Chapter 3
Materials & Methods
2.1: Polymerase Chain Reaction (PCR):

PCR was carried out in 50-100 µl with the following ingredients:

**100 µl reaction:**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X Buffer</td>
<td>10 µl</td>
</tr>
<tr>
<td>dNTP (genetix 10 mM)</td>
<td>8 µl</td>
</tr>
<tr>
<td>Primer Mix (50 ng/µl)</td>
<td>4 µl</td>
</tr>
<tr>
<td>25mM MgCl2</td>
<td>1 mM</td>
</tr>
<tr>
<td>Template</td>
<td>1 µl (50 pg)</td>
</tr>
<tr>
<td>Enzyme (Klen Teq)</td>
<td>2 U</td>
</tr>
<tr>
<td>H2O</td>
<td>Up to 100 µl</td>
</tr>
</tbody>
</table>

Drop of mineral oil was added and tubes were vortexed properly. Momentary spin was given to all the tubes.

Cycling parameters are as follows:

1. 95°C for 10 minutes Denaturation
2. 95°C for 1 minute Denaturation
3. 57.5°C for 30 seconds Annealing
4. 72°C for 1 minute Extension
5. 72°C for 10 minutes Final Extension

Primers used for PCR Reactions:

<table>
<thead>
<tr>
<th>Primer Identity</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3</td>
<td>5’ AATTAACCCTCACTAAA 3’</td>
</tr>
<tr>
<td>T7</td>
<td>5’ TAATACGACTCACTATA 3’</td>
</tr>
<tr>
<td>P3</td>
<td>5’ TAACCCTCACTAAAGGG 3’</td>
</tr>
<tr>
<td>P4</td>
<td>5’ TACGACTCACTATAGGG 3’</td>
</tr>
<tr>
<td>P1</td>
<td>5’ TCGAGGTCGACGGTAT 3’</td>
</tr>
<tr>
<td>P2</td>
<td>5’ CGCTCTAGAACTAGTGG 3’</td>
</tr>
<tr>
<td>M1</td>
<td>5’ CGAGGTCGACGGTATCG 3’</td>
</tr>
<tr>
<td>M2</td>
<td>5’ CTCTAGAACTAGTGGATC 3’</td>
</tr>
<tr>
<td>FP</td>
<td>5’ ACCATGATTACGCCAAG 3’</td>
</tr>
</tbody>
</table>
2.2: Purification and Precipitation of DNA:
One volume of TE- saturated phenol-chloroform was added in the DNA mix. Tube was vortexed for one minute and centrifuged at 12,000 rpm for 5 minutes. The upper aqueous phase containing DNA was transferred to another sterile eppendorf. One volume of chloroform (Qualigen) and isoamyl alcohol (Qualigen) [24:1] was added in the tube and vortexed for one minute. Tube was again centrifuged at 12,000 rpm for 5 minutes. The upper phase was collected in the fresh tube and DNA was precipitated by adding 1/10 volume of 1M NaCl (Sigma) and 2.5 volume of ethanol (Merck). Tubes were incubated in -20°C for 2 hours. Tube was then centrifuged at 12,000 rpm for 10 minutes. Supernatant was discarded; the DNA precipitate was washed with prechilled 70% alcohol. Pellet was dried and resuspended in TE (10mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0) or mili Q.

2.3: Preparation of Competent Cells:
Competent cells were prepared by two different methods.

2.3.1: Trituration Method of Competent Cells Preparation:
DH5α bacterial strain was used for competent cell preparation. DH5α competent cells are made according to the method described by Hanahan (1983) with some modification. DH5α cells were streaked from glycerol stock on fresh LB (Hi media)-Agar (BDH) plate containing 20μg/ml nalidixic acid (sigma, stock solution concentration 20 mg/ml). Plate was incubated overnight at 37°C. Next day 25 ml of Luria broth supplemented with same antibiotic was inoculated by single colony picked from overnight plate. This Preculture was grown at 30°C at 200 rpm for 16 hours (primary culture). After that 100 ml LB media (secondary culture) was inoculated with 1 ml inoculum. No antibiotic was added in the secondary culture. Culture is kept for shaking at 200 rpm at 37°C until the OD reaches to 0.45-0.55. Cells were chilled in ice for 2 hours. Cells were pelleted down by centrifugation at 4000 rpm for 15 minutes. Supernatant was discarded. Pelleted cells were then resuspended in freshly prepared trituration buffer (100 mM CaCl₂ (sigma), 70mM MgCl₂ (sigma), 40 mM sodium acetate (Qualigen), pH 5.5) with equal volumes of the starting culture. Cells were incubated in ice for 45 minutes. Cells were pelleted gently by centrifugation at 2500
rpm for 5 minutes (all the steps were strictly done in 4°C). Cells were resuspended in 5ml (1/20th volume of starting culture) of ice-cold trituration buffer with 15% glycerol and stored at -80°C in aliquots.

