MATERIALS

Genomic library: A genomic library of Arabidopsis thaliana constructed into the vector λ-EMBL-4 was kindly provided by Elliot Meyerowitz (California Institute of Technology).

cDNA heterologous probe: The CaM cDNA clone (pCM116) from electric eel, which was used as a probe, was obtained from A. R. Means (Baylor College of Medicine, Houston).

Radioactive chemicals: Alpha$^{32}$P-dCTP and alpha$^{32}$P-dATP (specific activity 3000 Ci/nmol) were obtained from BARC, Bombay and alpha$^{35}$S-dATP (1000 Ci/nmol) was procured from Amersham International.

Chemicals: DNase, RNase, Lysozyme, Chloramphenicol, Tetracycline, SDS, Tris, Dithiothreitol, Urea, BSA, Ammonium persulfate, 2-mercaptoethanol, ATP, Ethidium bromide, Acrylamide, bis-acrylamide etc., were obtained from Sigma chemical company. Sequencing grade acrylamide and urea were obtained from BRL. Yeast extract, bactoagar and bacto tryptone were obtained from DIFCO Laboratories. Luria broth mixture, agar and Murashige and Skoog medium were procured from Hi-Media Laboratories. Ficoll and polyvinyl pyrrolidone (PVP) were purchased from Pharmacia. TEMED was procured from Serva. Various Restriction enzymes, DNA polymerase I, T4 DNA ligase were purchased from Amersham International, New England Biolabs, United State Biochemicals.
(USB), BRL and Boehringer. The dideoxy sequencing kits were obtained from BRL, Sequenase version 2 sequencing and Random primer kits were purchased from USB. Membrane filters (Nitrocellulose and Nylon) were obtained from Schleicher and Schuell, Germany and Stratagene. Y-methacryloxy-Propyl Trimethoxy silane (antisiliconizing solution) was purchased from Sigma and Dimethyl-dichlorsilan (siliconizing solution) was obtained from Fluka. Other chemicals were purchased from various Indigenous sources like BDH, CDH, Glaxo, SRL, Spectrochem, Hi-media, SD-Fine chemicals and were of the highest purity available. some restriction enzymes were also obtained from Banglore Genie.

Seeds of Arabidopsis thaliana strain Columbia, C24 were procured from Prof. L. Wilmitzer’s Laboratory in Berlin.
## GENOTYPE OF BACTERIAL STRAINS

### BACTERIAL STRAINS

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>supE, thi, Δ(lac proA,B)/F', traD36, proA,B, lacIqZ M15</td>
</tr>
<tr>
<td>JM101</td>
<td>endA1, gyrA96, thi, hsdR17, supE44, relA1, traD36</td>
</tr>
<tr>
<td>JM107</td>
<td>Δ(lac proA,B)/F', proA,B, lacIqZ M15</td>
</tr>
<tr>
<td>RR1</td>
<td>supE44 hsdS20 ara-14 proA2</td>
</tr>
<tr>
<td></td>
<td>lacY1 galK2 rpsL20 xyl-5 mtl-1</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>supE44 hsdR17 recA1 endA1</td>
</tr>
<tr>
<td></td>
<td>gyrA46 thi relA1 lacF'[proAB⁺ lacIq lacZ M15 Tn10(tetR)]</td>
</tr>
<tr>
<td>MC1029</td>
<td>araD139, (ara,Leu)7697, Δ(lacZ)M15, galU, galK, strA, recA56</td>
</tr>
<tr>
<td>K803</td>
<td>hsdR⁺, hsdM⁺, gal⁻, met⁻, supE</td>
</tr>
</tbody>
</table>
METHODS

SCREENING OF GENOMIC LIBRARY BY PLAQUE HYBRIDIZATION

Preparation of bacterial cells for transduction: E. coli strain K803 was used as host for the genomic library screening. The library was constructed in vector λ-EMBL-4. A fresh bacterial colony from an agar plate was inoculated into 50 ml of LB broth supplemented with 0.2% filter sterile maltose and 0.01 M MgSO₄ and incubated overnight at 37°C. The culture was centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was discarded and the resulting pellet was suspended in 10 mM MgSO₄ at 1/10th of the original volume. A fraction of the cells was used for transduction and the remaining cells were kept at 4°C up to one week for further use.

