CHAPTER 2

MATERIALS AND METHODS
2.1 Materials

2.1.1 Source of chemicals and materials used

Agarose, aprotinin, β-mercaptoethanol, bovine serum albumin (BSA) (Fraction V), calcium chloride, Dulbecco’s modified Eagle’s medium (DMEM), dithiothreitol (DTT), ethidium bromide, fetal bovine serum, guanidium isothiocyanate, isopropyl β-D-thiogalactoside (IPTG), leupeptin, lithium chloride, magnesium chloride, O-nitrophenyl β-D-galactoside (ONPG), phenylmethane sulphonyl fluoride (PMSF), PEG 8000, piperidine, pepstatin, sodium dodecyl sulphate (SDS), sucrose, N,N,N’,N’-tetramethyl ethylene diamine (TEMED), trypsin and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) were procured from the Sigma Chemical Company, USA; acrylamide and bis-acrylamide were from Serva Chemical Company, Germany; Sephadex G-50, Sephadex G-25 and poly(dl.dC).poly(dl.dC) were from Pharmacia Biotech, Sweden. Bacto-agar, bacto-tryptone and yeast extract were obtained from Difco Laboratories, USA, proteinase K from Boehringer-Mannheim, Germany, and lipofectamine from Life Technologies, USA. All restriction enzymes were purchased from New England Biolabs Inc., USA; Klenow polymerase, polynucleotide kinase, T4 DNA polymerase, T4 DNA ligase, Taq DNA polymerase and reverse transcriptase were obtained from either New England Biolabs Inc., USA, or Promega Corporation, USA. The DNA size markers and protein molecular mass markers were purchased from Bangalore Genei, India. The DNA multiprime labelling kit was procured from BRIT, India; the core footprinting kit, gel shift assay kit, Erase-a-Base kit, Taq track sequencing kit and luciferase assay kit were from Promega Corporation, USA, and Qiagen DNA purification columns were from Qiagen Inc., USA. The rat genomic library was obtained from Stratagene Company, USA; custom made oligonucleotides were purchased either from Bangalore Genei, India, or from Life Technologies, USA; HeLa nuclear extracts and purified recombinant Sp1 protein were purchased from Promega Corporation, USA, and antibodies to the Sp1 and AP1 family of proteins were purchased from Santa Cruz Biotechnology Inc., USA. Hybond N and Hybond N+ membranes were procured from Amersham International, UK, and Whatman filter paper from Whatman International Ltd., USA. X-ray films were obtained from Konica Corporation, Japan, and radiolabelled nucleotides were procured from BRIT, India. All other chemicals were purchased from local manufacturers and were of analytical grade.
2.1.2 Bacterial strains

_E. coli_ LE392

*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*

This is a suppressing strain commonly used to propagate bacteriophage vectors and their recombinants. It was used for growing the genomic library.

_E. coli_ DH5-α

*supE44 ΔlacU169 (φ80lacZ ΔM15) hsdR17 recA1 end A1 gyrA96 thi-1 relA1*

This is a recombination-deficient suppressing strain used for plating and growth of plasmids and cosmids. The φ80 lacZ ΔM15 permits α-complementation with the amino terminus of β-galactosidase encoded in pUC vectors. It was used for all routine transformations, plasmid isolation and selection of recombinants.

_E. coli_ JM109

*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiΔ(lac-proAB)F’[traD36 proAB+ lacF lacZΔM15]*

It is a recombination-deficient strain that supports the growth of vectors carrying amber mutations and can modify, but not restrict transfected DNA. It has an F’episome that is required for single-stranded DNA production. This strain was used for transformation with pGL3 vectors. The bacterial strains were maintained on LB plates or stored in LB containing 15% glycerol at -70°C.

2.1.3 Plasmid vectors

_pUC18:_

This is a high copy plasmid vector derived from pBR322. It has an ampicillin-resistance marker and a lac Z gene and can display α-complementation in appropriate hosts, which allows the identification of recombinants by histochemical staining.

_pBSII KS +/- and pBSII SK +/- vectors:_

These two vectors are identical except that they contain the multiple cloning sites arranged in opposite orientations. They are high copy phagemid vectors containing an ampicillin-
resistance marker gene, a lac Z gene and an F1 origin of replication for production of single-stranded DNA.

pGL3 vectors:
These vectors are high copy number plasmids carrying an ampicillin-resistance marker and an F1 ori for single-stranded DNA production. They contain a modified firefly luciferase cDNA (luc⁺) as a reporter gene. A series of three pGL3 luciferase reporter vectors which are structurally identical except for the inclusion of promoters and enhancers were used.

(i) pGL3-Basic: This vector lacks any eukaryotic promoter and enhancer sequences. Expression of luciferase activity in cells transfected with this vector depends on insertion of a functional promoter (upstream of luc⁺) in the proper orientation. It is used in the identification of putative regulatory sequences.

(ii) pGL3-Promoter: It contains an SV40 promoter upstream of the luciferase gene, and can be used to identify putative enhancer elements that can be inserted either upstream or downstream of the promoter - luc⁺ transcriptional unit.

(iii) pGL3-Control: This vector contains SV40 promoter and enhancer sequences, resulting in strong expression of luc⁺ in many mammalian cell types, and is useful in monitoring transfection efficiency and serves as a convenient internal standard for promoter and enhancer activities expressed by pGL3 recombinants.

pCMV.SPORT-βgal vector:
It is a plasmid vector (obtained from Life Technologies, USA) containing the E. coli lac Z gene cloned downstream of the strong CMV promoter for high expression of β-galactosidase in mammalian cells and an ampicillin resistance marker for selection. This reporter vector is used as an internal control to monitor the efficiency of transfection.

pCH110:
This plasmid vector (obtained from Pharmacia Biotech, Sweden) contains a function lac Z reporter gene expressed from SV40 early promoter for expression of β-galactosidase in mammalian cells and an ampicillin marker for selection. It is used as an internal control to monitor transfection efficiency.
2.1.4 *Rat genomic library*

A rat genomic library constructed in the Lambda DASH II vector using DNA from testis of Sprague Dawley male rat was procured from Stratagene Company, USA. It carries inserts of 9-22 kb, which can be released using Eco RI. This library is amplified and grown on the *E. coli* host strain LE392.

2.1.5 *Laboratory animals and cell lines*

**Animals:**
An inbred strain of Wistar rats from the CCMB animal house was used in the experiments reported in this study.

**Cell lines:**
(a) HeLa: a cervical carcinoma derived human epithelial-like cell line.
(b) F111: a rat lung embryo fibroblast cell line
(c) NIH 3T3: a mouse embryo fibroblast cell line
(d) PCC-4: a mouse embryonal carcinoma cell line

2.1.6 *Bacterial media, antibiotics and commonly used solutions*

1. **Ampicillin:** A 1000X stock solution of 100 mg/ml ampicillin was made in sterile double-distilled water.
2. **LB (Luria Bertani) broth:** 1% bacto-tryptone, 1% sodium chloride, 0.5% bacto-yeast extract pH 7.0.
3. **LB agar:** LB containing 1.5% agar
4. **Top agar:** LB containing 0.7% agar
5. **IPTG:** 200 mg/ml stock of isopropyl β-D-thiogalactoside in sterile double-distilled water.
6. **X-Gal:** 20 mg/ml stock of 5-bromo-4-chloro-3-indolyl-βD-galactoside in dimethyl formamide.
7. **SM:** 0.1M NaCl, 50 mM Tris.HCl pH 7.5, 0.01% gelatin, 0.2% MgSO₄ in sterile double-distilled water.
8. **1X TE:** 10 mM Tris.HCl pH 8.0, 1 mM EDTA
9. **1X TBE:** 89 mM Tris-Borate pH 8.3, 2 mM EDTA
10. **1X SSC:** 0.15 M NaCl, 0.015 M sodium citrate pH 7.0
11. Phenol : Chloroform: Phenol was prepared by the equilibration of liquified phenol containing 0.1% hydroxyquinoline with 0.1M Tris.HCl pH 8.0. It was mixed in equal volume with chloroform : isoamyl alcohol (24:1) and stored in a dark bottle, at 4°C.

12. PBS: 2.7 mM potassium chloride, 1.4 mM potassium dihydrogen phosphate, 137 mM sodium chloride and 4.3 mM disodium phosphate made in double-distilled water and sterilized by autoclaving.