2.3.2: Na-MOPS Method of Competent Cells Preparation:
This method essentially combines Hanahan's high efficiency procedure with the traditional calcium-shock methods (Hanahan D, 1983). This method provides very high efficiency (10^7-10^8). After inoculating the secondary media as described in above method, the cells were grown at 200rpm at 37°C until OD reaches to 0.5-0.6. Cell pellet was collected by centrifugating the culture at 2500 rpm for 5 minutes. Supernatant was discarded and cells were resuspended in 40 ml per 100 ml of starting culture of ice cold transformation buffer Tfbl (30mM CH_3 COOK (Qualigen), 50mM MnCl_2 (Qualigen), 100mM KCl (Sigma), 10mM CaCl_2 (sigma), also containing 150 g/l glycerol (Qualigen) final pH 5.0). Cells were incubated in ice for 5-10 minutes. Cells were recollected by centrifugating at 2000 rpm for 5 minutes at 4°C. Cells were finally resuspended in 4 ml per 100ml starting culture of ice-cold Tfb2 (10mM Na-MOPs (sigma) pH 7.0, 75mM CaCl_2, 10mM KCl, final pH 7.0 also containing 150 g/l glycerol and stored at -80°C in aliquots.

2.4: Preparation of Vector and Insert DNA for Cloning:
For cloning purposes, both insert and vector DNA was digested with appropriate restriction enzymes (Genetix) to generate compatible ends. 10 X Y²/tango buffer (33mM Tris acetate pH 7.9, 10mM magnesium acetate, 60mM potassium acetate, 0.1mg/ml BSA) is used for maximum enzyme activity. Digestion was carried out at 37°C. After digestion the DNA of both insert and vector is purified by phenol-chloroform method and then precipitated as described above (2.2). After that ligation of insert and vector DNA (1:3) is done by T4 DNA ligase (genetix or Bangalore genei) and 5x ligation (400mM Tris Cl, 100mM MgCl_2, 100mM DTT, 5mM ATP; final pH 7.8). The reaction is incubated overnight at 16°C. After overnight incubation the ligase is heat inactivated by heating the ligation mix at 65°C for 10 minutes. 4 µl of ligation mix was used for transformation.
2.5: Transformation of Competent Cells:
After competent cell preparation, transformation of DH5α is done. Competent cells were thawed in ice. 1-5ng of plasmid DNA was added to the 100μl of competent DH5α cells. Cells were tapped gently and incubated in ice for 30 minutes. The cells were subjected to heat shock at 42°C in a water bath for 90 seconds and then chilled on ice for 5 minutes. 900μl of LB is added to the cell and the transformation mix was allowed to revive for 40 minutes at 37°C.

2.6: Plating of Transformation Mix:
In the case of plasmid transformation only 100μl from the transformation mix was plated on to the antibiotic containing LB-Agar plate. In the case of transformation of a ligation reaction (vector and insert) the transformation mix was centrifuged at 2000rpm for 5 minutes at 4°C, and cell pellet was resuspended in 100μl of LB and the entire mix was plated on antibiotic containing LB-Agar plate. The plates were incubated at 37°C for 12-16 hours until the colonies were apparent. If ligation reaction is used for transformation, a positive control of transformation was carried out with 2.5 ng of pBluescript vector and a negative control was kept with out any DNA (only competent cells were plated). Digested and then self-ligated vector is also used as a ligation control. Transformation efficiency was determined by counting the number of colonies/μg of DNA on selection plate.

2.7: Screening of Recombinants:
20 μl of 40 mg/ml X-gal (genetix) and 100μl of 100mM IPTG (genetix) was spread on LB-agar plate containing 100 μg/ml ampicillin. In order to absorb this selection mix, plate was incubated at 37°C for 30 minutes. The transformation mix was then plated on to this plate and allowed to grow at 37°C for 16 hours. All recombinant colonies will be of white color (because recombinants lack β-galactosidase activity) while non recombinants will be of blue color. Alternatively individual colonies were picked up by a tooth prick, resuspended in 50 μl H2O, heat denatured at 95°C for 10 minutes, and then centrifuged for 5min at 14000 rpm. Supernatant was used for PCR using appropriate primers.
2.8: Storage of Bacterial Strains:
All the glycerol stocks were made in 15% (w/v) glycerol. From the overnight culture of the bacteria, cells were taken, and sterile glycerol was added to a final concentration of 15% (w/v) vortexed and stored at -80°C with proper labeling.

2.9: Plasmid DNA Isolation:
Plasmid DNA was isolated either on mini scale or midi scale. The plasmid mini-prep method allows for the rapid isolation of small amounts of plasmid DNA while plasmid midi prep method allows isolation of DNA in large amount.

2.9.1: Isolation of Plasmid DNA by Miniprep Method:
1 ml of LB medium containing the appropriate antibiotic was inoculated with a single colony and grew overnight at 37°C with vigorous shaking. Overnight culture was transferred in an eppendorf tube. Tube was centrifuged at 5000rpm for 5 minutes. The supernatant was drained out, leaving 50μl of LB in the tube. The pellet was resuspended in the remaining LB by vortexing. 300μl of miniprep lysis buffer (0.2N NaOH (BDH), 1% SDS (Sigma)) was added in the tube and mixed by inverting. Tube was then incubated on ice for 5 minutes. 150μl of ice cold 5M Potassium acetate pH 4.8 was added and incubated on ice for 5 minutes. Tubes were strongly vortexed and centrifuged at 13000 rpm for 5 minutes. Supernatant was transferred to a fresh tube, avoiding the white precipitate which is cell debris. RNAse A (Sigma) is then added to a final concentration of 20μg/ml and incubated at 37°C for 20 minutes. 900μl of 100% ethanol was added, mixed properly and allowed to precipitate at room temperature for 5 minutes. DNA was collected by centrifugating at 13,000 rpm for 15 minutes. The pellet was washed with 70% ethanol, dried and resuspended in 10-20μl of sterile mili Q or TE (10 mM Tris.Cl, 1 mM EDTA pH 8.0).