Estimation of titer of the genomic library: In order to determine the number of phage particles, the phage lysate was serially diluted into phage buffer and mixed with 0.2 ml of the above bacterial cells. The mixture was incubated for 30 min at 37°C and plated with molten top agar (45-50°C) onto LB plate and incubated at 37°C overnight. The number of plaques were counted and total plaque forming units (pfu) were determined taking the dilution factor into consideration.

Plating of genomic library and transfer of plaques on to nitrocellulose for hybridization: To screen a library of
Arabidopsis (genomic size 7 x 10^7 bp), a total of 1600 plaques should be examined for one genome representation. Since the host strain of \(\lambda\)-EMBL 4, K803 does not discriminate between recombinant and nonrecombinant phages, a higher number of plaques of the Arabidopsis genomic library (50,000) was plated. The approximate number of pfu (after determining the titer of the genomic library) in a volume of 50 ul or less was mixed with 0.2 ml plating bacterial cells and incubated at 37\(^\circ\)C for 20 min. Three ml of top agarose (0.7% in LB) at 50\(^\circ\)C was added and poured after mixing onto a 82 mm plate containing LB medium. The plates were incubated at 37\(^\circ\)C until the plaques visible but confluent lysis of the lawn was avoided. The plates were chilled at 4\(^\circ\)C for an hr to allow the top agar to harden for transfer of the plaques, nitrocellulose circles were carefully placed onto the agarose surface avoiding trapping of any air bubbles. Three asymmetric marks were made through the nitrocellulose using a sterile needle. The position of the punctures was marked on the bottom side of the plates using a marker. After a min of laying the filter on the plaques surface, the filter was removed carefully using a fine forcep and transferred to a Whatman 3 MM paper with their plaques surface side up. Immediately after the transfer of every filter, the plate as well as filter were numbered using a marking pencil. The filters were then transferred to a plastic tray containing two layers of 3 MM papers soaked with denaturing buffer (1.5 M NaCl and 0.5 M NaOH). After 5 min, the filters were transferred to a tray containing
two papers of 3MM paper soaked with neutralizing solution (1.5 M NaCl, 0.5 M Tris-Cl, pH 7.6) and left for 5 min. The filters were then rinsed in 2X SSC and placed on Whatman 3 MM paper to dry at room temp. They were baked at 80°C for 2 hrs and stored under vacuum till use.

**Plaque hybridization:** The baked filters were floated on 6 X SSC in tray until they become thoroughly wet from beneath and then submerged for 5 min. The filters were transferred one by one to a circular glass dish and stacked one over the other. About 50 ml of free washing solution (50 mM Tris-Cl, pH 8.0, 1 M NaCl, 1 mM EDTA and 0.1% SDS) was added and incubated at 42°C, with shaking for one hr to remove sticking debris from the filters. The filters were removed and transferred to a sterile 82 mm petriplate containing the prehybridization solution (1 ml per nitrocellulose circle) (50% formamide, 5X Denhardt's solution, 5X SSPE, 0.1% SDS and 100 ug/ml denatured salmon sperm DNA) and incubated at 42°C with slow shaking for 2-4 hrs. The 32p-labelled DNA probe was denatured by heating at 100°C for 5 min and added to the prehybridization solution and incubated at 42°C for 16-24 hrs. During hybridization the petriplates were kept tightly covered and sealed with Saran wrap to prevent the evaporation of fluid.

After the hybridization period, the filters were quickly transferred to a tray containing large volume of 2X SSC containing 0.1% SDS at room temperature and washed in the same
solution with 3-4 changes for 5-10 min each time. The filters were then washed twice in 0.5X SSC and 0.1% SDS for 1-1.5 hr at 50°C. (moderate stringency). The filters were wrapped between Saran wrap and after applying radioactive ink as small dots at three asymmetric places outside the area of filters, they were exposed to Indu X-ray film at -70°C with an intensifying screen for two days. After developing, the films were aligned with the filters using the marks left by the radioactive ink on the Saran wrap. By aligning these dots on the X-ray film with the three asymmetric dots on the plates, the areas of positive plaques were identified. A small area containing several plaques was picked up for each positive signal using a sterile Pasteur pipette. They were transferred to microfuge tubes containing phage buffer and a drop of chloroform. It was left at room temperature for 2 hrs for the phages to diffuse out. The phages from these suspensions were replated and screened again by hybridization. Less than 500 plaques per plate were plated and the positive clones after the second round of screening were separated by the same manner as described above. Usually more than one plaques were collected in the agar plug of the second round. A third round of screening of the plaques collected from the second round was done by plating 100-200 phages per plate. After the third round, a well-defined positive recombinant phage clone was picked up and its homogeneity was confirmed by re-plating and screening it for the fourth time. The positive recombinant phage clones were picked and DNA from each clones was isolated for further analysis.
MEDIUM SCALE ISOLATION OF λ-PHAGE DNA