2.1.7 Sterilization

All glassware and plasticware was sterilized by autoclaving at a steam pressure of 15 psi at 120°C for 20 min. Solutions were prepared in double-distilled water and generally sterilized by autoclaving. Some solutions such as RF1, RF2 and 20% maltose were sterilized by filtering through a sterile 0.45 μm nitrocellulose filter (Millipore). Bacterial growth media were autoclaved at 15 psi at 120°C for 40 min. Cell culture media, antibiotics and trypsin were sterilized by filtration through 0.45μ filters. For experiments with RNA, glassware was sterilized by baking at 180°C for 24 hrs followed by autoclaving, and all solutions were prepared in diethyl pyrocarbonate (DEPC)-treated autoclaved water.

2.2 Methods

2.2.1 Isolation of nucleic acids

2.2.1.1 Plasmid DNA

Three different procedures were employed for the isolation of plasmid DNA depending on the quantity and purity of DNA that was required.

(i) Small scale isolation of plasmid DNA

Small scale preparation of plasmid DNA was made by the alkaline lysis method (Birnboim and Doly, 1979) as described by Sambrook et al. (1989). A volume of 5 ml of LB medium containing 100 μg/ml ampicillin was inoculated with a single colony of E. coli DH5-α bearing the plasmid and incubated overnight at 37°C with shaking at 200 rpm. Bacterial pellet from 3 ml of the overnight culture was resuspended in 100 μl of ice-cold solution I (50 mM glucose, 25 mM Tris.HCl pH 8.0, 10 mM EDTA pH 8.0) by vortexing. 200 μl of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added and the contents were mixed by inverting the tube several times. This was followed by the addition of 150 μl of ice-cold
solution III (3 M potassium - 5 M acetate) and gentle mixing. The tube was incubated on ice for 5 min and centrifuged at 12,000 X g for 10 min at 4°C. The supernatant was extracted with an equal volume of phenol : chloroform and precipitated with two volumes of 95% ethanol for 20 min at -20°C. The nucleic acids were pelleted by centrifugation at 12,000 X g for 20 min at 4°C, washed with 70% ethanol, vacuum-dried and dissolved in 30 μl of TE pH 8.0 containing 20 μg/ml of DNase-free pancreatic RNase (prepared by boiling RNase for 20 min). The plasmid DNA was then checked on a 0.8% agarose gel (as described in section 2.2.18.1) and stored at -20°C. The typical yield from 3 ml of culture was about 12-16 μg and the DNA was suitable for routine procedures such as restriction digestion, preparation of radiolabelled probe and manual sequencing.

(ii) Medium scale isolation of plasmid DNA

Plasmid DNA was isolated on a medium scale by the alkaline lysis method as described by Sambrook et al. (1989), with a few modifications. A volume of 100 ml of LB containing 100 μg/ml of ampicillin was inoculated with 1 ml of an overnight culture of E. coli DH5-α carrying the plasmid and incubated overnight at 37°C with shaking at 200 rpm. The cells were harvested by centrifugation at 3,000 X g for 15 min at 4°C in a Sorvall SS34 rotor. The pellet was resuspended in 7.5 ml of ice-cold solution I (50 mM glucose, 25 mM Tris.HCl pH 8.0). 15 ml of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added, and the contents were mixed and stored at room temperature for 5 min. This was followed by the addition of 10 ml of ice-cold solution III (3 M potassium-5 M acetate) and mixing by gentle inversion. The tubes were chilled on ice for 15 min, and the bacterial debris and genomic DNA was pelleted by centrifugation at 12,000 X g for 30 min at 4°C. The plasmid DNA in the supernatant was precipitated with 0.6 volumes of isopropanol for 20 min at room temperature. The nucleic acids were pelleted at 12,000 X g for 30 min at room temperature. The pellet was air-dried and dissolved in 500 μl of TE pH 8.0.

The plasmid DNA was further purified by precipitation with polyethylene glycol (PEG). A volume of 500 μl of 5 M lithium chloride was added to 500 μl of the nucleic acid solution, mixed well and centrifuged at 12,000 X g for 20 min at 4°C, to pellet the RNA. The DNA in the supernatant was precipitated with an equal volume of isopropanol and pelleted at 12,000 X g for 20 min at room temperature. The pellet was washed with 70% ethanol, vacuum-dried and dissolved in 500 μl of TE pH 8.0 containing 20 μg/ml of DNase-
free pancreatic RNase. The DNA was incubated at room temperature for 30 min, after which 500 μl of 1.6 M NaCl containing 13% (w/v) PEG 8000 was added, mixed and the tube was kept on ice for 1 hr. The DNA was recovered by centrifugation at 12,000 X g for 15 min at 4°C. The resulting pellet was dissolved in 400 μl of TE pH 8.0 and extracted once with phenol, once with phenol: chloroform and once with chloroform. The plasmid DNA in the aqueous phase was precipitated with 100 μl of 7.5 M ammonium acetate and two volumes of ethanol for 20 min at room temperature and recovered by centrifugation. The pellet was washed with 70% ethanol, air-dried and dissolved in 200 μl of TE pH 7.6. The plasmid DNA preparation was checked on an agarose gel, quantitated by measuring the OD_{260} and stored at -20°C. The typical yield from 100 ml of culture was about 400 μg and the DNA was suitable for transient transfection into cultured mammalian cells and preparation of DNA footprinting probe.

(iii) Preparation of plasmid DNA using the Qiagen column

A small scale preparation of plasmid DNA was made by the alkaline lysis method and purified on a Qiagen column. Bacterial pellet from 3 ml of culture was resuspended in 0.3 ml of solution P1 (50 mM glucose, 25 mM Tris.HCl pH 8.0), 100 μg/ml DNase-free pancreatic RNase) and 0.3 ml of solution P2 (0.2 M NaOH, 1% SDS) was added and mixed. Then 0.3 ml of chilled solution P3 (3 M potassium-5 M acetate) was added and the contents were mixed again by gentle inversion and chilled on ice for 10 min. The tube was centrifuged at 12,000 X g for 10 min at 4°C and the resulting supernatant containing the plasmid DNA was purified on a Qiagen tip-20 column as described below. The Qiagen tip-20 was equilibrated by applying 1 ml of QBT buffer (750 mM NaCl, 50 mM MOPS, pH 7.0, 15% ethanol, 0.15% Triton X-100). Then the supernatant containing the plasmid DNA was applied to the column and the protein and RNA contaminants were washed off with 4 ml of QC buffer (1 M NaCl, 50 mM MOPS, pH 7.0, 15% ethanol). The bound plasmid DNA was eluted from the column with 0.8 ml of QF buffer (1.25 M NaCl, 50 mM Tris.HCl, pH 8.5, 15% ethanol), precipitated with 0.7 volumes of isopropanol for 15 min at room temperature and pelleted at 12,000 X g for 30 min in a microfuge. The pellet was washed with 70% ethanol, air-dried, dissolved in 30 μl of TE, pH 8.0, checked on a 0.8% agarose gel and stored at -20°C. The typical yield from 3 ml of culture was about 8-12 μg and the DNA was suitable for automated DNA sequencing.
2 Isolation of bacteriophage DNA

Bacteriophage DNA was prepared by a modified version of the plate lysate method described in Sambrook et al. (1989).

Plating of lambda bacteriophage

About $10^4$-$10^5$ plaque forming units (pfu) of the recombinant lambda clone were infected at $37°C$ for 20 min with 100 μl of an overnight culture of E. coli LE 392 grown in LB medium containing 0.2% maltose and 10 mM MgSO₄. The infected bacteria were mixed with melted top agar and spread over a 90 mm plate. After the agar had set, the plate was incubated overnight at $37°C$.

Extraction of bacteriophage DNA

A volume of 5 ml of SM buffer (0.1 M NaCl, 50 mM Tris.HCl, pH 7.5, 0.01% gelatin, MgSO₄) was added to each plate and the plates were rocked gently for 2 hrs on a warm shaker, to allow elution of the bacteriophage into the SM solution. Bacterial debris was eliminated by the addition of 2-3 drops of chloroform and centrifugation at 3,000 X g for 30 min at room temperature. The bacteriophage in the supernatant was precipitated by the addition of 0.5 g of PEG 8000 and 0.3 g of NaCl, and incubation on ice for 1 hr and pelleted by centrifugation at 12,000 X g for 30 min at $4°C$. The supernatant was drained and the pellet suspended in 750 μl of LB medium. An equal volume of DE52 slurry equilibrated in water (prepared as described below) was added and the contents was mixed thoroughly by shaking the tube several times. The sample was centrifuged at 12,000 X g for 5 min at room temperature and the supernatant was collected. This step was repeated once more. Then 17.5 proteinase K (0.1 mg/ml) and 42.5 μl of 10% SDS was added to the supernatant and the mixture was incubated at room temperature for 5 min. This was followed by the addition of 3 μl of 3 M potassium acetate and incubation at $88°C$ for 20 min. The sample was cooled on ice for 10 min and the debris was removed by centrifugation at 12,000 X g for 20 min at $4°C$. The bacteriophage DNA was precipitated at -70°C for 10 min after addition of equal volume of isopropanol to the supernatant. The DNA pellet was recovered by centrifugation, washed with 70% ethanol, dried and dissolved in 50 μl of TE pH 8.0. The DNA preparation was checked on a 0.7% agarose gel and stored at -20°C.