2.9.2: Midi-Prep Method (Alkali Lysis) of Plasmid Isolation:
Midi preparation of plasmid DNA was done by alkaline lysis method described by Sambrook et al., (1989). 100 ml LB medium containing the appropriate antibiotic was inoculated by 1ml of inoculum from an overnight culture of DH5α cells containing the desired plasmid. This was incubated at 37°C overnight with vigorous shaking. Cells were harvested by centrifugation at 4000 rpm at 4°C for 10 minutes. Pellet was
resuspended in 10 ml of ice-cold Solution I (50mM glucose (Qualigen), 25mM Tris. Cl (Sigma pH 8.0), 10mM EDTA (Sigma)) and incubated in ice for 8 minutes. 20 ml of freshly prepared Solution II (0.2 N NaOH, 1% SDS) was added to the suspension and mixed gently by inverting the tube and incubated in ice for 8 minutes. 15 ml of solution III (5M potassium acetate pH 4.8) was added and mixed thoroughly by inverting the tube gently. The tube was incubated in ice for 8 minutes. After incubation, the tube was strongly vortexed, lysate was centrifuged at 14,000 rpm for 25 minutes, at 4°C and supernatant was carefully transferred to a fresh tube avoiding any cell debris. RNase was then added to a final concentration of 20μg/ml and incubated at 37°C for 60 minutes. DNA was precipitated by adding 0.6 volume of isopropanol (Qualigen) and incubating the tube at room temperatures for 15 minutes. DNA was recovered by centrifugation at 4°C at 12,000 rpm for 15 minutes. Pellet was washed with 70% ethanol and then it was dried and resuspended in sterile mili Q or TE. If DNA is used for sequencing purpose it was purified by PEG 8000 (Sigma). For that, DNA was resuspended in TE. Equal volume of 13% PEG (dissolved in 1.6M NaCl) was mixed with DNA solution and incubated in ice for 30 minutes. DNA was recovered by centrifugating at 13000 rpm for 10 minutes. DNA precipitate was washed with 70% alcohol and dried.

2.10: Quantitation of Nucleic Acids:
The quantity and purity of nucleic acids in solution was determined by measuring absorbance at 260nm and 280nm. The concentration of double stranded DNA was calculated by taking the Abs\textsubscript{260} 1OD = 50μg/ml. The concentration of single stranded RNA was calculated by taking the Abs\textsubscript{260} 1OD = 40μg/ml. The concentration of single stranded DNA was calculated by taking the Abs\textsubscript{260} 1OD = 33μg/ml.

2.11: Restriction Digestion:
DNA was digested by different restriction enzymes for different purposes. Restriction digestions were carried out in the appropriate buffer (such as R+, O+, etc) according to the manufactures instructions (MBI Fermentas).

2.12: Gel Electrophoresis:
Gel electrophoresis was done to see the DNA obtained from amplification, digestion etc.
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2.12.1: Agarose Gel Electrophoresis:
Agarose (Sigma) gel electrophoresis was done as described in Sambrook et al., 1989. Generally, 0.8 % and 2% gels were used for various purposes. Routinely 0.5 X TBE buffer (0.045M Tris-borate (Sigma), 0.001M EDTA pH 8.0) was used as running buffer. DNA was visualized by adding ethidium bromide to a concentration of 0.5µg/ml in the gel before pouring.

2.12.2: Polyacrylamide Gel Electrophoresis:
Polyacrylamide gel electrophoresis was done as described in Sambrook et al., 1989. 30% acrylamide solution (29 gm acrylamide (sigma) and 1 gm N-N' bis acrylamide (sigma) made it 100 ml) was prepared. 6-8% gel and 0.5X TBE (0.045M Tris-borate, 0.001M EDTA) running buffer was used in most of the experiments.

2.12.3: Electrophoresis:
The gel was run at 200 V for 3 hours. Before loading the samples, all the lanes of gel were flushed with the needle of a syringe to prevent the uneven loading. For gel shift assays the gel was run at 250V for 3 hour in 0.5X TBE buffer. Bromophenol blue and Xylene cyanol were used as tracking dyes. After complete run the gel was stained with ethidium bromide solution and visualized under transilluminator.

2.13: Labeling DNA Fragments with Radioactive Nucleotides:
DNA fragments were labeled with either α\(^{32}\)P-dCTP or γ\(^{32}\)P-ATP. After labeling, the unincorporated nucleotides were removed by passing through a Sephadex G-50 spun column. DNA was labeled by different methods.

Probe Preparation by PCR
Polymerase Chain Reaction was carried out in 20µl volume with the following ingredients (If radiolabel is α\(^{32}\)P-dCTP)

- 10X Buffer: 2µl
- α\(^{32}\)P-dCTP: 2µl
- dNTP-dCTP (100µM): 1µl
- dNTP (10µM): 2µl
- 25mM MgCl\(_2\): 1.2µl
- Primer Mix (50ng/µl): 1µl
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Template DNA 50pg
Klen Taq 1U
Water Up to 20μl

(If radiolabel is α\(^{32}\)P-dATP), dNTP-dATP mix was used in place of dNTP-dCTP.

