at 37°C in LB containing 10mM MgSO4 and 0.2% maltose, till an A660 of 0.6 was reached. The cells were harvested by centrifugation in a sterile polypropylene tube and resuspended in 0.3 volumes of cold, sterile 10mM MgSO4 solution.

Plating of library
20μl of the packaged products were mixed with 300μl of the XL1Blue plating cells and incubated at 37°C for 15 min. 7ml of top agarose (0.7% in LB) was added and mixed with the infected cells and poured onto a basal predried LB agar plate. The plates were incubated at 37°C for 6-7h, or till the plaques began appearing.

Southwestern screening of the expression cDNA libraries.
The cDNA library was plated at a density of 20-30,000 plaques per 150mm plate. After about 3-4 h, when minute plaques began appearing a nitrocellulose filter presoaked in 10mM IPTG was layered onto the surface and the plaques were allowed to grow at 37°C for further 5-7h. Prior to removal of the membrane the plates were chilled for a minimum of 2h. The membrane was air-dried for 10-15min and later used for the southwestern binding (Singh et al., 1988).

Genomic DNA isolation.
1g tissues was homogenised in 10ml lysis buffer (75mMNaCl, 2mM EDTA pH8.0), in a hand held glass homogenizer. Proteinase K and SDS were added to the final
concentration of 100μg /ml and 2% respectively. The samples were mixed and incubated at 37°C for 4-6 h. The proteins were extracted with phenol (Tris-saturated pH 8.0), phenol:chloroform (1:1 v/v) and chloroform:isoamyl alcohol (24:1 v/v) and the aqueous phase was separated by centrifugation at 10,000 rpm, 15°C, 10 min (in a Sorvall RC5B). The DNA that partitioned in the aqueous phase was precipitated by adding 1/10 volume of 3M sodium acetate pH 5.2 and 0.6 volume of isopropanol, and the gelatinous precipitate of the high molecular weight DNA was spooled on a sterile glass rod, washed three times in 70% ethanol, dried and dissolved in TE (pH 8.0).

**Release and purification of cloned insert**

The products of the end-filling reaction were electrophoresed in a 5% native polyacrylamide gel in 1xTBE. The gel was exposed to a X-ray film and the insert band was excised. The polyacrylamide pieces were crushed by passing through a 1ml syringe and DNA was extracted in 1ml of water at 37°C. The solution was concentrated by extracting with butanol and purified by passing through a Sephadex G-50 spun column.

**Sephadex G-50 spun columns**

Sephadex G-50 spun column chromatography was used as a rapid single step purification procedure to purify the high molecular weight DNA from contaminants like nucleotides, salts or organic solvents such as butanol.

A sterile 2ml disposable plastic syringe (Steriware), plugged with sterile glass wool was filled with autoclaved slurry of Sephadex G-50 in water. The column was placed in a 15ml Falcon tube and centrifuged in a Remi table top centrifuge at 3,000 rpm for 4 min, till it almost became dry. Under the same conditions, the compacted column was equilibrated for the desired volume (200μl) by repeating the cycles of loading and centrifugation (till the volume recovered equalled the volume loaded). The DNA was then loaded onto the column and centrifuged under the same conditions.

**Agarose gel electrophoresis**

Routine electrophoresis was performed in 1% Agarose in 1x TBE.

**Sequencing of DNA**

Nucleotide sequencing was performed following Sanger's dideoxy-termination method (Sanger *et al.*, 1977), modified by Chen and Seeburg (1985), by using the TaqTrack kit from Promega, Inc.

The sequencing (M13 forward or reverse) was end-labelled using T4 PNK. The
reaction contained 10pmoles of primer, 10pmoles γ-32P ATP, 5 U of T4 PNK enzyme in a 10μl reaction. The mix was incubated at 37°C for 10min and transferred to 90°C for 2min for enzyme inactivation and stored at -20°C.

Prior to annealing the plasmid and the primer, the plasmid was alkali denatured. 2pmoles of plasmid taken in 18μl volume to which 2μl of 2M NaOH, 2mM EDTA solution was added and incubated at room temperature for 5min. The alkali was neutralised with 2μl of 2M ammonium acetate and DNA was precipitated with ethanol.