Preparation of phage stock: About 10^5 pfu of bacteriophage were mixed with 0.1 ml of K-803 bacterial cells prepared as described earlier and incubated at 37°C for 20 min. After the pre-adsorption step, 3 ml of molten top agar (0.8% agar in LB medium) at 45°C was added, mixed well and poured onto a LB plate. After the top agar was solidified, the plate were inverted and incubated for 8-12 hr or until confluent lysis appeared. Five ml of SM was added and the plate was left at 4°C for several hours with intermittent gentle shaking.

The E.coli strain K803 was grown overnight in LB medium containing 0.2% maltose. One ml of this was used as an inoculum for 100 ml of LB medium containing 5 mM CaCl_2 and 10 mM MgCl_2 and grown till OD_{550} reached 0.2. At this point the phages were added at an moi of 0.01 (10^9 phage particles for 1 liter) and incubated at 37°C with vigorous shaking for 3-5 hrs or until visible lysis occurred. One ml of chloroform was added to each flask and shaken at 37°C for 15 min. The lysate was centrifuged at 10,000 rpm for 15 min to remove cell debris. The phage titer of the supernatant was usually between 10^{10} to 10^{11}/ml. Pancreatic DNase and RNase both to a final concentration of 1 ug/ml were added to the supernatant and incubated at 37°C for 30 min. Six grams of solid NaCl was added and dissolved. PEG 6000 was added to give a final concentration of 10% (w/v), mixed by swirling and left on ice for atleast 2 hr. The phage pellet was collected by centrifuging at 5000 rpm for 20 mins and resuspended in 10 ml of solution.
containing 100 mM NaCl, 50 mM Tris-HCl, pH 7.5 and 10 mM MgSO₄. To
the phage suspension, solid KCl was added in 4 equal aliquots,
mixing well each time to a final concentration of 1 M and left on
ice for 15-30 min. It was centrifuged at 10,000 rpm for 10 min at
4°C in Sorvall SS34 rotor to pellet down the PEG that was
precipitated by KCl. A step gradient of CsCl in TM (10 mM Tris-
HCl pH 7.5 and 10 mM MgSO₄) in an ultracentrifuge tube (SW41
rotor) was made as follows:

<table>
<thead>
<tr>
<th>CsCl Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.45 g/ml CsCl</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>1.50 g/ml CsCl</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>1.70 g/ml CsCl</td>
<td>2.0 ml</td>
</tr>
</tbody>
</table>

The phage suspension was loaded on the top of the gradient
and centrifuged at 25,000 rpm at 15°C for 2 hr. The phages that
banded at the interphase of the 1.45 g/ml and the 1.5 g/ml
solution of CsCl were collected by aspirating from the top. The
phages were dialysed against 1000 fold volume of 10 mM Tris-HCl,
ph 8.0 and 10 mM MgCl₂ for 2 hr with one change of the buffer.
The DNA from the purified phages was extracted with equal volume
of phenol, by agitating gently for 20 min and centrifuging at
10,000 rpm for 10 min. The aqueous phase was extracted with
phenol twice more and then twice with an equal volume of
chloroform. The aqueous phase precipitated with two volumes of
cold ethanol. The fibrous DNA precipitate was spooled out using a
glass rod or a sealed capillary tube and washed with 70% 
ethanol. After drying briefly, the DNA was dissolved in TE (10 mM
Tris-HCl, pH 8.0 and 1 mM EDTA). The DNA obtained by this procedure was digestible by various restriction enzymes and the yield was remarkably high (about 400 ug of DNA/100 ml phage lysate).