Preparation of DEAE cellulose (Whatman DE52)

To 2.5 g of DEAE cellulose DE52 resin, 100 ml of 0.05 M HCl was added and the mixture was shaken till the pH equilibrated to approximately pH 4.5. Then a solution of 5 M
NaOH was added with constant gentle stirring till the pH of the supernatant reached pH 7.5. The supernatant was decanted and the resin was washed several times with LB medium pH 7.5. It was finally suspended in one-fourth the volume of LB medium to give a slurry containing 75% resin and 25% LB.

2.2.1.3 Isolation of total RNA

Total RNA was isolated from F111 fibroblasts by the method described by Chomczynski, and Sacchi (1987). About $5 \times 10^6$ cells (grown as described in section 2.2.17.1) were scraped into ice-cold PBS, washed once and suspended in 0.5 ml of solution D (4 M guanidium-thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M β-mercaptoethanol). This was followed by the sequential addition of 50 μl of 2 M sodium acetate pH 4.0, 500 μl of water-saturated phenol and 100 μl of chloroform with thorough mixing between each addition. The tubes were placed on ice for 15 min and centrifuged at 12,000 X g for 20 min at 4°C. The aqueous layer containing RNA was precipitated with 500 μl of isopropanol at -70°C for 1 hr and RNA was pelleted by centrifugation. The pellet was dissolved in 300 μl of solution D and precipitated again with 0.3 ml of isopropanol for 1 hr at -20°C. Finally, RNA was recovered by centrifugation, and stored at -70°C in 95% ethanol. One aliquot of the sample was dissolved in DEPC-treated water and its integrity was checked on a 1% agarose gel.

2.2.2 Digestion of DNA with restriction endonuclease

An aliquot of 500 ng to 2 μg of DNA was digested with 5 units of restriction endonuclease per μg of DNA in a final volume of 10 to 20 μl. The reaction was carried out for 3 hrs using suitable buffers and assay conditions specified by the manufacturers. The enzyme was heat-inactivated by heating the digested samples at 65°C for 10 min. The digested DNA fragments were analyzed by agarose gel electrophoresis.

2.2.3 Ligation of DNA fragments

For ligation of plasmid vector to insert DNA, 50-100 ng of digested vector DNA was mixed with a 3-fold molar excess of the insert DNA in 10 μl of buffer containing 50 mM Tris.HCl pH 7.5, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP and 25 mg/ml BSA. 200 units of T4 DNA ligase was added and the ligation reaction was carried out at 16°C for 16 hrs. In case
of blunt-end ligation, PEG 8000 was added to a final concentration of 15%. The ligated DNA was directly used for transformation or stored at -20°C.

2.2.4  **Transformation of E. coli**

2.2.4.1  **Preparation of competent cells**

Depending on the efficiency of transformation required for various cloning procedures, competent cells were made by three different methods.

(i)  **Preparation of competent E. coli DH5-α cells using rubidium chloride**

Competent cells were made according to the method described by Hanahan (1985) with a few modifications. A single colony of *E. coli* DH5-α, maintained on a fresh LB agar plate was inoculated into 5 ml of LB medium and incubated at 37°C with shaking at 200 rpm for 16 hrs. One ml of this overnight culture was inoculated into 35 ml of LB medium and incubated at 37°C at 200 rpm till the OD$_{600}$ reached 0.55. The culture was then chilled on ice for 15 min and the cells were pelleted at 1,500 X g for 15 min at 4°C in an HB-4 rotor in a Sorvall centrifuge. The supernatant was completely drained and the pellet was gently resuspended in 10 ml of ice-cold RF1 buffer (100 mM RbCl, 50 mM MnCl$_2$.4H$_2$O, 30 mM potassium acetate, 10 mM CaCl$_2$.2H$_2$O, 15% glycerol) and incubated on ice for 20 min. The cells were then distributed into prechilled microfuge tubes in 100 μl aliquots, flash frozen in liquid nitrogen and stored at -70°C. The competent cells prepared by this method generally yielded a transformation efficiency of 5 x 10$^6$ to 1 x 10$^7$ colonies/μg of pUC18 DNA and were stable upto three months. They were used for most of the routine cloning experiments involving ligations of compatible, cohesive termini.

(ii)  **Preparation of ultracompetent cells**

Ultracompetent cells were prepared according to the method described by Inoue *et al.*, (1990) . A single colony of *E. coli* DH5-α maintained on a fresh LB agar plate was inoculated into 5 ml of LB and incubated at 37°C at 200 rpm for 16 hrs. One ml of this overnight culture was inoculated into 100 ml of LB medium and incubated at 18°C at 200 rpm, till the OD$_{600}$ reached 0.6. The culture was chilled on ice and centrifuged at 1,500 X g for 15 min at 4°C to pellet the cells. The cells were then resuspended in 32 ml of ice cold buffer I (10 mM PIPES pH 6.7, 15 mM CaCl$_2$, 250 mM KCl, 55 mM MnCl$_2$) and incubated
on ice for 10 min. The centrifugation was repeated and the cells were resuspended in 8 ml of ice cold buffer I containing 7% DMSO, distributed into 100 μl aliquots, flash frozen in liquid nitrogen and stored at -70°C. Ultracompetent cells prepared by this method yielded a transformation efficiency of 1 x 10^8 colonies/μg of pUC18 DNA and were stable for three months at -70°C. Ultracompetent cells were used for cloning experiments involving ligation of DNA fragments with blunt-ended termini.

(iii) Preparation of competent cells for electroporation

Competent *E. coli* DH5-α to be used for electroporation were prepared according to the method described by the manufacturer of the electroporator, ECM600, (BTX Company, USA). A single colony of *E. coli* DH5-α maintained on a fresh LB agar plate was inoculated into 10 ml of LB medium and incubated overnight at 37°C at 200 rpm. This culture was then inoculated into 1000 ml of LB medium and incubated at 37°C at 200 rpm till it attained an OD₆₀₀ of 0.5 to 1.0. The cells were then chilled on ice, pelleted by centrifugation at 1,500 X g at 4°C for 15 min and washed once with 1000 ml, twice with 500 ml and once with 20 ml of chilled sterile double-distilled water. Finally, the cells were resuspended in 2 ml of ice cold 10% glycerol, distributed into 50 μl aliquots, flash frozen in liquid nitrogen and stored at -70°C. These cells were used for electroporation in experiments where a transformation efficiency of > 10^8 cells/μg of DNA was required.

2.2.4.2 Transformation of competent and ultracompetent *E. coli* cells by heat shock

Competent cells were removed from the -70°C freezer and thawed on ice. Ligated DNA sample (3 μl) was added to the competent cells and mixed gently. The cells were incubated on ice for 30 min, following which they were subjected to heat shock at 42°C for 90 sec. After the heat shock, 400 μl of LB was added to the cells and the tube was incubated at 37°C for 1 hr. The cells were then plated on 90 mm LB agar plates containing 100 μg/ml of ampicillin. In cases where the plasmid vector displayed the property of α-complementation, the cells were plated on LB agar plates containing 100 μg/ml ampicillin and coated with 40 μl of 20 mg/ml X-Gal and 4 μl of 200 mg/ml IPTG. The plates were incubated at 37°C for 14-16 hrs to enable the growth of transformants.
2.2.4.3 Electroporation of *E. coli*

Electroporation was carried out in an electrocell manipulator, ECM600, according to the procedure recommended by the manufacturer. A 50 µl aliquot of frozen competent *E. coli* DH5-α cells, prepared as described in section 2.2.4.1, was thawed on ice and mixed gently with 1 µl of the ligation mixture (~10 ng of DNA). The cells were transferred to a disposable pre-chilled cuvette and a pulse of current was applied for 5-6 msec, at a strength of 12.25 kv/cm, at electroporation settings specified by the manufacturer. Immediately after electroporation, 950 µl of LB medium was added, mixed, and the cells were incubated at 37°C for 1 hr at 200 rpm. Appropriate dilutions of the transformed cells were plated on LB agar plates containing 100 µg/ml ampicillin, and incubated at 37°C for 14 to 16 hrs to allow transformants to grow.