The cycling condition was as follows:

- 95°C for 10 minutes
- 95°C for 1 minute
- 57.5°C for 30 seconds
- 72°C for 1 minute

40 times step 2-4.

72°C for 10 minutes

The PCR product was then passed through Sephadex G50 Spun column to remove the unincorporated nucleotides.

End Labeling Reaction:

10 ng of double stranded oligo DNA was incubated with 20μCi of γ-\(^{32}\)dATP and T4 polynucleotide kinase in a 20μl reaction at 20°C overnight. The labeled probe was purified by passing through Sephadex G50 spun column to remove unincorporated free nucleotides.

End Filling Reaction:

End filling reaction was carried out with Klenow fragment according to manufacturer’s (MBI Fermentas) instructions. 2U of Klenow fragment was used for 10 ng of DNA. Unincorporated nucleotides were removed by Sephadex G50 Spun column.

Labeling of cDNA by Random Oligonucleotide Primers:

cDNA labeling was done by HexaLabel DNA labeling kit from Fermentus life sciences, according to the manufacture’s instructions. 100 ng DNA was labeled. 10 μl of hexanucleotide in 5X buffer was added in the tube. The tube was vortexed and spun down for 3-5 seconds. The reaction mix was boiled for 10 minutes and cooled in ice.

Based on choice of labeled dATP or dCTP, 3 μl of mix A (dNTP- dATP) or mix C (dNTP- dCTP), 50μCi of α\(^{32}\)P-dATP or α\(^{32}\)P-dCTP, 5U of Klenow fragment was added in the tube. The reaction mix was vortexed and spun down. The reaction was
incubated at 37°C for 2 hours. Reaction was chased for 5 minutes by adding 4μl of dNTP mix. Reaction then finally stopped by adding 0.2mM EDTA, pH 8.0 and passed through Sephadex G50 Spun column to remove the unincorporated nucleotides.

2.14: Preparation of Nuclear Extracts from Chick Embryos:
12-days-old chick embryos were taken for nuclear extract preparation. All steps were conducted at 4°C. Brain, heart, liver and skeletal tissues were taken out from embryo and added to the prechilled hand homogenizer. One ml of buffer A (20mM Hepes (Sigma), 20% glycerol (Qualigen), 10mM NaCl (Sigma), 1.5mM MgCl2 (Sigma), 0.2mM EDTA (Sigma) 0.1% triton-X100 (Sigma), 100mM PMSF (Sigma), 1mM DTT (Sigma), 10mg/ml Leupeptin (Sigma), 10 mg/ml Aprotenin (Sigma)) was added in each homogenizer having individual tissue. The tissues were homogenized in ice. The homogenates were centrifuged at 2500 rpm at 4°C for 15 minutes. Supernatants were discarded, pellets were then resuspended in 300-500 μl of buffer B (20mM Hepes, 20% glycerol, 500mM NaCl, 1.5mM MgCl2, 0.2mM EDTA, 0.1% TRITON-X, 100mM PMSF, 1mM DTT, 10mg/ml Leupeptin, 10mg/ml Aprotenin) and incubated in ice for 60 minutes with intermittent tapping (at every 10 minutes interval). The homogenates were then centrifuged at 13,000 rpm at 4°C for 15 minutes. The supernatants were aliquoted and snap freezed at -70°C. Bradford method (Bradford et al., 1976) was used to estimate the protein concentration in the samples.

2.15: Protein-DNA Binding Assay
Protein-DNA Binding reactions were carried out in 40 μl volume. Binding reactions contain (20mM HEPES pH 7.9, 5% glycerol, 60mM NaCl, 1.5 mM MgCl2, 1mM EDTA, 1mM DTT), 4μl (20 μg) nuclear extract, 1μg poly dIdC and 32P- labeled probe (40,000 cpm). All the components were mixed thoroughly and incubated in ice for 30-45 minutes. For competition 100 fold molar excess of cold (unlabeled) DNA (self or nonself) was added to the binding reaction prior to the addition of the labeled probe. Cold competitor was first added, incubated in ice for 10 minutes and then radiolabeled probe was added in the reaction tube. DNA-protein complexes were separated from the free probe by electrophoresis in 6-8% native acrylamide gel.
2.16: Fixation and Autoradiography:
After the complete gel run, plates were separated with the help of spatula. The gel is transferred to the Whatmann 3mm paper and dried in gel dryer at 75°C for 60 minutes. The dried gel was either exposed to phosphorimager for quick image view or exposed to the XAR (KODAK) X-ray film and kept in -70°C. After proper exposure the film was developed by developer (KODAK) and fixed in fixer (KODAK), dried and saved.

2.17: Elution of DNA:
DNA was either eluted from acrylamide gel or from agarose gel.

2.17.1: Elution of DNA from Acrylamide Gel:
Piece of gel harboring DNA was cut from the gel and eluted. For elution the gel pieces containing DNA or DNA-protein complexes were chopped, put in a tube and elution buffer (500mM, Ammonium acetate, 10mM MgCl₂, 1mM EDTA, and 0.1% SDS) was added in the tube. The tube was kept overnight on shaker. After over night incubation the supernatant was transferred in to another tube. DNA precipitation was done by standard method as described above (2.2); DNA was dried and resuspended in sterile mili Q or TE.