**SMALL SCALE ISOLATION OF λ-PHAGE DNA**

Growth of bacteria and infection with the phage were as described above for the medium scale preparation of phage DNA. Five ml of the lysate after treating it with chloroform was centrifuged and the supernatant was treated with pancreatic DNase and RNase (both at a final concentration of 1 ug/ml) at 37°C for 30 min. An equal volume of 20% (w/v) PEG 6000, 2 M NaCl/10 mM MgSO₄ was added, mixed and left on ice for one hr. The phage pellet was recovered by centrifuging at 10,000 xg for 20 min at 4°C in the Sorvall SS34 rotor. Supernatant was aspirated out and the phage pellet was suspended in 0.3 ml of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl and 10 mM MgSO₄ and transferred to a microfuge tube. It was extracted twice with equal volume of phenol and the phases were separated by brief centrifugation. The resulting aqueous phase was extracted with chloroform. To the aqueous phase, 30 ul of 3 M sodium-acetate was added and mixed. The DNA was precipitated with 600 ul of chilled ethanol. The DNA was washed with 70% ethanol, dried briefly under vacuum and dissolved in 50 ul TE. Five ul was used for restriction enzyme digestion.
LARGE SCALE PREPARATION OF PLASMID DNA:

The alkaline lysis method of Birnboim and Doly, (1979) was followed for the isolation of plasmid DNA as described by Maniatis et al., 1982, while the Kreig and Melton protocol (Promega Biological Research Products) was used for the isolation of supercoiled plasmid DNA for double stranded sequencing. Fifty ml of LB medium containing appropriate antibiotics was inoculated with a single bacterial colony and was grown overnight at 37°C with vigorous shaking. This overnight culture was used to inoculate 500 ml of LB medium with the antibiotics to get a initial OD$_{600}$ of 0.05 and then allowed to grow till OD$_{600}$ was approximately 0.4. For amplification of the plasmid DNA, chloramphenicol was added to a final concentration of 170 ug/ml from a stock of 34 mg/ml in ethanol. It was further incubated at 37°C with vigorous shaking for additional 12-16 hrs. The bacterial cells were harvested from the culture by centrifugation at 4000 xg for 10 min at 4°C and the cells were washed in 100 ml of ice cold STE (0.1 M NaCl, 10 mM Tris-Cl, pH 7.8 and 1mM EDTA).

The cell pellet was resuspended in 10 ml of solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA), and 5 mg/ml of lysozyme was added to it, and was incubated at room temperature for 5 min. To this 20 ml of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added and the contents were gently mixed by inverting the capped tube several times and left on ice for 5 min. Fifteen ml of an ice-cold solution of 3 M potassium acetate
(pH 4.8) was added and contents were mixed by inverting the tube sharply several times and kept on ice for 10 min.

The lysate was centrifuged at 10,000 rpm for 20 min in a Sorvall SS34 rotor. The supernatant was collected by filtering through a layer of miracloth to retain the floating debris. To the clear supernatant, 0.6 volume of isopropanol was added, mixed well and left at room temperature for 15 min. The DNA precipitate was collected by centrifuging at 12,000 rpm for 20 min at room temperature and washed once with 70% cold ethanol. The pellet was dried briefly under vacuum and dissolved in 8ml of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA).

CsCl-density gradient centrifugation: For every ml of the DNA solution one gram of solid CsCl was added and mixed to dissolve the salt. Ethidium bromide was added from a stock of 10 mg/ml to get a final concentration of 600 ug/ml. The final density of the solution was 1.55 g/ml ($\gamma = 1.3860$). The cesium chloride-DNA solution was transferred to TV865 rotor tubes and the tubes were filled to it capacity with liquid paraffin oil. Centrifugation was carried out in Sorvall OTD-75B ultracentrifuge for overnight (16 hrs) at 45,000 rpm at 15°C. Upon long UV illumination of the tube after centrifugation, two bands were usually visible, the upper band consisting of linear bacterial DNA and nicked circular plasmid DNA and the lower band consisting of closed circular plasmid DNA. The lower band was collected by carefully aspirating it from above. Ethidium bromide was removed by
extraction with TE saturated n-butanol and the DNA was precipitated with ethanol after three fold dilution. The precipitate was collected by centrifugation, washed with 70 % ethanol, dried in vacuum and dissolved in TE.