2.2.5 Preparation of radiolabelled DNA probes

2.2.5.1 Random primer labelling of DNA

Double-stranded DNA was radiolabelled using random primers as described by Feinberg and Vogelstein (1983) using a multiprime labelling kit. About 50-100 ng of double-stranded DNA was denatured in a volume of 20 µl by boiling for 5 min and quick chilling on ice. This was followed by the sequential addition of 5 µl of random primers solution, 5 µl of 10X reaction buffer, 5 µl each of dCTP, dGTP, dTTP, 40 µCi of α-[32P]-dATP and 2 units of Klenow enzyme. The reaction volume was made upto 50 µl and the reaction was carried out at 37°C for 2 hrs. The enzyme was inactivated at 65°C for 10 min and the probe was separated from the unincorporated nucleotides by Sephadex G-50 spun column chromatography as described in section 2.2.18.5, denatured in boiling water for 5 min and rapidly chilled on ice.

2.2.5.2 End-labelling DNA with T4 polynucleotide kinase

Single-stranded oligos used for Southern hybridization and primer extension as well as double-stranded oligos used in EMSAs were end-labelled using T4 polynucleotide kinase according to the following procedure. About 1 to 3 pmoles of the oligonucleotide was labelled with 5 units of T4 polynucleotide kinase and 10 µCi of γ[32P]-ATP in 10 µl of buffer containing 50 mM Tris.HCl pH 7.5, 10 mM MgCl₂, 5 mM DTT and 0.1 mM spermidine. The sample was incubated at 37°C for 15 min and then heated at 90°C for 2 min.
to stop the reaction. The sample was made upto 100 µl with TE pH 8.0, and the unincorporated radionucleotides were separated out by Sephadex G-25 spun column chromatography.

2.2.5.3 End-filling recessed 3' DNA ends

Double-stranded DNA having recessed 3' ends generated by restriction digestion were filled in using Klenow enzyme as described by Sambrook et al. (1989). About 2 µg of double-stranded DNA was labelled with 2.5 units of Klenow, 80 µM each of dGTP and dTTP, and 50 µCi each of α-[32P]-dATP and α-[32P]-dCTP in 10 µl of buffer containing 10 mM Tris.HCl pH 7.5, 5 mM MgCl₂ and 7.5 mM DTT at 37°C for 30 min. Labelling was enhanced by the addition of a second aliquot of Klenow and further incubation for 30 min. This labelling step was followed by a 15 min chase with Klenow and all four unlabelled dNTPs to counteract the 3' to 5' exonuclease activity of Klenow and generate completely blunt-ended fragments of homogeneous size. The reaction was stopped by inactivating the enzyme at 75°C for 10 min, the volume was made upto 100 µl with TE pH 8.0 and the probe was purified on a Sephadex G-50 column.

2.2.6 Selection of recombinant clones by colony hybridization

When recombinants could not be selected by blue-white colony selection due to lack of α-complementation by the plasmid vector, colony hybridization was carried out to identify the positive clones. The recombinant colonies were replica plated on LB agar plates containing 100 µg/ml ampicillin and incubated overnight at 37°C. They were then transferred to Hybond-N nylon membranes according to the method of Buluwela et al. (1989). The nylon membrane was laid over the plate and left for 2 min to allow the transfer of colonies. The orientation was marked by piercing the filter and the underlying agar with a needle dipped in Indian ink at asymmetric positions. The filter was then peeled off and laid on a Whatman 3 MM filter soaked in 2X SSPE (0.3 M NaCl, 20 mM sodium phosphate pH 7.4, 2 mM EDTA) and 5% SDS for 2 min, following which it was heated at 650 W for 2.5 min in a microwave oven, to denature and fix the bacterial DNA to the membrane. The filters were wetted in 5X SSPE and 0.1% SDS for 5 min and prehybridization was carried out at 65°C for 2 hrs in 3 ml/90 mm filter of 0.5 M sodium phosphate and 7% SDS. The filters were hybridized at 65°C for 16 hrs by the addition of denatured radiolabelled probe (prepared by random primer labelling
as described in section 2.2.5.1). The filters were washed in 100 ml of 2X SSC, 0.5% SDS for 20 min at room temperature, in 1X SSC, 0.5% SDS for 20 min at 65°C and 0.5X SSC, 0.5% SDS for 20 min at 65°C. The washed filters were wrapped in saran wrap and exposed to X-ray film in cassettes with intensifying screens for 16 hrs at -70°C. The autoradiograms were developed and positive colonies were selected by aligning the signals on the autoradiogram to the filters and colonies on the plate.

2.2.7 Southern hybridization

The DNA samples to be hybridized were digested with appropriate restriction enzymes and resolved on 0.8% to 1% agarose gels alongside DNA markers, as described in section 2.2.3. The gel was stained with ethidium bromide, photographed and the DNA was transferred to Hybond N+ membrane by vacuum blotting in a TE 80 Transvac vacuum blotter (Hoefer Scientific Instruments, USA) for 1.5 hrs in 400 mM NaOH. The blot was then rinsed in 6X SSC, air-dried and subjected to prehybridization, hybridization, and washing as described in section 2.2.18.1. The hybridized blot was exposed to X-ray film at -70°C for 1 to 12 hrs.

2.2.8 Screening of genomic library

The rat genomic library was screened according to the procedure of Benton and Davis (1997) as described by Sambrook et al. (1989).

2.2.8.1 Plating of bacteriophage

After determining the titre of the library, 100 μl of a mid-log phase culture of E. coli LE392 grown in LB medium containing 0.2% maltose and 10 mM MgSO4 was infected with 5 x 10⁴ pfus of λ DASH bacteriophage at 37°C for 20 min. The infected bacteria were mixed with 6 ml of melted top agar and spread on 150 mm LB plates. The plates were incubated for 8 to 10 hrs at 37°C till the edges of the plaques started touching each other, and then chilled for 2 hrs at 4°C.

2.2.8.2 Transfer of plaques

Each plate was overlaid with a nitrocellulose or nylon filter and left for 2-3 min to allow adsorption of the phage to the filter and the orientation of the filter was marked. The filters were peeled off from the plate and laid face up on a Whatman 3 MM filter paper
soaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 min. The filters were then neutralized by transferring them to a Whatman 3 MM filter soaked in 2X SSC for 5 min. The filters were air-dried for 30 min, placed between two Whatman 3 MM papers and baked for 2 hrs at 80°C in a vacuum oven.

2.2.8.3 Plaque hybridization

The filters were washed in 200 ml of prewashing solution (50 mM Tris.HCl pH 8.0, 1M NaCl, 1 mM EDTA and 0.1% SDS) for 30 min at 65°C and prehybridized in 6 ml/150 mm filter of prehybridization solution (50X Denhardt's solution, 5X SSC, 1% SDS, and 100 µg/ml of salmon sperm DNA). Hybridization was carried out at 65°C for 16 hrs after the addition of denatured probe (prepared by random primer labelling) of high specific activity (5 X 10^8 to 1 X 10^9 cpm/µg) at a concentration of 5 X 10^6 to 1 X 10^7 cpm/ml of hybridization solution (5X Denhardt's solution, 5X SSC, 1% SDS, 10 mM EDTA). After hybridization, the filters were washed as described in section 2.2.6. The washed filters were exposed to X-ray film for 48 hrs at -70°C. The positive plaques were picked and eluted into 200 µl of SM containing 10 µl of chloroform.

2.2.8.4 Purification of positive plaques

The mixture of plaques picked after primary screening was analysed by additional rounds of screening by replating at lower densities of 500-2000 pfus/90 mm plate for secondary screening and 50-200 pfus/90 mm plate for tertiary screening. Finally, single plaques were picked and suspended in 0.2 ml of SM with 10 µl of chloroform and stored at 4°C. Purity of the plaque was confirmed by an additional round of hybridization.