Denatured plasmid (approximately 1.5-2pmoles of ds DNA) was dissolved in 8μl volume and Taq DNA polymerase buffer, labelled primer (2 pmoles) were added to make up final volume 25μl. The mix was pre-incubated at 37°C and 1 μl of appropriate ddNTP mix was added separately into tubes labelled A, T, G, C. 1.6μl of Taq DNA polymerase (2.5 U/l) was added to the annealing reaction mix and 6μl of this mix was added to each tube. The reaction was incubated at 70°C for 10 min. Stop solution was added and the products were denatured at 94°C for 2min, chilled quickly and loaded onto a 8% denaturing poly acrylamide gel.

A typical sequencing gel composition was 10 ml 40% (38:2) Acrylamide, 8M Urea, 1x TBE in a total volume of 50ml.

**Preparation of nuclear extracts.**

The nuclear extracts were prepared by the procedure of Andrews and Faller (1992). Approximately one gram tissue was minced in STM buffer (0.25M Sucrose, 50mMTris-HCl, pH 7.5, 25mM KCl, 5mM MgCl2, 0.5mM PMSF and 0.5mM DTT) and the supernatant was discarded. The tissue was homogenised in homogenisation buffer (same as STM buffer) in a glass homogeniser. The tissue homogenate was filtered through six layers of cheese cloth to remove the cellular debris and connective tissue. The filtrate was centrifuged in a Sorvall SS-34 rotor at 4000 rpm at 4°C for 10min. The pellet was resuspended in lysis buffer (10mM HEPES pH7.4, 1.5mM MgCl2, 10mM KCl, 0.5mM DTT, 0.5mM PMSF) and left on ice for 10 min following which the samples were vortexed for 10 sec. The lysed nuclei were pelleted and subjected to extraction buffer (20mM HEPES pH7.4, 0.42M NaCl, 10mM MgCl2, 10% glycerol) for 20min at 4°C. The chromatin was separated by centrifugation at 14,000 rpm in a Sorvall SS-34 rotor at 4°C for 30min. The supernatant was dialysed against a buffer containing 20mM Tris-HCl, pH7.4, 100mM NaCl, 10mM MgCl2, 0.5mM DTT, 0.5mM PMSF and 10% glycerol overnight at 4°C and used in mobility shift and Southwestern assays.
Protein estimation
Proteins were estimated by the Bradford assay (Bradford et al., 1976) that was adapted for a microtiter plate-based procedure. This comparison based assay involved calibration with known concentrations of a standard protein like bovine serum albumin. The linear range of this assay was between protein concentrations, 100ng to 3μg. Concentrated protein solutions thus had to be diluted prior to the assay and the actual concentration was determined after correcting for the dilution factor.

In the actual protocol, the protein solution, in 200μl volume was pippeted out into each cavity of the microtiter plate, to which 50μl of undiluted commercially available Bradford's reagent (Coomassie blue R-250 solution) was added. The samples were mixed thoroughly by pipetting and the absorbance of the color developed was estimated at 590nm wavelength in an ELISA reader.

Concentration of proteins
Concentration of the dilute proteins was achieved by solution precipitation with saturated ammonium sulfate (to a final concentration of 60%). This procedure was preferred over vaccuum drying as it was not affected by the high glycerol or NaCl concentrations usually present in various buffers. The precipitate could be rapidly recovered by centrifugation at 15,000 rpm at room temperature and was readily soluble. Removal of ammonium sulfate from the pellets was achieved by overnight microdialysis in microcentrifuge tube lids.

SDS PAGE of proteins
SDS-polyacrylamide gel electrophoreses were carried out by the method described by Laemmli (1970) using a discontinuous buffer system. The final concentrations of the buffers used were as follows:

- Stacking gel: 0.125 M Tris-HCl, pH 6.8
- Resolving gel: 0.35M Tris-HCl, pH 8.8
- Electrophoresis buffer: 0.25M Tris-HCl, 0.192 M glycine, pH 8.3

A stock solution containing 30% acrylamide w/v and 0.8% N-N’ methylene bisacrylamide w/v were used. The resolving gel had 10% acrylamide and the stacking gel had 4.5% acrylamide. The gel and the buffers contained 0.1% SDS. The gels were polymerised using TEMED (20 ml) for 30 ml of gel mixture and freshly prepared Ammonium persulphate (200 ml of 10% solution). The gels were cast in a vertical gel apparatus (Hoefer Instruments, CA, USA). Before loading the protein samples were not heat denatured as common. Electrophoresis was carried out at a constant current of 20mA, till the samples crossed the stacking gel and then at 40 mA through the resolving
Polyacrylamide gels of the proteins were stained by soaking the gel for 3 hours in a fresh 0.2% solution of Coomassie Brilliant Blue prepared in the fixative (45: 10: 45:: methanol : acetic acid : water). The polypeptides resolved in the gel were visualised by destaining the gel overnight with several changes of the fixative solution.