2.17.2: Elution of DNA from Agarose Gel:
The DNA was electrophoresed on a 0.8-2.0% agarose gel. The desired fragment was excised from the gel. The gel slice was placed in a 0.5ml eppendorf tube with a pinhead hole at the bottom. This hole was covered with thin mesh of glass wool. The 0.5ml eppendorf was placed in to a 1.5ml eppendorf tube and centrifuged at 4000rpm for 5 minutes. This step is repeated 3-4 times until 80-90% DNA recovered. The eluent collected in the 1.5-ml eppendorf was precipitated with 1/10 th volume of 1M NaCl and 2.5 volume of 100% alcohol and precipitated.

2.18: South Western Blotting:
South western analysis was first described by Bowen et al (Bowen et al., 1980) to identify the DNA binding proteins by protein blotting.

12-days-old chick embryonic heart, liver and brain nuclear extracts were prepared and quantitated according to the Bradford method (Bradford et al., 1976). Bovine serum albumin (BSA) was used as the standard. Polyacrylamide gel electrophoresis under denaturing condition (in the presence of 0.1% SDS) was performed according to
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...the method of Laemmili et al., 1970. 40 μg of nuclear extracts from 12-day-old chick embryonic brain, liver and heart were loaded with equal amount of loading dye (5% SDS, 5mM Tris.Cl pH 6.8, 2M DTT, 20% glycerol) on to the gel. The proteins were stacked at pH 6.8 in stacking gel containing 4% acrylamide (Sigma), 0.106% N, N’ methylene bis acrylamide (Sigma), 0.125 M Tris.Cl, pH 6.8, 0.01% TEMED (Amresco) and 0.1% ammonium per sulphate (Amresco). The resolving gel was made of 12% acrylamide, 0.33% N, N’ methylene bis acrylamide, 0.375 M Tris.Cl pH 8.8, 0.01% TEMED and 0.1% ammonium per sulphate. The protein samples were electrophoresed in running buffer consisting of 0.025M tris base, 0.192 M glycine, pH 8.3 and 0.1% SDS. After complete run the gel was transferred to nitrocellulose (Hybond R+) membrane for 16 hours in transfer buffer containing 0.025M tris base, 0.192 M glycine (Merck), pH 8.3 and 15% methanol (Qualigen). After complete transfer the membrane was removed from transfer apparatus and blocked by blotto (50mM Tris.Cl pH 7.5, 50mM NaCl, 1mM EDTA, 1mM DTT and 5% skimmed milk powder (Anikspray)) for 2 hours at 4°C with gentle agitation. Blot was then washed thrice in washing solution (50mM Tris.Cl pH 7.5, 50mM NaCl, 1mM EDTA, and 1mM DTT). After that membrane was blocked in binding buffer (10mM Tris.Cl, pH 7.5, 25mM NaCl, 10mM MgCl₂, 5mM EDTA, 1mM DTT) with 200 fold molar excess of poly dIdC for 2 hours at 4°C. After poly dIdC incubation, membrane was soaked in binding buffer for 8 hours and then soaked in binding buffer containing 10⁶cpm/ml radioactive probe for overnight at 4°C. Membrane was finally washed four times in binding buffer (Each wash for 8 minutes at room temperature). After washing the membrane was air-dried and exposed on x-ray film or phosphorimagener.

2.19: DNA Sequencing:
The nucleotide sequences of selected binding sites were manually done by dideoxy sequencing using Sequi Therm EXCEL II DNA sequencing kit from Epicenter Biotechnologies; USA. DNA quantity was taken according to the manufacturers instructions (For 3kb plasmid, 400ng DNA was used). Cyclic sequencing was done. For that reagents were thawed in ice. Premix was prepared by adding following components in a 0.5-ml eppendorf.

15pmole of unlabelled T3/T7 primer
10μCi of [α-\textsuperscript{35}S]-dATP at 1,000Ci/mmole
7.2 μl of Sequi Therm EXCEL II Sequencing buffer
400 ng DNA template (3 Kb)
Sterile water to 17μl
5U of Sequi Therm EXCEL II DNA polymerase
Four 0.5 ml eppendorf were labeled as G, A, T, C. 4 μl of premix was added in each tube. 2 μl of Sequi Therm EXCEL II Termination mixes (containing dideoxy dNTP and dNTP) were added in the corresponding tube (For Example. G termination mix in G tube, A termination mix in the A tube etc.) Reaction mixes in each tube were mixed thoroughly. Each reaction was overlaid with mineral oil and the tubes were centrifuged briefly in a microcentrifuge to separate the mineral oil layer from the reaction component.

Reaction conditions were as follows:
1. Preheated the thermocycler to 95°C
2. Heated the reactions for 5min at 95°C.
3. Cycled the reactions 30X for
   - 30sec at 95°C
   - 30sec at 55°C
   - 1 min at 70°C

Upon completion 3 μl of stop/loading buffer (95% Formamide, 0.1% EDTA and standard tracking dyes) was added to each reaction, and preceded with electrophoresis. Samples were run in denaturing gel containing 6% acrylamide and 8M urea (Sigma). 1X TBE buffer was used as running buffer. Gel was run at 3000V, 60W, and 300mA. Before loading samples were preheated at 95°C. After the complete run, gel was treated with the fixer containing 10% methanol and 10% acetic acid for 30 minutes. Gel is then dried and exposed to phosphorimager.