2.2.9 Exonuclease III mapping

2.2.9.1 Generation of nested deletions with exonuclease III

Nested deletions of the 2 kb Sac I DNA fragment were generated using the Erase-a-Base kit. Approximately 8 µg of supercoiled plasmid DNA carrying the insert to be deleted was digested successively with two enzymes, one that generated a 4 bp 3’ overhang protecting the vector sequence and the other which generated a 5’ overhang adjacent to the insert. An aliquot of the digested DNA was checked on a 0.8% agarose gel at each step, to confirm that digestion was complete. After digestion, the DNA was extracted once with phenol :
chloroform, precipitated with ethanol and centrifuged. The pellet was dried and resuspended in 60 μl of exonuclease buffer containing 6.6 mM Tris.HCl pH 8.0 and 0.66 mM MgCl₂. The tube was warmed to 30°C and 300 units of exonuclease III was added and mixed. After 20 sec, 2.5 μl aliquots were taken out at 30 sec intervals (exonuclease III digestion at the rate of 200 bp/min at 30°C), mixed with 7.5 μl of S1 nuclease mix [172 μl of double-distilled water + 27 μl of S1 nuclease 7.4X buffer (300 mM potassium acetate pH 4.6, 2.5 M NaCl, 10 mM ZnSO₄, 50% glycerol) + 60 units of S1 nuclease] and incubated at 37°C for 30 min. The reaction in each tube was stopped by the addition of 1 μl of S1 nuclease stop buffer (0.3 M Tris base, 50 mM EDTA) and heating to 70°C for 10 min. The extent of digestion was checked by analysing 2 μl aliquots of DNA from each time point on a 1% agarose gel. Meanwhile, 1 μl of Klenow mix (5 units of Klenow enzyme + 30 μl of Klenow buffer) was added and incubated at 37°C for 3 min. This was followed by the addition of 1 μl of dNTP mix and further incubation at 37°C for 5 min, after which the Klenow was inactivated at 65°C for 10 min. The samples were ligated for 1 hr at room temperature by the addition of 40 μl of ligase mix (790 μl of double-distilled water + 100 μl of ligase 10X buffer + 100 μl of 50% PEG + 10 μl of 100 mM DTT + 5 units of T4 DNA ligase). 10 μl of ligated product from each time point was used to transform E. coli DH5-α cells and the transformants were plated on LB plates containing 100 μg/ml ampicillin and incubated at 37°C for 12-14 hrs. Five or six transformants from each time point were analysed by the rapid screening procedure described below.

2.2.9.2 Rapid screening of exonuclease III deletion clones

Isolated colonies from each deletion time point were streaked on fresh LB plates and grown at 37°C for 14 hrs. Each bacterial streak was scraped and resuspended in 40 μl of cracking solution (10 mM Tris.HCl pH 8.0, 100 mM NaCl, 10 mM EDTA pH 8.0) by vortexing. The nucleic acids were extracted with an equal volume of phenol : chloroform and the aqueous layer was treated with 1 μl of 1 mg/ml RNase A at room temperature for 10 min. 10 μl of DNA loading dye was added and 20 μl of sample was checked on a 1% agarose gel with appropriate supercoiled DNA markers. The suitable clones, differing from each other by 100 to 200 bp were selected for further analysis.
2.2.10 DNA sequence analysis

2.2.10.1 DNA sequencing by dideoxy ribonucleotide method

DNA sequencing by the dideoxyribonucleotide termination method of Sanger et al., (1977) was carried out using the Taq track sequencing kit, as described below.

(i) Labelling primer with T4 polynucleotide kinase:

Ten pmoles of primer was labelled with 10 pmoles of $\gamma^{32P}$-ATP and 5 units of T4 polynucleotide kinase in 10 µl of buffer containing 50 mM Tris.HCl pH 7.5, 10 mM MgCl$_2$, 5 mM DTT and 0.1 mM spermidine. The reaction was incubated at 37°C for 15 min and stopped by inactivating the enzyme at 90°C for 2 min.

(ii) Denaturation of template DNA:

About 4 µg (2 pmoles) of plasmid DNA was made upto 18 µl with water and denatured by the addition of 2 µl of 2 M NaOH, 2 mM EDTA for 5 min at room temperature. The reaction was neutralised with 2 µl of 2 M ammonium acetate pH 4.6 and precipitated with 75 µl of ethanol for 30 min at -70°C. The denatured DNA was pelleted at 12,000 X g for 20 min at 4°C. The pellet was washed with 200 µl of 70% ethanol, dried and resuspended in 18 µl of double-distilled water.

(iii) Annealing of template and primer:

Two µl (2 pmoles) of primer was annealed with 18 µl (4 µg) of the denatured template DNA in 25 µl of buffer containing 50 mM Tris.HCl pH 9.0 and 10 mM MgCl$_2$ at 37°C for 20 min.

(iv) Extension/termination reaction:

In each set of sequencing reactions 1 µl each of deaza ddNTP mix G, A, T and C was aliquoted into four separate tubes on ice. Five units of sequencing grade Taq DNA polymerase was added to the annealed primer-template solution and mixed. A 6 µl aliquot of this reaction mix was added to each of the tubes containing A, C, G and T ddNTP mixes. The contents were mixed and extension was carried out at -70°C for 20 min. The reaction was stopped by the addition of 4 µl of stop solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol).

(v) Electrophoresis of sequencing reactions:

The sequencing reactions were denatured at 75°C for 5 min and 2 µl of each reaction was loaded on an 8% denaturing urea-polyacrylamide gel of 0.35 mm thickness which had been prerun for 45 min. Electrophoresis was carried out at a constant power of 70 W in 1X
TBE buffer. A second loading of samples was done after 3 hrs and they were electrophoresed for an additional 90 min. After electrophoresis, the gel was fixed in 10% methanol, 10% acetic acid solution for 45 min, dried and autoradiographed overnight at -70°C. The autoradiograms were developed and sequences were read manually.

2.2.10.2 Automated DNA sequencing

Automated DNA sequencing was carried out with the Dye primer cycle sequencing ready reaction kit from Perkin Elmer, USA, on a model 377 version 2.1.1 automated sequencer (Applied Biosystem, USA).

Plasmid DNA template of high purity (purified on a Qiagen column) was used for automated sequencing. Four µl each of A and C reaction mix and 8 µl each of G and T ready reaction mix was aliquoted into two 0.2 ml microfuge tubes. This was followed by the addition of 1 µl (250 ng) each of plasmid template DNA to the tubes containing A and C mix and 2 µl (500 ng) each of DNA to the tubes containing G and T mix. The contents were mixed and overlaid with 20 µl of light mineral oil. The PCR based sequencing reaction was carried out in a Gene Amp PCR System model 9600 thermal cycler under the following conditions:

Step 1: Rapid thermal ramp to 96°C, 96°C for 10 sec, rapid thermal ramp to 55°C, 55°C for 5 sec, rapid thermal ramp to 70°C, 70°C for 1 min. This step was repeated for 15 cycles.

Step 2: Thermal cycling after step 1 was continued by rapid thermal ramp to 96°C, 96°C for 10 sec, rapid thermal ramp to 70°C and 70°C for 1 min. Step 2 was repeated for a total of 15 cycles followed by rapid thermal ramp to 4°C.

2.2.10.3 Computer analysis of DNA sequence data

The DNA sequence data was analysed using the EMBL and TRANSFAC databases and the software PCGENE release 6.70 using the following programmes:

(i) SEQUIN: for entry and editing of DNA sequence

(ii) NMANIP: to manipulate the DNA sequence using the INVERSE and COMPLEMENT programmes.

(iii) RESTRI: to carry out a restriction analysis of the sequence

(iv) SECONDARY STRUCTURE ANALYSIS: to perform a secondary structure analysis of oligonucleotide primers using the HAIRPIN programme
FASTA: to search the EMBL database in order to identify homologous sequences

TFSEARCH: to analyse the sequence for the presence of putative transcription factor binding sites

2.11 Polymerase chain reaction

DNA was amplified by PCR according to the method described by Saiki et al. (1987). About 10 ng of template DNA was mixed with 250 ng of each of the two primers, 0.2 mM NTPs and 2 units of deep vent polymerase (which generates a blunt-ended PCR product) in 0 µl of reaction buffer containing 10 mM KCl, 10 mM Tris.HCl pH 8.8, 10 mM ammonium sulfate, 2 mM MgSO₄, and 0.1% Triton X-100. The reaction mixture was overlaid with 50 l of mineral oil (saturated with sterile water) and heated to 95°C for 2 min to denature the DNA. The samples were subjected to 35 cycles of PCR amplification under the following conditions: annealing at 68°C for 30 sec, extension at 72°C for 30 sec and denaturation at 4°C for 30 sec. At the end of the last cycle, an additional extension was carried out at 72°C or 5 min. At the end of the PCR reaction, the products were analysed by checking a 5 µl aliquot on a 1.5% agarose gel. For cloning of PCR products, the following strategy was employed. The PCR products generated by using the deep vent polymerase generally have 95% blunt ends. Therefore, they were cloned into Sma I-digested, dephosphorylated plasmid vector. One µl of the ligated product was used to transform competent E. coli DH5-α by electroporation.