Silver staining
Silver staining of proteins being 10-100 times more sensitive than Coomassie method, was used for visualisation of the dilute protein solutions recovered during various stages of purification and characterisation. The sensitivity of the technique demands extreme care in handling the gel and use of chloride-free water in all ingredients (preferably MilliQ or double distilled water).

The SDS -Polacrylamide gels were fixed in a 50% methanol solution for a minimum of 3-4 h and subsequently washed extensively with 3-4 changes of water. The gel was then soaked in 0.02% (w/v) solution of sodium thiosulphate for 60 seconds. (timing critical) and once again washed with water. The gel was then transferred into a freshly prepared stain solution (0.2g of silver nitrate, 75μl of formaldehyde (37% stock) in 100ml of water) and incubated for 20min in dark with shaking. The stained gel was rinsed three to four times in water and transferred to a new tray. The gel was developed by immersing the gel in a 6% solution of sodium carbonate containing 50μl of the 37% formaldehyde stock.

Transfer of proteins
The protein samples separated on the SDS PAGE were left in the renaturation buffer (50mM NaCl, 20mM Tris-HCl pH7.4, 10mM MgCl2). The proteins in the gel were transferred onto the nitrocellulose membrane by semi-dry graphite method described by Towbin et al. (1979) as follows: The gel containing the protein, the nitrocellulose membrane and 6 sheets of Whatman 3mm were cut according to the gel size, were soaked in transfer buffer (39mM glycine, 48M Tris base, 0.0375% SDS and 20% Methanol) for 30 min. Three pieces of the soaked Whatman 3mm paper were placed on the anode plate onto which the nitrocellulose membrane was placed. The gel was aligned onto the membrane on top of which 3 more pieces of whatman 3mm were placed. Care was taken to remove air bubbles from the Whatman sheets as it impedes transfer. The cathode plate was then placed on top of the assembly and were connected to the respective electrodes. The transfer was performed for 150min. at 0.8 mA per cm². After transfer the efficiency was checked by staining the membrane by Ponceau-S
as well as staining the transferred gel. Usually the efficiency was 80-90%.

**South-western assay**
The membrane with transferred proteins was soaked in the binding buffer (10mM Tris-HCl pH7.0, 50mM NaCl, 10mM MgCl2, 1mM EDTA) without BSA for 10min. Prebinding was performed in binding buffer containing 3% BSA and 20-50 µg of sheared *E.coli* DNA for 2h at room temperature. To this $10^5$ cpm per ml of the end filled Bkm 2(8) probe (end-labelled was added and DNA-protein binding was performed for 90 min at room temperature). The membrane was washed in binding buffer with 3 changes of the buffer for 15 min each, dried and exposed for autoradiography.

**DNA slot blots**
Appropriate amounts of DNA samples were made upto 100µl (in TE pH8.0). The DNA samples were denatured with an equal volume of 1N NaOH at room temperature for 30min. The samples were neutralised with 2 volumes of neutralising solution (1 : 1 : 1 mixture of 1M Tris-HCl pH 7.5; 3M NaCl and 1N HCl). The tubes were vortexed. The DNA samples were loaded onto the nylon membrane to their respective slots by using a Manifold II slot blot apparatus under vacuum. The slots were washed with 200µl of neutralising solution. The membrane was air-dried and the DNA was fixed by baking at 80°C, for 2h.

**Nucleic acid hybridisation**
Hybridisation of the membrane immobilised DNA to various denatured, radiolabelled probes (10ng/ml or $10^6$ cpm/ml) was performed in the phosphate SDS buffer (0.5M sodium phosphate pH 7.5, 7% SDS) at the hybridisation temperature for 12-16h. A prior prehybridisation step of 2h in the same buffer without the probe was always included.