**Rapid isolation of plasmid DNA:** The procedure was adapted from the protocol of Kreig and Milton (Promega Protocol, 2nd edition). A single bacterial colony was inoculated in 250 ml of LB medium containing 50 ug/ml appropriate antibiotic and was grown overnight on a shaker at 37°C. The overnight culture was suspended thoroughly in 6 ml of ice-cold freshly prepared lysis buffer (25 mM Tris-HCl, pH 8.0, 10 mM EDTA and 50 mM glucose). The suspended mixture was incubated on ice water for 10 min, 12 ml of freshly prepared solution containing 0.2 N NaOH and 1% SDS was added and it was mixed gently and further incubated on ice water for 10 min. To the above solution, 7.5 ml of 3 M sodium acetate, pH 4.6 was added and mixed by inversion and was incubated on ice water for 20 min. The above lysate was centrifuged at 12000 x g for 15 min and supernatant was transferred to another tube. DNase-free RNase was added to a final concentration of 20 ug/ml and it was incubated at 37°C for 20 min. Supernatant was extracted twice with 1 volume of TE saturated phenol/chloroform and finally once with one volume of chloroform : isoamyl alcohol (24 : 1). The aqueous phase was transferred to a corex tube and the DNA was precipitated with ethanol. The DNA pellet was dissolved in 1.6 ml of sterile water.
and 0.4 ml of 4 M NaCl added and mixed. The DNA was precipitated by addition of 2 ml of 13% PEG. It was mixed and incubated on ice water for 60 min. The DNA precipitate was collected by centrifugation at 12,000 x g for 10 min and pellet was washed thoroughly with 5 ml of 70% ethanol. The pellet was dried under vacuum and was dissolved in appropriate volume of TE buffer.

**Small scale isolation of plasmid DNA:** The procedure is based on the alkaline lysis method of Birnboim and Doly (1979). All operations were carried out at room temperature in an Eppendorf microfuge tube. Bacteria were grown to saturation in 5 ml of LB with selective antibiotics. Cells were harvested by 2 min centrifugation and resuspended in 200 ul of solution I (50 mM glucose, 10 mM EDTA, and 25 mM Tris-HCl, pH 8.0). They were lysed by the addition of 400 ul of freshly prepared solution II (0.2 N NaOH and 1% SDS), mixed gently and cooled on ice for 5 min. Three hundred ul of solution III (3 M potassium acetate pH 4.8) was added, mixed well and left on ice for 30 min. The precipitate was removed by 5 min centrifugation. The supernatant was mixed with 0.6 volume of isopropanol and left on ice for 5 min. The nucleic acid pellet was collected by a 5 min centrifugation, washed with 70% ethanol and the pellet was dried and dissolved in 50 ul of 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1mM EDTA and 20 ug/ml of RNase A. After 30 min of incubation at 37°C, the sample was extracted with phenol, phenol:chloroform:isoamylalcohol (25:24:1) and chloroform, successively. To the aqueous phase, two volumes of ethanol was added, mixed and
incubated at -70°C for 10-30 min. The precipitate was collected by centrifugation for 5 min, washed with 70% (v/v) ethanol, dried briefly under vacuum and dissolved in 20 ul of TE. Four ul was used for restriction enzyme digestion.

**ISOLATION OF DNA FROM ARABIDOPSIS SEEDLINGS**

**Plant material:** Arabidopsis seeds were surface sterilized in 80% ethanol followed by sterilization in 5% sodium hypochlorite for 10-15 min and after thorough rinsing in sterile water the seeds were dispersed into flasks containing half strength MS medium (Murashige and Skoog, 1962) with 0.8% agar. The seeds were vernalized by keeping flasks at 4°C for 4 days and grown under continuous light at 25°C. DNA was isolated from four weeks old plants.