Automated sequencing was done in the sequencing facility available at the Department of Biochemistry, University of South Campus, New Delhi and at the Human Genome Center, School of Life Sciences, Jawaharlal Lal Nehru University, New Delhi.
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2.20: DNAse I Foot Printing Assay:
Bovine pancreatic deoxyribonuclease I (DNAse I; Sigma) was stored as a 30 unit/μl solution in 5mM sodium acetate (pH 4.0), 1mM CaCl2 and 50% glycerol. DNA was amplified with pBluescript specific T3 and T7 primers. DNA was digested with Paul enzyme, electroeluted and then klenow labeled. The stock solutions of DNAse I were kept at -20°C and freshly diluted to the desired concentration immediately prior to use. Foot printing experiments were performed in 40μl reaction volume. 40-50μg of 12-day-old chick nuclear heart and liver extracts were incubated with the labeled DNA fragments (Klenow labeled) for 60 minutes. Poly dIdC was added to avoid nonspecific binding. After 60 minutes incubation at 4°C, digestion was initiated by the addition of DNAse I solution whose concentration was adjusted to yield a final enzyme concentration of about 0.01 unit/ml in the reaction mixture. For DNAse I activity, cofactor solution containing 10mM MgCl2, 5mM CaCl2 was added in the reaction mix. DNAse I treatment was given at 37°C. After the required period of time (varies from 30-70 seconds), the reaction was stopped by adding stop buffer (1% SDS, 200mM NaCl, 20 mM EDTA). Samples were then phenol-chlorophorm extracted and precipitated by 0.1 vol of 1M NaCl and 2.5 vol of absolute alcohol. Samples were resuspended in loading dye containing 95% formamide solution, 0.1mM EDTA and tracking dyes (bromophenol blue and xylene cyanol.), heat denatured at 95°C for 5 minutes and electrophoresed in 6% acrylamide gel containing 5M urea.

2.21: Isolation of RNA from Tissue Samples:
Neonatal mouse and 12-day-old chick heart, brain and liver tissues were used for RNA preparation. For RNA work all the solutions were made in DEPC treated water. All the glassware were backed at 200-250°C for more then 5 hours. Sterile conditions were maintained to a great extent. All precautions were taken to prevent RNase contamination. Tissues were dissected and immediately homogenized in RNA Wiz (Sigma) in glass homogenizer. 1ml RNA Wiz was used for 100mg tissue. Homogenates were transferred in to 1.5 ml eppendorfs. The homogenates were incubated at room temperature for 5 minutes. Proteins were removed from nucleic acids by adding chloroform (0.2X of starting volume) to the homogenate. Tubes were shaked vigorously for 20 seconds and incubated at room temperature for 10 minutes.
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Tubes were then centrifuged at 10,000g for 15 minutes at 4°C. The upper aqueous phase was transferred in a sterile RNAse free tube. 0.5X starting volume of RNAse free mili Q was added in the tube and mixed well. Nucleic acids were then precipitated by adding isopropanol (1X of starting volume) and incubated at room temperature for 10 minutes. RNA was then pelleted by centrifugating at 13,000 rpm for 15 minutes at 4°C. The supernatant was discarded and pellet was washed with 70% ice chilled absolute alcohol. The pellet was air dried and resuspended in appropriate amount of RNAse free water.

2.21: Northern Blotting:
RNA samples were prepared by mixing 20μg of RNA from different tissues. 1X MOPS buffer (0.02M MOPS, 8mM Sodium Acetate, 1mM EDTA) was added in the sample. 7μl of formaldehyde (Qualigen) and 15μl of formamide (Qualigen) per 20μg RNA was also added. Samples were mixed well and incubated at 65°C for 15 minutes, followed by chilling on ice. 2μl of standard loading dye and 1μl Ethidium Bromide was added in the sample. 1.2% formaldehyde gel was prepared as described by Sambrook et al., 1989. RNA samples were loaded based on equal RNA concentrations and electrophoresed at 80V. 1X MOPS (0.02M MOPS, 8mM Sodium Acetate, 1mM EDTA, prepared in DEPC treated water) buffer was used as the running buffer. After initial run of 60 minutes, the running buffer was exchanged in both the chambers. Electrophoresis continued till the bromophenol blue dye reached to the bottom of the gel. Running buffer changes improves the quality of bands. The gel was visualized under UV transilluminator and quality of RNA was determined. Gel was transferred carefully in the baking glass tray and rinsed in DEPC treated water twice (each wash was for 10 minutes), which help in the removal of formaldehyde. The gel was then equilibrated in 10X SSC for 10 minutes. Following which the RNA was transferred on to Nylon membrane by the capillary method as described in Sambrook et al., 1989. Upward capillary blotting was performed to transfer RNA on to a positively charged membrane. The well portion of gel was cut off to aid even transfer of the RNA on to the membrane. A piece of positively charged nylon membrane (Hybond R+) was cut exactly to the gel dimension, wetted briefly with 10X SSC and placed on to the gel, the imaginary well facing upwards. Above the nylon membrane two layers of 3mm
Whatman strips equals to the gel size, paper layers, and 1mm Whatman strips were kept to form a stack of 3 cm height. Transfer was carried out with 10XSSC, for more than 16 hours. The nylon membrane was then removed and RNA was UV cross linked to the membrane in a UV cross linker (Stratalinker, Stratagene) and stored at room temperature until probed. Prehybridization was done in heat-sealed autoclaved bags. Membrane was prehybridized in prehybridization buffer (0.5 M Sodium phosphate, 7% SDS, 1mM EDTA, pH 8.0, 100µg/ml sheared denatured Salmon sperm DNA) at 65°C for 3 hours. α-^{32}P dATP/α-^{32}P dCTP labeled DNA probe (10^6 cpm/ml) was added to the prehybridization solution. Hybridization was carried out overnight at 55°C. Filters were washed sequentially as follows:

- 2X SSC  0.5% SDS; room temperature for 10 minutes
- 1X SSC  0.5% SDS; 65°C for 10 minutes
- 0.5X SSC 0.5% SDS; 65°C for 10 minutes
- 0.2X SSC 0.5% SDS; 65°C for 10 minutes

Washing at 65°C is essential to avoid nonspecific signals. Membrane was then air dried and exposed to phosphorimager (Fugi) for quick view and then exposed on X-ray film.

2.22: cDNA Library Screening:
For screening purpose Uni- Zap® XR Library (Stratagene) prepared from 72 hour White leghorn was used. Expression library was screened using γ^{32}P labeled trimerized oligo (Sigma) probe. XL-1 Blue MRF^ cells were grown overnight in LB medium containing tetracycline in the presence of 10mM MgSO_4 (Qualigen) and 0.2% maltose (Qualigen) at 30°C. Cells were harvested at 2500 rpm for 10 minutes. Supernatant was discarded and cells were resuspended in 10mM MgSO_4. Cells were diluted to OD_600 = 0.5 with 10mM MgSO_4. 0.12X10^6 pfu (plaques forming units) of bacteriophage were mixed with XL-1 Blue MRF^ cells (OD_600 = 0.5). The mix was incubated at 37°C for 15 minutes to allow phage adsorption to the cells. Mean time top agarose (0.7% agarose in LB) was melted and equilibrated at 55°C. Top agarose was added in the infected cells, mixed by inverting and spreaded quickly on a prewarmed (42°C) and dried 150mm NYZ (LB and 10 mM MgSO_4/Tetracycline) plates. The plates were incubated at 37°C till tiny plaques were visible. In the meantime
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Nitrocellulose membrane (Hybond) were soaked in 10mM IPTG solution for 30 minutes and then air-dried. Each plate was overlayed by nitrocellulose membrane and incubated for 6-8 hrs at 37°C. Air bubbles trapping between the filter and the top agarose was avoided. Plates were cooled for 30 minutes. Plates and membranes were marked by asymmetric pricks for the orientation. The membranes were lifted off from the plates and immersed in blocking solution (50mM KCl, 15mM HEPES, pH 7.5, 1mM EDTA, 1mM DTT, 1% BSA (Sigma)). Blocking was carried out at 4°C for one hour, followed by membranes wash with TNE buffer (50mM KCl, 15mM HEPES pH 7.5, 1mM EDTA, 1mM DTT, 5% glycerol) thrice (each wash for 10 minutes). Membranes were then hybridized for 12-16 hours. Hybridization was carried out at 4°C in TNE containing denatured sheared salmon sperm DNA (100μg/ml, Amresco) and trimerized probe (2x 10^6 cpm/ml). The membranes were then washed thrice in TNE buffer (each wash for 8 minutes). After that membranes were dried briefly on Whatman sheets and exposed to phosphorimager for quick view and then exposed on Kodak BioMax MS film for 2-5 days depending on radioactive counts in membranes. Once the filters were removed from the plates, plates were stored at 4°C to recover positive plaques after screening. The plaques identified as positive in the first round of screening were picked up from the plates and transferred to 1.5 ml eppendorf containing 200-400 μl (depends on plaques number and size of plaques) SM buffer (50mM Tris.Cl, pH 7.5, 100mM NaCl, 8mM MgSO4, 0.01% gelatin (Qualigen)). 20μl of chloroform was added in the tube to kill XL-1 blue cells. The tubes were vortexed well and left overnight at room temperature. This helps to recover the phage in the SM buffer. This phage suspension was used for subsequent round of screening. Phages were further diluted in SM buffer such that 2000 and 500 plaques were used in secondary and tertiary rounds of screening respectively. After tertiary round of screening positive plaques were picked up and in vivo excised.