**Western assay**
The proteins separated by SDS-PAGE were tansferred onto the HyBond-C (nitrocellulose) membrane using the graphite dry transfer method described above. Upon ascertaining the efficacy of transfer by reversible staining with Ponceau-S, the membrane was blocked with TNT (20mM Tris-HCl pH 8.0, 150mM NaCl, 0.05% Tween-20) containing 3% BSA. Antibody binding was performed in plastic bags, wherein the membrane was incubated with the appropriate dilution (1:500) of antibody solution in TNT (100ml per sq.cm surface area) at room temperature for 2 h with continuous shaking. The blots were washed in TNT, for 45min (3 washes, 15min each) and subsequently bound to the second antibody solution (alkaline phosphatase
conjugated rabbit anti-mouse IgG; (1:3000 dilution in TNT containing 1.5% BSA) at room temperature for 1 h. with shaking. The blots were washed again with TNT for 30 min (3 washes, 10 min each), rinsed in the alkaline phosphatase buffer (100mM Tris-HCl pH 9.0, 100mM NaCl, 5mM MgCl₂) and antibody binding was detected by the color development assay for its conjugated alkaline phosphatase activity.

**Color development:**

For color development the western blots after binding to the second antibody, were incubated in minimum volume of the colour reagent for 10 min, with constant shaking.

Color reagent: 33 ml of BCIP (from a 50mg/ml stock in dimethyl formamide) and 66ml of NBT (from a 50mg/ml stock prepared in DMSO) per 10ml of AP (alkaline phosphatase) buffer.

Upon color development, the developing was stopped by washing the blot with 10mM EDTA solution.

**Electrophoretic mobility shift assay**

This rapid electrophoretic assay for detection of sequence specific DNA binding proteins from relatively crude protein extracts, is based on the differential migration of free DNA than that, which is complexed with proteins (Fried and Crothers, 1981). In this a short DNA fragment or an oligo of defined sequence, radiolabeled at its 5' end by T4 polynucleotide kinase or Klenow serves as a probe. Specific DNA binding involves complex formation with the proteins in presence of excess unlabelled nonspecific DNA. The relative affinities of specific DNA binding being higher, is favored over complexing with the non-specific DNA, even though the latter is in 100-1000 fold higher quantity.

In a typical assay, 10-50μg of crude protein extract was incubated with an optimal amount of unlabelled non specific competitor DNA (500ng - 2μg of sheared E.coli DNA or poly dl:dC) at 25°C for 20 min, in a minimal volume 30-50μl of the reaction buffer (10mM Tris-HCl pH 7.5, 150mM NaCl, 5mM EDTA). Subsequently 15-30,000 cpm of radiolabelled Bkm 2(8) or GATA₁₆ probe was added and incubation was carried out for further 30-45 min. The reaction was mixed with the sample loading buffer (0.025% bromophenol blue, 0.025% xylene-cyanol, 40% glycerol in TE) mix was loaded onto a 5% polyacrylamide gel (native) in 1x TBE and electrophoresed at 125V (in a Hoefer's gel apparatus) till either the bromophenol blue dye (for the GATA₁₆ probe) or the xylene cyanol (for Bkm 2(8) probe) migrated up to the end of the gel. The gel was dried and exposed to X-ray film or phosphorimager plate.
Preparation of Bkm - Sepharose

Bkm-Sepharose column was prepared by coupling the 545 bp cloned Bkm 2(8) insert to activated -CNBr Sepharose (Pharmacia), following the procedure of Kadonaga and Tjian (1984).

The freeze dried, activated -CNBr Sepharose was swollen in water (1g gel swells to about 3ml volume) and washed several times with water on a sintered glass filter to remove the preservation additives. The gel was subsequently washed with 0.1M HCl followed by several washes with water. The coupling efficiency being maximum at alkaline pH, the concentrated DNA solution in water (DNA should not be dissolved in Tris or any -NH2 containing buffer as these react with the -CNBr) was adjusted to pH 8.0 by addition of 1/100 vol.of 1M potassium phosphate buffer. The DNA was incubated overnight with the activated Sepharose at 4°C on a rotatorque. Upon completion of the cross-linking, the gel was allowed to stand and the supernatant was collected for estimating the uncoupled DNA. Usual coupling efficiency was about 90%. The gel was washed with water and the unreacted -CNBr sites were blocked by incubating the gel with 1M Ethanolamine-HCl, pH 8.0, for 4-6 h at room temperature. This was followed with alternate washes with low and high salt buffers (10mM and 1M potassium phosphate buffer, both at pH 8.0), 1M KCl and water, and finally stored in the TNE buffer (20mM Tris-HCl, pH 7.5, 150mM NaCl and 5mM EDTA).