2.12 Preparation of nuclear extracts

2.12.1 Isolation of nuclei from rat liver

Rat liver nuclei were purified as described by Kaufmann et al. (1983). The liver was dissected, rinsed in ice-cold STM solution (0.25 M sucrose, 50 mM Tris.HCl pH 7.4, 5 mM MgCl₂, 1 mM EGTA pH 7.4, and 0.5 mM PMSF), weighed and homogenised in eight volumes of ice-cold homogenising buffer (0.25 M sucrose, 50 mM Tris.HCl pH 7.4, 5 mM MgCl₂, 25 mM KCl, 0.5 mM PMSF and 2 mM DTT). The homogenate was filtered through 8 layers of cheese cloth and centrifuged at 800 X g for 10 min at 4°C in a Sorvall SS-34 rotor. The pellet was washed with STM solution and centrifuged again as above. The crude nuclear pellet was suspended in 5 ml (per 4 g of tissue) of dense sucrose solution (2.1 M sucrose, 50 mM Tris.HCl pH 7.4, 1 mM EGTA pH 7.4, 5 mM MgCl₂ and 0.5 mM PMSF)
layered over a cushion of 6 ml of dense sucrose in an ultracentrifuge tube and centrifuged in an SW41 swing out rotor at 70,000 X g for 1 hr at 4°C in a Beckmann ultracentrifuge (model L8-80M). After centrifugation, the top layers were discarded, and the nuclear pellet was suspended in 5 ml of STM and centrifuged at 1,000 X g for 10 min at 4°C in a Sorvall SS-34 rotor. The pellet was washed twice with STM as described above and the washed nuclei were suspended in 1 ml of STM and checked for purity and integrity under a phase contrast microscope. The total number of nuclei was estimated by making an appropriate dilution and counting on a hemocytometer.

2.2.12.2 Preparation of nuclear extract from rat liver nuclei

Nuclear extract was prepared from purified rat liver nuclei by a modified version of the method described by Dignam et al. (1983). The nuclei were pelleted, washed once with low salt buffer (20 mM HEPES pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 0.02 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT and 10 μg/ml each of leupeptin, pepstatin and aprotinin) and resuspended in 332 μl (per 2 X 10⁸ nuclei) of the same low salt buffer. To this nuclear suspension, 166 μl of high salt buffer (20 mM HEPES pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT and 10 μg/ml each of leupeptin, pepstatin and aprotinin) was added dropwise, with gentle mixing to give a final salt concentration of 0.4 M KCl. Extraction was allowed to proceed for 30 min on ice with occasional mixing of the contents. The extract was then centrifuged at 12,000 X g for 20 min at 4°C to pellet the membrane fraction. The supernatant was dialysed against 200-fold excess of dialysis buffer (20 mM HEPES pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT and 1 μg/ml each of leupeptin, pepstatin and aprotinin) at 4°C for 1 hr, distributed in 20 μl aliquots after the addition of leupeptin, pepstatin and aprotinin to final concentrations of 10 μg/ml, and frozen at -70°C.

2.2.12.3 Preparation of nuclear extract from cultured cells

Nuclear extracts from cultured mammalian cell lines were prepared according to the procedure described by Andrews (1991). Cell lines were maintained as described in section 2.2.17.1. Approximately 1 to 5 X 10⁶ cells were harvested by scraping the culture with a rubber scraper, washed once with PBS and suspended in 400 μl of hypotonic buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF and
The cells were allowed to swell on ice for 30 min, vortexed for 2 min and passed rapidly through a 21 gauge needle 10-15 times to lyse the cells. The nuclear pellet was collected by centrifugation at 12,000 X g for 20 min at 4°C and resuspended in 100 μl of cold high salt buffer C (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF and 10 μg/ml of pepstatin, leupeptin and aprotinin). The tube was incubated on ice for 20 min. Cellular debris was removed by centrifuging as above and the supernatant was stored at -70°C in 25 μl aliquots.

2.2.13 Electrophoretic mobility shift assay

EMSAs were carried out according to the procedure of Chodosh et al. (1986) as described in Ausubel et al., (1989), with a few modifications. The sequences of the oligonucleotides used are given in Table 1. The labelled oligonucleotide probe (prepared as described in section 2.2.5.2) was mixed with 5 μg of nuclear extract (made as described in section 2.2.12) in 20 μl of gel shift binding buffer containing 4% glycerol, 1 mM MgCl₂, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris.HCl pH 7.5 and 1 μg of poly(dI.dC).poly(dI.dC). The sample was incubated at room temperature for 30 min to facilitate binding of protein to the DNA probe. The reaction products were resolved on a 6% native polyacrylamide gel, cast and run in 0.5X TBE at 150 V for 3 hrs at 4°C. The gel was dried and subjected to autoradiography. The autoradiogram was developed after overnight exposure at -70°C. In competition experiments 50 to 100-fold molar excess of cold competitor DNA was preincubated with the nuclear extract for 5 min before addition of the labelled oligo. In antibody supershift experiments, 2 μg of antibody was added and the reaction mixture was further incubated for 1 hr at 4°C before loading onto a 5% native polyacrylamide gel.

2.2.14 UV crosslinking of protein-DNA complexes

UV crosslinking of protein-DNA complexes to determine the size of the protein bound to the DNA-template was carried out according to the method of Chodosh et al. (1986) as described in Ausubel et al., (1989). An EMSA binding reaction was carried out as described above and the protein-DNA complexes in the tubes were irradiated with UV light at 305 nm from a distance of 5 cm for 30 min using a UV cross linker (model Stratalinker 2400 from Stratagene Company, USA). Four identical samples were pooled together, 4X SDS-PAGE
<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>+109/+80</td>
<td>5'-CTCACCATACCTGCCTCCGCCCCTTTGGGTCTAG-3'</td>
</tr>
<tr>
<td>DS1</td>
<td>+3/+28</td>
<td>5'-GTTCGGCGGAACGCTGCCTCAGCCTC-3'</td>
</tr>
<tr>
<td>DS2</td>
<td>+29/+50</td>
<td>5'-AACACCAGCCAACCCAGATCCC-3'</td>
</tr>
<tr>
<td>DS3</td>
<td>+51/+72</td>
<td>5'-GAGGTGCAGCGCCAGCCAGCCCAGCC-3'</td>
</tr>
<tr>
<td>AP-1 wild-type</td>
<td>-19/+15</td>
<td>5'-CCCAGCTGACTGCACTAGGTCTGCGGGAACG-3'</td>
</tr>
<tr>
<td>AP-1 mutant 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP-1 mutant 2</td>
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<td></td>
</tr>
<tr>
<td>AP-1 consensus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC box wild-type</td>
<td>-114/-81</td>
<td>5'-CTCACCATACCTGCCTCCGCCCCTTTGGGTCTAG-3'</td>
</tr>
<tr>
<td>GC box mutant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sp1 consensus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GT box wild-type</td>
<td>-65/-41</td>
<td>5'-AGCAGTGAGATCCACCGTCTAGGAGGAGGAG-3'</td>
</tr>
<tr>
<td>GT box mutant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCC wild-type</td>
<td>-135/-102</td>
<td>5'-GGGGTTAGAGCTGCTGCTACTGCTTCTCCAGCCTACCTG-3'</td>
</tr>
<tr>
<td>TCC mutant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP2 consensus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oct1 consensus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VK2</td>
<td></td>
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</tr>
</tbody>
</table>
loading dye was added, and boiled for 5 min. The samples were resolved on a 8% SDS-polyacrylamide gel alongside appropriate protein markers. The gel was stained, destained, dried and exposed to X-ray film for 24 to 72 hrs. The autoradiogram was developed and the molecular mass of the crosslinked protein was determined by comparing its size to that of protein markers on a semi-log plot of molecular mass versus distance migrated.

2.2.15 DNase I footprinting assay

DNase I footprinting assays were carried out using a DNase I footprinting kit as described in the Promega technical bulletin with a few modifications as explained below.

2.2.15.1 Preparation of single end-labelled DNA probe

About 15 μg of plasmid DNA carrying the insert to be footprinted, was digested with the appropriate restriction enzyme to release the insert. The 5' overhangs were labelled by end-filling with Klenow enzyme in the presence of α-[32P]-dATP and α-[32P]-dCTP. The end-filled probe was purified by Sephadex G-50 spin column chromatography and digested with a second restriction enzyme to exclude one labelled end. The single end-labelled probe was purified as follows. The labelled DNA was mixed with loading dye and loaded on a 6% native polyacrylamide gel and electrophoresed at 150 V for 2 hrs in 0.5X TBE till the bromophenol blue dye reached the bottom. The gel was subjected to autoradiography to visualise the bands. The autoradiogram was aligned with the gel and the DNA bands were cut out, crushed thoroughly and eluted overnight into 4 ml of 0.1X TE pH 8.0 at 37°C. The eluate was concentrated to a volume of 100 μl by 8-10 rounds of extraction with an equal volume of butanol, purified on a Sephadex G-50 spin column and stored at -20°C.