The procedure used for DNA isolation was adapted from the method of Murray and Thompson, (1980). Seedlings were ground to fine powder using a mortar and pestle in the presence of liquid nitrogen. The powder was transferred to a solution (2ml/g of tissue) containing 100 mM Tris-HCl, pH 8.0, 100 mM EDTA, 250 mM NaCl and 100 ug/ml of proteinase K, and was swirled to suspend the tissue thoroughly. The suspension was made 1% with respect to Sarkosyl by adding a 10% of its stock solution and mixed gently. It was then left at 50°C for one hr with intermittent mixing by gentle shaking. The lysate was centrifuged for 10 min at 7500x g at 4°C. Isopropanol (0.6 volume) was added to the supernatent
and mixed gently to precipitate the nucleic acids. Nucleic acids precipitate was recovered by centrifugation at 7500x g for 15 min at 4°C, and dissolved in TE. The DNA solution was adjusted to a final concentration of 0.7 M NaCl by addition of 5 M NaCl and mixed thoroughly. To it, 10% CTAB solution containing 0.7 M NaCl was added to produce a final concentration of 1% CATB, mixed well and incubated at 65°C for 10 min. It was extracted with an equal volume of chloroform : isoamyl alcohol (24:1) and after centrifugation (13,000x g, 10 min) the aqueous phase was removed with a large bore pipette. One tenth volume of 10% CATB containing 0.7 M NaCl was added and the chloroform/isoamyl alcohol step was repeated. The final aqueous phase was clear and it was transferred to a fresh tube. Nucleic acids were precipitated by adding 0.6 volume of isopropanol. The tube was gently shaken back and forth until a white fibrous DNA precipitate became clearly visible and it was transferred to a fresh tube containing 70% ethanol by hooking the precipitate onto the tip of a Pasteur pipette. DNA was pelleted down by centrifugation, dried briefly, dissolved in TE and its concentration was determined spectrophotometrically by measuring the absorbence at 260 nm. The purity of the DNA was monitored by measuring OD at 260 and 280 nm.

**SUB-CLONING OF DNA FRAGMENTS INTO PLASMIDS**

In order to construct recombinant DNA molecules, the DNA was digested with appropriate restriction enzyme and the fragments
were separated on gel electrophoresis. The fragments of interest for subcloning were isolated by either of the following two methods: (i) electroelution onto DEAE cellulose paper and (ii) agarose freezing - thawing method.

**Purification of DNA fragments by electrophoresis onto DEAE-cellulose paper:** Strips of DEAE cellulose paper (DE-81) were soaked in 2.5 M NaCl for several hr and washed several times in water. The fragments of the digested DNA were separated by electrophoresis on agarose gel and visualized under long wave UV-illumination after staining with ethidium bromide (0.5 ug/ml). While visualizing the fragments a small piece of the gel was cut out just in front of the band(s) to be recovered and slightly larger than the band size. The gel piece was taken out and a piece of soaked DEAE cellulose paper was vertically inserted into the slit adjacent to the DNA band and the gel piece was placed back into the slit such that the DEAE paper is fixed inside the incision close to the DNA band. Electrophoresis was resumed until DNA had transferred onto the paper strip. Then the agarose piece was removed, DEAE cellulose paper taken out carefully, washed in sterile water and transferred to an microfuge vial containing 0.5 ml of the DEAE elution buffer (1.5 M NaCl, 1mM EDTA and 20 min Tris-HCl, pH 7.5). The tube was vortexed to shred the paper and incubated at 37°C for 2 hr. The suspension was centrifuged. Loose DEAE cellulose paper was compressed to the bottom with the help of a sterile Pipetman tip and centrifuged again. The eluate was extracted with one volume of buffered
phenol and the DNA was precipitated with ethanol and washed with 70% ethanol. The DNA pellet was dried and dissolved in desired volume of TE or sterile water.

**Agarose freezing-thawing method:** The fragments of digested DNA were separated by electrophoresis on low percentage (0.8%) agarose gel and visualized under long wave UV-illumination after staining with ethidium bromide. The desired DNA band with agarose gel was cut out and transferred to an microfuge vial containing sufficient buffered phenol to cover the gel piece and was incubated at -70°C for 30 min. After the completion of incubation time, the vial containing the gel was microfuged for 20 min at 4°C. The aqueous phase was recovered and once it was extracted with chloroform-isoamyl alcohol (24:1). The DNA was precipitated with 2 volumes of ethanol dried and dissolved in desired volume of sterile water.

**Ligation of the insert fragments into the vector DNA:** The vector and the insert fragments were prepared by digesting with appropriate restriction enzyme and purifying by electroelution as described in the previous section. Alternatively, a shot-gun cloning strategy was followed when more than one fragments were to be sub-cloned.

The ligation reaction was set up as follows:

1 ul  10X ligation buffer
1 ul  10 mM ATP
200-500 ng target and the vector DNA
1 unit T4 DNA ligase (5 unit for the blunt end ligation)

The concentration of the total target and vector DNA in the ligation reaction was kept at 20-50 ng/ul and the vector to target DNA ratio was maintained between 1:2 to 1:5.

The ligation reactions were incubated at 12-15°C overnight and were used for transformation directly or after diluting the reaction mixes appropriately. Selection of the transformants was made by virtue of the antibiotic marker present in the vector.