Two dimensional electrophoresis of proteins

This method, devised by O’Farrell (O’Farrell, 1975), permits resolution of proteins on the basis of their isoelectric point and denatured molecular weight. The separation in the first dimension is performed in 1.5mm tubes, where the protein sample is isoelectrically focussed through a low percentage polyacrylamide gel containing non-ionic detergents like urea and NP-40 and a mixture of ampholyte resins in the pH range 3-10.

Gel Composition for 10 ml:

5.5g Urea
1.33ml of acrylamide mix (28.83:1.62, acrylamide: bis-acrylamide)
0.5ml Ampholine pH 3-10, degassed
200μl of NP40
TEMED (7μl)
10% APS (10μl)
Catholyte solution: degassed 0.02M NaOH.
Anolyte solution: 0.01M Phosphoric acid.

The tube gels were layered with the gel overlay solution of 8M Urea and the ampholines were allowed to prefocuss in the following sequence:
200V for 15min, 300V 30min, 400V for 30min.

The crude oocyte extracts (300-500μg) prepared in the lysis buffer (9.5 M Urea, 2% w/v NP40, 2% Ampholine (200μl of pH 5-8; 50μl of pH 3-10 for 9.5 ml solution) containing 5% mercaptoethanol) were layered onto the tube gels using a hamilton syringe or a micropipette tip and electrophoresis was continued for 12h at 400V with a final focussing at 800V for 1h.

The tubes were removed and frozen at -20°C till further use. For the second dimension analysis the gels were extruded out by applying pressure at one end and the tube was incubated in the SDS PAGE sample loading buffer for 30min at room temperature. The tubes were placed on a 10% SDS PAGE slab gel, and subsequently electrophoresed.

One of the tube gels that was electrophoresed in the same run was finely cut into serial 5mm pieces and immersed in 500μl MilliQ water (Millipore Corp., USA) in a microcentrifuge tube. The pH in each of these tubes was measured with a micro pH meter (Orion Scientific) and was used as a reference for the actual estimation of pl of the proteins.

**Glycerol gradient centrifugation**

Density gradient centrifugation facilitates differential resolution of macromolecules on the basis of their molecular densities or molecular weights. The separation involves formation of stable step or linear gradient of dense matrices like glycerol, sucrose, ficoll etc. The separation can be controlled by fixing the two extremes densities. In this case a linear gradient between 15% and 35% glycerol was selected. Equal volumes of the two solutions (25mM HEPES pH 7.6, 100mM KCl, 0.1% NP-40 containing 15% and 35% glycerol respectively) were filled in the two arms of the automatic gradient former (Pharmacia), in such a manner that the denser solution was towards the outlet. Using the controllable peristaltic pump and the adjustable syringe with a level control, a bottom to top linear gradient was formed in the 13ml polyallomer SW41 tubes. The tubes were almost completely filled. 500μg (100μl of 5mg/ml solution) of the crude snake oocyte extract in buffer containing 10% glycerol was gently layered on this. After accurately balancing the tubes, these were subjected to centrifugation at 38,000 rpm, for 24h at 8°C, under vacuum.
Upon completion of the run, 400µl fractions were collected from the top by reversing the peristaltic flow of the gradient former. Proteins were estimated in individual fractions by the Bradford's rapid protein estimation assay and equal quantities of proteins from various fractions were concentrated. 50µg protein from each sample was separated on the SDS-PAGE and assayed for Bkm 2(8) binding protein in the Southwestern procedure.

**Immunisation of animals**

The antigenic emulsion for immunisation was prepared by repeatedly passing a (1:1 ratio v/v) mixture of the protein solution and Freund's complete adjuvant, through a 24 gauge hypodermic needle. The emulsion was administered by the subcutaneous route in 2-3 depots on the back. About 10mg dose of protein was used per animal per injection. The primary injections were followed by 3 boosters at 10 day intervals. In the booster doses, the antigen was prepared with Freund's incomplete adjuvant.

Upon completion of the immunisation schedule the 0.5 ml of blood was collected from the animals by the retro-orbital puncture procedure. The blood collected in a microcentrifuge tube was allowed to clot in an upright position and later centrifuged at 6,000 rpm for 10min. The serum thus separated, was stored at 4°C by addition of sodium azide (final concentration 0.02%). Various dilutions of the serum were later assayed in a western blot procedure, using the blots prepared with crude snake oocyte extracts.