2.2.15.2 DNase I footprinting reaction

Approximately 20,000 cpm of probe was mixed with 5 to 10 μg of nuclear extract in 40 μl of binding buffer containing 4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris.HCl pH 7.5 and 2 μg of poly.(dl.dC).poly(dI.dC). Binding was allowed to proceed at room temperature for 30 min after which 40 μl of Ca2+/Mg2+ solution (5 mM CaCl2, 10 mM MgCl2) was added and the contents were mixed. After 1 min at room temperature, DNA was digested with 0.25 units of RQ1 DNase (1 unit/μl diluted 5-fold in 10 mM Tris.HCl pH 8.0). The reaction was stopped after exactly 1 min by the
addition of 90 µl of stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS and 100 µg/ml of yeast RNA). The digested DNA was vortexed, extracted once with 200 µl of phenol : chloroform (equilibrated with TE pH 8.0 containing 0.5 M NaCl), and precipitated with ethanol. The pellet was washed twice with 70% ethanol, dried and resuspended in 5 µl of loading dye (0.1 M NaOH : formamide (1:2 v/v), 0.1% xylene cyanol and 0.1% bromophenol blue). A negative control reaction lacking protein was processed in an identical manner. The radioactivity in the above reaction products was determined accurately and samples containing equivalent counts were denatured at 80°C for 5 min, chilled and resolved on an 8% denaturing urea-polyacrylamide gel. A Maxam and Gilbert chemical cleavage sequencing reaction (A+G) of the same probe was carried out as described in the next section and loaded in parallel with the footprinting reactions. The samples were electrophoresed at 70 W for 1.5 hrs till the bromophenol blue reached the bottom. The gel was fixed, dried and autoradiographed at -70°C overnight. The autoradiogram was developed and the protected regions were identified by comparison of the reaction containing protein with the negative control and subsequent alignment with the A+G sequence ladder.

2.2.15.3 Preparation of the A+G ladder by chemical sequencing

The A+G sequencing ladder to be run in parallel with DNase I footprinting reactions was prepared according to Maxam and Gilbert (1977) by a modified version of the procedure described in Sambrook et al. (1989). Approximately 20,000 cpm of the single end-labelled DNA was mixed with 4 µg of sonicated salmon sperm DNA in 14 µl of water. Three µl of freshly dilute 8.8% formic acid was added and the mixture was incubated at 37°C for 7 min and chilled in ice. This was followed by the addition of 150 µl of 1 M piperidine. The reaction was heated at 90°C for 30 min and again chilled in ice. The sample was distributed into two tubes and the DNA was precipitated for 30 min at -70°C with 2.5 volumes of ethanol after the addition of 170 µl of water and 28 µl of 3 M sodium acetate. The DNA was recovered by centrifugation at 12,000 X g for 20 min at 4°C, and dissolved in 90 µl of water. The contents of the two tubes were pooled and precipitated with ethanol once again. The pellets were washed with 70% ethanol, dried, resuspended in 5 µl of loading dye and loaded on an 8% denaturing urea polyacrylamide gel, in parallel with the footprinting reactions.
2.2.16 Primer extension analysis of RNA

Primer extension of RNA transcripts was carried out according to the protocol described by Sambrook et al. (1989). Twenty pmoles of primer (Table 1) was end-labelled with T4 polynucleotide kinase and purified on a Sephadex G-25 column. The specific activity of the labelled primer was determined by Cerenkov counting and $10^5$ cpm of primer was mixed with 30 μg of total RNA extracted from F111 cells (as described in section 2.2.1.3). The mixture was precipitated with ethanol, and the pellet washed with 70% ethanol, dried and resuspended in 30 μl of hybridisation buffer (40 mM PIPES pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% formamide). The nucleic acids were denatured at 85°C for 10 min and transferred to 30°C for 12 hrs, to enable hybridisation of the labelled primer and RNA template. The primer-RNA hybrids were precipitated with ethanol once again, the pellet washed with 70% ethanol, dried and dissolved in 20 μl of reverse transcriptase buffer (10 mM Tris.Cl pH 8.8, 50 mM KCl, 0.1% Triton X-100, 1 mM each of the 4 dNTPs and 20 units of placental RNase inhibitor). Forty units of AMV reverse transcriptase was added and the reaction was carried out at 42°C for 2 hrs. It was stopped by the addition of 1 μl of 0.5 M EDTA pH 8.0 and RNA was digested by treatment with 1 μl of DNase-free pancreatic RNase (5 mg/ml) at 37°C for 30 min. Then 150 μl of TE pH 7.6 containing 0.1 M NaCl was added and the reaction products were extracted with an equal volume of phenol : chloroform, and precipitated with ethanol. The pellet was washed with 70% ethanol and dissolved in 3 μl of formamide loading dye. The extended products were denatured at 95°C for 5 min, chilled rapidly and loaded on an 8% denaturing urea-polyacrylamide gel. A dideoxy ribonucleotide sequencing reaction of a genomic subclone, (spanning the primer binding site and upstream regions) was carried out with the same primer and run in parallel with the extended primer. The samples were electrophoresed till the bromophenol blue reached the bottom, fixed, dried and autoradiographed. The autoradiogram was developed and the transcription start site was identified by aligning the position of the extended product with the sequencing ladder of the lamin A subclone.

2.2.17 DNA transfection into mammalian cells
2.2.17.1 Maintenance of cell lines

HeLa, PCC-4, NIH 3T3 and F111 cell lines used in this study were maintained as monolayer cultures in DMEM supplemented with 10% fetal bovine serum (FBS) and
antibiotics at 37°C in a humidified 5% CO₂ incubator. The cells were subcultured upon attaining 70% confluency. The medium was aspirated and the cells were washed with phosphate-buffered saline (PBS), after which they were trypsinized with 200 to 400 µl of 0.1% trypsin and 0.1% EDTA, centrifuged briefly and suspended in fresh DMEM containing 10% FBS and antibiotics. The suspended cells were re-seeded at a 1:5 dilution and incubated at 37°C in the CO₂ incubator.

2.2.17.2 Transient transfection of DNA

Polycationic lipid-mediated transient transfections for functional analysis of the lamin A promoter were carried out using lipofectamine (Life Technologies, USA). About 0.5 X 10⁶ cells were seeded per 60 mm dish containing 5 ml of DMEM supplemented with 10% FBS and incubated for 24 hrs, till the cells were 60-80% confluent. Two µg of pGL3 luciferase reporter construct and 1 µg of β-galactosidase reporter construct (pCMV.Sport-βgal for HeLa cells and pCH110 for NIH 3T3 and PCC-4 cell lines) was mixed with 300 µl of serum-free DMEM and added to a solution containing 18 µl of lipofectamine in 300 µl of DMEM. The mixture was incubated at room temperature for 45 min to allow the DNA-liposome complexes to form. The cells were washed once with serum-free medium. Then 600 µl of the DNA-lipid complex was added. After 5 hrs of incubation, 3 ml of DMEM containing 20% FBS was added and incubation continued. After 20 hrs the cells were washed with PBS and fresh DMEM containing 10% FBS was added. The transfected cells were grown for an additional 24 hrs and assayed for luciferase and β-galactosidase reporter enzymes as described in the next section.

2.2.17.3 Luciferase assay

Luciferase assay was carried out using the luciferase assay kit. The growth medium from the transfected cells was discarded and the cells were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH₂PO₄, 1.4 mM KH₂PO₄ pH 7.3). A volume of 300 µl of 1X reporter lysis buffer was added and the dishes were incubated for 15 min at room temperature to allow cell lysis to occur. The cells were then scraped into a tube, vortexed and centrifuged at 12,000 X g for 2 min at 4°C to pellet the cell debris. The cell extract was assayed for luciferase activity by rapidly mixing an aliquot (10-20 µl) of the supernatant with the luciferase assay reagent (20 mM tricine, 1.07 mM (MgCO₃)₄Mg(OH)₂. 5 H₂O, 2.67 mM
MgSO₄, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP pH 7.8) in a total volume of 100 μl and immediately measuring the light output in a luminometer (Turner Systems, USA).

2.2.17.4 β-galactosidase assay

The E. coli β-galactosidase gene was used as an internal reference in reporter gene transfection studies to assay promoter function. The assay for β-galactosidase in cell extracts was carried out according to the method described in Sambrook et al. (1989). An aliquot (20 to 50 μl) of the cell lysate was made up to 300 μl with 0.1 M sodium phosphate pH 7.5 and this was followed by the addition of 3 μl of 100 X Mg solution (0.1 M MgCl₂, 4.5 M β-mercaptoethanol) and 66 μl of 1X ONPG solution (4 mg/ml ONPG in 0.1 M sodium phosphate pH 7.5). The contents were mixed and incubated at 37°C for 15 min. The reactions were stopped by the addition of 500 μl of 1 M Na₂CO₃ and the OD₄₂₀ nm values of the samples were read.