2.23: In Vivo Excision of pBluescript-SK (+/-) Phagmid Using the ExAssist/SOLR System:
The cDNA clone in pBluescript- SK (+/-) was recovered by in vivo excision from Uni Zap XR vector using the ExAssist/SOLR system. The XL-1 Blue MRF’ and ExAssist helper phage (SOLR) cells were grown in LB containing 10mM MgSO4 and 0.2%
maltose and appropriate antibiotic (tetracycline for XL-1 Blue and kanamycin for SOLR cells) at 30°C overnight. Cells were harvested by centrifuging at 2500 rpm for 10 minutes at 4°C and resuspended to an OD₆₀₀ = 1.0 in MgSO₄. XL-1 Blue MRF’ cells and 1 x10⁵ pfu (plaque forming units) from the positive plaques were mixed with 1 x10⁵ pfu of ExAssist helper phage. The mixture was incubated at 37°C for 15 minutes. This was mixed with 3ml of freshly prepared LB and grown for 3 hours at 37°C with continuous shaking at 200rpm. The culture was then heated at 65-70°C for 20 minutes and centrifuged at 2500rpm for 15 minutes. The supernatant containing the rescued phagmids was collected in sterile tubes and mixed with SOLR cells (OD₆₀₀ = 1.0) and incubated at 37°C for 15 minutes. The cells were plated on to LB agar plates containing 100µg/ml ampicillin and incubated overnight at 37°C. Colonies appeared on the plate. A single colony was then picked and inoculated in 1ml of LB containing ampicillin and grown overnight. The overnight grown culture was used to isolate plasmid DNA. Plasmids DNA were digested with appropriate restriction enzymes (EcoR1 and Xho1) and run on agarose gel to see the size of the released fragment. Alternatively these plasmids DNA were also used to sequence the clones.

2.24: Visualization of Proteins on SDS-PAGE:
SDS- Polyacrylamide gels containing more than 2µg protein concentration were visualized by standard Coomassie Brilliant Blue (CBB) staining; 0.1% w/v CBB dissolved in 25% (v/v) methanol and 10% (v/v) acetic acid in water, followed by destaining in 25 % (v/v) methanol and 10% (v/v) acetic acid in water.

2.25: Protein Expression:
Glutathione S. Transferase (GST) gene fusion is an integrated system for the expression, purification and detection of fusion proteins produced in E. coli.

a) Construction of GST Fusion Proteins:
pGEX 4T-1, 4T-2, and 4T-3 (GST vectors) were digested by EcoRI (MBI fermentus) and XhoI (MBI fermentus). Novel cDNAs were also digested with the same restriction enzymes. Both vectors and inserts were ligated (1:3) with ligase enzyme (MBI fermentus), and then ligation mixes were transformed in competent E. coli DH5α or BL21 cells.

b) Expression and Purification of GST Fusion Protein:
E. coli BL21 (DE) pLys cells harboring the appropriate recombinant plasmid construct were grown overnight at 30°C in Luria Broth (LB) containing 0.1 mg ml⁻¹ ampicillin. From overnight culture, fresh LB was inoculated with the same antibiotics. After incubation, to an OD₆₀₀ of 0.4, IPTG was added to a final concentration of 0.5 mM in the culture and the cells were incubated for 3 hours at 37°C with continuous shaking. Following this the cells were harvested by centrifugation at 5000g for 10 minutes at 4°C. The harvested cells were then washed with PBS buffer (140mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.8mM KH₂PO₄, and final pH of 7.4). The cells were then resuspended at 4°C in lysis buffer (50mM HEPES, 500mM NaCl, 2mM EDTA pH 8.0, 10mM DTT, 100μM of PMSF and bacterial Protease inhibitor Cocktail), equivalent to 2% of the original culture volume. Cell lysis was performed by first incubating the cells at 10°C for 30 minutes with 0.1 mg ml⁻¹ lysozyme followed by sonication of 3 cycles (each comprising of a 30 seconds of pulse at 4W followed by incubation on ice for 1 minutes) using sonicator (150M SONIPREP). The lysed cell suspensions were further incubated with 0.1% Triton-X-100 with continuous rotation at 4°C for 1 hour. The soluble fractions were separated from insoluble fractions by centrifugation at 13000 rpm for 30 minutes at 4°C. The soluble fraction was further incubated with Glutathione Sepharose 4B gel matrix (pre-equilibrated first with PBS buffer containing 10mM DTT and 100μM PMSF and then with lysis buffer containing 10mM DTT and 100μM PMSF). After 45 minutes of incubation, the bound fraction was separated with unbound fraction by centrifugation at 5000g for 3 minutes. The column was washed thrice with washed buffer containing PBS, 100mM NaCl, 10mM DTT and 100μM PMSF and protease inhibitor cocktail (as mentioned earlier) until the solution absorbance at 214/280nm reached zero. GST fused proteins were eluted with two column volumes of elution buffer (10mM glutathione in 50Mm Tris.Cl pH 8.0). The protein was stored at −80°C until further use.

2.26: Transfection:
EcoR1 and Xho1 digested GFP-C1, C2 and C3 vectors were ligated with EcoR1 and Xho1 digested 54.12 DNA and then ligation mixes were transformed in competent E. coli DH5α cells. DNA from positive clones were made by midi prep method and used for transfection study. Rat cardiac muscle cells, H9c2 (from ATCC) and Chinese
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Hamster ovary cells were maintained in DMEM (Sigma) with 10% Serum (Gibco BRL), Penicillin-Streptomycin (100 μg/ml) and Amphotericin B (50 μg/ml). Cells were grown on glass coverslips for 24 hours till the cells were 50-60% confluent. Lipofectamine 2000 (Gibco BRL) kit was used for transfection according to the manufacturer’s instructions. H9c2 (for GFP-jun) and CHO (for GFP C1, 2 3 and 54.12 constructs) cells were transfected after serum free treatment for 12 hours with 1μg DNA (per 35 mm dish with cover slips). The cells were seen for the expression of the green fluorescence after 48 hours under the blue filter of the fluorescence Microscope (Zeiss).