**In situ localisation of Bkm DNA and anti-BBP antibodies**

**Preparation of nuclei**

Nuclei from somatic tissues were prepared by a procedure similar to that for preparation of nuclear protein extracts, except that after the hypotonic treatment, the nuclear pellet was suspended and washed with 3:1 methanol:acetic acid fixative. The oocyte nuclei were prepared in a slightly different manner.

Ovaries were crushed with the aid of toothed forceps in PBS and after removal of the debris, transferred into a 15 ml falcon tube and were pelleted by low speed centrifugation 1,000rpm, 5min. The pellet was suspended in 15 ml of methanol acetic acid fixative and pelleted by low speed centrifugation. The procedure was repeated twice when finally a concentrated suspension of the nuclei were dropped onto prewarmed acid cleaned, grease free slides.
DNA in situ hybridisation

The procedure described by Jones (1973) and Singh et al. (1977) was used with minor modifications. The oocyte nuclear preparations were treated with RNase A (50 μg/ml) and RNase T1 (2 units/ml) in 2x SSC at 37°C for 1 h, washed in 2x SSC at room temperature (3 changes, 10 min. each), dehydrated through ascending grades of alcohol (50%, 70%, 90% and 100%), air-dried, heat denatured in a simmering solution of 10 mM Tris-HCl, pH 7.4, 5 mM MgCl₂ for 1 min. followed by dehydration in ice cold 70%, 90% and absolute ethanol for 4, 2 and 2 min. respectively and the slides were air-dried. Hybridisation was done in presence of formamide.

15 μl of hybridisation mix was used for each slide covering a 22 x 22 mm area. The hybridisation mix consisted of 2x SSC, 10% dextran sulphate, 500 μg/ml of sheared and denatured fish DNA, 50% formamide and 5 x 10⁴ cpm/μl of ¹²⁵I labelled Bkm 2(8) probe (Specific activity 10⁷ cpm/μg). Hybridisation was carried out at 37°C for 14 h. The coverslips were removed and the slides were rinsed in 2x SSC at room temperature and washed at 45°C in 50% Formamide and 2x SSC pH 7.0 for 10 min, at 4°C for 5 h, in 2x SSC and then dehydrated through a series of alcohol grades, and finally air dried. Hybridised slides were coated with Ilford K₂ nuclear emulsion, and exposed for 6-8 weeks at 4°C, developed in Kodak D19B developer on ice for 12 min. and fixed for 5 min. in high speed fixer. Slides were stained in Gurr's Giemsa stain (2 ml Giemsa stain in 50 ml of 0.1 M phosphate buffer, pH 6.8) for 20-30 min, rinsed in phosphate buffer, pH 6.8 and air-dried. Photographs were taken on a slow speed 35mm film using a Zeiss photo microscope with camera attachment.

Antibody localisation

The slides were rehydrated in PBS through descending grades of methanol solutions prepared in PBS (90%, 70%, 50%, 30%). The nuclei were permeabilised by immersing PTX (PBS + 0.1% Triton X-100) for two minutes each and subsequently blocked by incubation in PBTX (PBS + 0.1% Triton X-100 and 1% BSA) for 3-4 h at room temperature on a rotary shaker. The slides were removed from the coplin jars and 80 μl of 1:250 dilution of anti-BBP antiserum prepared in PBTX was layered onto each slide and covered with a strip of parafilm. The slides were incubated for 12 h at 4°C in a tray containing moistened tissue paper. The antibody solution was washed off by rinsing twice in PBTX followed by an extended wash in PBTX for 2 h at room temperature on a rotary shaker. The slides were incubated with a 1:400 dilution of biotin-conjugated goat antimouse anti-IgG (Vectastain kit) at room temperature for 2 h, following which the unbound antibody was washed off by rinsing twice with PTX and a 1 h wash with the same at room temperature, with shaking. The PTX wash were followed by two changes with PT (PBS containing 0.1% Tween-20) of 30 min each.
The avidin-HRP conjugate was prepared fresh by incubating 5 µl of reagent A (Avidin) + 5 µl of reagent B (HRP) in 1 ml of PT for 30 min at room temperature. 80 µl of the conjugate was layered onto each slide and incubation was carried out for 45 min at room temperature. The slides were rinsed and washed with PT for an additional 30 min and the color was developed by adding a drop of freshly prepared solution of the diaminobenzidine (DAB) reagent containing trace quantity of hydrogen peroxide. The slides were thus stained for 5 min. The stain solution was washed off and the slides were dried and mounted in glycerol.