2.2.18 General methods

2.2.18.1 Agarose gel electrophoresis

The DNA samples were suspended in water containing one-sixth the volume of 6X DNA loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol). Depending upon the size of the fragments to be resolved, the samples were loaded on 0.7% to 1.5% agarose gels cast in 0.5X TBE containing 0.5 μg/ml ethidium bromide. Electrophoresis was carried out in 0.5X TBE at 5 V/cm. Standard DNA size markers were run alongside for estimation of DNA fragment sizes. The ethidium bromide stained DNA samples were visualised on a UV transilluminator and photographed.

2.2.18.2 Purification of DNA fragments from agarose gels by electroelution

Insert DNA required for cloning and radiolabelling was purified by electroelution from agarose gels. The digested DNA sample containing 500 ng - 1 μg of the insert was resolved on a 1% high purity agarose gel as described in section 2.2.18.1. The DNA bands were visualised on a UV transilluminator after staining with ethidium bromide and the DNA band to be electroeluted was excised. The gel slice was placed on a dialysis bag containing 1 ml of 0.5X TBE and electrophoresed at 100 V in 0.5X TBE buffer for 1.5 hrs till the DNA
sample was eluted from the gel. Then the direction of current was reversed for 20 sec to enable the DNA to be released from the walls of the dialysis bag. The bag was gently tapped and the 0.5X TBE containing the eluted DNA was recovered. It was extracted 4-5 times with an equal volume of butanol to remove the ethidium bromide and concentrate the DNA solution. This was followed by two extractions with phenol : chloroform. Finally, the eluted DNA was precipitated with ethanol, dissolved in 10 µl of TE pH 8.0, and checked on an agarose gel.

2.2.18.3 Quantitation of nucleic acids

The concentration of nucleic acids was estimated by measuring the OD at 260 nm (Sambrook *et al.*, 1989). The following empirical relationships were used to calculate the concentrations. An OD of 1 corresponds to approximately 50 µg/ml of double-stranded DNA, 40 µg/ml of RNA and 33 µg/ml of single-stranded oligonucleotides. The purity of nucleic acids was estimated by calculating the OD$_{260}$ : OD$_{280}$ ratio. Pure DNA and RNA preparations have OD$_{260}$/OD$_{280}$ of 1.8 and 2.0 respectively.

2.2.18.4 Precipitation and desalting of nucleic acids

In all the routine experimental procedures followed, DNA was precipitated and desalted as described in Sambrook *et al.* (1989). The nucleic acid solution was made upto 200 µl with TE pH 8.0 and one-tenth the volume of 3 M sodium acetate pH 5.2 was added. The contents were mixed and the nucleic acids were precipitated at -70°C for 1 hr or -20°C overnight after the addition of 2.5 volumes of 95% ethanol, and pelleted by centrifugation at 12,000 X g for 20 min at 4°C. The pellet was washed with 70% ethanol to remove any salts and dissolved in an appropriate volume of TE pH 8.0 or water.

2.2.18.5 Sephadex G-50 and G-25 column chromatography

Sephadex gel filtration column chromatography was employed to separate out salts, contaminants and unincorporated radionucleotides from DNA solutions. Sephadex G-25 was used for the purification of oligonucleotides of length 15 to 150 bp, and Sephadex G-50 was used when the DNA to be purified was longer than 150 bp. A sterile 1 ml disposable plastic syringe was plugged with sterile glass wool and filled with the Sephadex G-25 or G-50 slurry previously equilibrated with TE pH 8.0. The column was packed by centrifugation at 1,000
X g for 5 min in a Sorvall HB-4 rotor at room temperature. Then 100 μl of the DNA solution to be purified was loaded and the column was centrifuged at 1,500 X g for 5 min at room temperature to elute the purified DNA.

2.2.18.6 Measurement of radioactivity in nucleic acids

For routine checking of labelling efficiency, samples were counted by Cerenkov's method. An aliquot of 1 μl of labelled DNA was spotted on a Whatman 3 MM filter and counted in the [\(^{3}\text{H}\)] channel of a liquid scintillation counter. The value was multiplied by four to correct for the difference in efficiency between the [\(^{3}\text{H}\)] and [\(^{32}\text{P}\)] channels. For a more accurate measure of radioactivity, the filter was suspended in scintillation fluid and the sample was counted in the [\(^{32}\text{P}\)] channel. Specific activity was expressed in terms of cpm/μg of DNA.

2.2.18.7 Purification of oligonucleotides

The single-stranded oligonucleotides (obtained from Bangalore Genei) used in PCR and primer extension experiments, were purified by ether extraction in order to remove any organic impurities. The DNA solution was made up to 500 μl with TE pH 8.0 and an equal volume of ether was added. The samples were vortexed, centrifuged at 12,000 X g for 2 min at room temperature and the ether layer was discarded. This step was repeated five times. The oligonucleotides were then concentrated to 100 μl by centrifuging the sample in a speed-vac and purified on a Sephadex G-25 column.

2.2.18.8 SDS-polyacrylamide gel electrophoresis

SDS-PAGE was carried out by the method described by Laemmli (1970) in a discontinuous buffer system. Gels of 1.5 mm thickness were cast and run on a vertical gel apparatus (GIBCO-BRL, USA). The resolving gel (8% acrylamide, 0.375 M Tris.HCl pH 8.8, 0.1% SDS) and stacking gel (5% acrylamide, 0.125 M Tris.HCl pH 6.8, 0.1% SDS) were polymerised by the addition of TEMED (12 μl for a 40 ml gel) and freshly prepared ammonium persulphate solution (400 μl of a 10% solution for 40 ml of gel mixture). The protein samples were boiled in 4X SDS-PAGE loading dye (200 mM Tris.HCl pH 6.8, 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol) for 5 min and loaded on the gel. Electrophoresis was carried out in electrophoresis buffer (0.025 M Tris.HCl, 0.192 M glycine pH 8.3) at a constant current of 20 mA till the samples entered the stacking gel and then at
40 mA through the resolving gel.

After the run, the gel was stained with 0.25% Coomassie brilliant blue R250 in 100 ml of methanol : glacial acetic acid : water (10:10:80) for 2-3 hrs and destained in 200 ml of methanol : acetic acid : water (30:10:60) for 3-4 hrs on a shaker, with several changes of the destaining solution. The destained gels were photographed, dried and stored.

2.2.18.9 Native polyacrylamide gel electrophoresis

Native polyacrylamide gel electrophoresis was performed as described by Sambrook et al. (1989). A 1.5 mm thick vertical slab gel was cast with 45 ml of gel mix containing 6% acrylamide, 2% glycerol and 0.1% ammonium persulphate in 0.5X TBE. The gel was polymerised by the addition of 12 μl of TEMED. DNA samples were prepared by the addition of 6X DNA loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol) and loaded onto the gel. Electrophoresis was carried out at 150 V in 0.5X TBE till the bromophenol blue almost reached the bottom of the gel. The DNA samples were analysed either by ethidium bromide staining or by autoradiography of the dried gel in case of radiolabelled samples.

2.2.18.10 Denaturing urea - polyacrylamide gel electrophoresis

Denaturing urea PAGE was carried out as described by Sambrook et al. (1989). A 0.4 mm thick gel was cast in a vertical slab gel apparatus with 40 ml of gel mix containing 24 g urea, 8% acrylamide, 0.1% ammonium persulphate and 15 μl of TEMED in 1X TBE. The gel was pre-electrophoresed at 70W for 40 min. The DNA samples were dissolved in formamide loading dye (10 mM NaOH, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol) denatured at 80°C for 5 min and loaded on the gel. Electrophoresis was carried out at 70W in 1X TBE. The gel was fixed for 45 min in 10% methanol, 10% acetic acid solution, vacuum-dried and subjected to autoradiography.

2.2.18.11 Estimation of protein concentration

The protein content of nuclear extracts was quantitated by the method described by Lowry et al. (1951). An aliquot of protein sample was made upto 250 μl with water and mixed with 1 ml of Lowry’s reagent (1 ml of sodium potassium tartarate + 19 ml of sodium carbonate in 0.1 N NaOH + 0.1 ml of 0.4% copper sulphate) and incubated for 20 min at
room temperature. This was followed by the addition of 0.1 ml of 1 N Folin's reagent and further incubation at room temperature for 30 min. The OD of the resultant solution was measured. A standard curve was generated using 10-100μg of BSA. The concentration of protein in the extract was estimated from the standard curve.