Transformation of *E. coli* with plasmid DNA: For the pUC plasmids, *E.coli* strain MC1029 or JM101, for Bluescript phagemid DNA, *E. coli* strain XL1-Blue, for pBR322 plasmid DNA, *E.coli* strain RR-1 and for M13 RF DNA, *E.coli* strain JM101 were used for transformation. The LB plates used for transformation contained 100 ug/ml of ampicillin, 20 ug/ml of X-gal and 32 ug/ml of IPTG, or 15 ug/ml of tetracycline, depending on the type of the plasmid used.

Preparation of competent cells used for transformation: The competent cells of the bacteria were prepared by the CaCl₂ treatment (Mandel and Higa, 1970) with minor modifications. Bacterial cells were grown to saturation density at 37°C, overnight. Flask containing 100 ml of LB medium was inoculated with one ml of the overnight culture and grown with vigorous shaking at 37°C till OD₆₀₀ was 0.3-0.4. The culture was chilled
on ice for 15-20 min, transferred to a 50 ml Sorvall centrifuge tube and centrifuged at 5000 rpm for 5 min at 4°C. The cell pellet was gently resuspended in half of the original volume of ice cold 100 mM MgSO₄ and recentrifuged at 4°C. The pellet was gently suspended in half of original volume of the ice-cold 50 mM CaCl₂, 10 mM Tris-HCl (pH 8.0) by gently swirling the tube and was incubated on ice for 30 min. The cells were then collected by centrifugation at 5000 rpm for 5 min at 4°C and were resuspended in 1/10th the original volume of ice cold 50 mM CaCl₂, 10 mM Tris-HCl (pH 8.0) by gently swirling the tube, and stored on ice till use. The competent cells were used for transformation up to 2 days after the preparation of the cells.

**Transformation protocol:** Sterile 10 ml glass test tubes were labelled appropriately and chilled on ice. The competent cells (0.2 ml) of appropriate *E.coli* host strain were aliquoted into each tube. DNA in the ligation buffer or in TE was added to the cells, mixed and stored on ice for 30 min. The tubes were heat-shocked at 42°C for 2 min. When pUC, bluescript or pBR plasmids were used for transformation, 1 ml of LB medium was added subsequently to each tube and incubated at 37°C for 30 min (tetracycline selection) or one hour (ampicillin selection) without shaking. An appropriate quantity of cells (0.1-0.4 ml) were plated onto selective media and incubated at 37°C for bacterial colonies to appear.

**Top agar plating protocol for JM101 cells transformed with M13 RF DNA:** To the JM cells and M13 RF DNA after the heat shock
treatment as described above, 3 ml of melted top agar (0.8% agar in LB), 10 ul of 100 mM IPTG, 50 ul of 2% X-gal and 200 ul of an exponential lawn culture of the same strain were added, mixed well and poured onto LB plates. After the solidification of the top agar the plates were incubated at 37°C overnight for the M13 plaques to appear.

TRANSFER OF DNA FROM AGAROSE GELS TO NITROCELLULOSE PAPER

The protocol for Southern transfer of DNA followed was essentially as described by Maniatis et al, 1982. The DNA fragments were separated by electrophoresis on agarose gel and the gel was photographed after staining it with 0.5 ug/ml of ethidium bromide. The gel was then transferred to a tray containing 0.25 M HCl and agitated for 15 min. This acid depurination step brings about partial hydrolysis of the DNA before alkali denaturation and helps in the transfer of large DNA fragments. The DNA was denatured by soaking the gel in several volumes of 1.5 M NaCl and 0.5 M NaOH for 1 hour with constant shaking. The gel was then neutralized by soaking in several volumes of 1 M Tris-HCl, pH 8.0, and 1.5 M NaCl for 1 hr with constant shaking. Meanwhile a sponge of desired dimensions and with a width of 1 and 1/2" was placed in a dish and 10 x SSC solution was poured to one half of the height of the sponge. Two layers of Whatman 3 MM paper cut into the size of the gel and wetted with 10x SSC were placed on the sponge.
The gel, after neutralization, was placed upside down on these 3 MM papers. A piece of nitrocellulose filter or nylon paper cut into desired size was floated on a solution of 2X SSC until it became completely wet. The wet nitrocellulose paper was then placed carefully on top of the gel, such that one end extended just over the lines of slots at the top of the gel and avoiding trapping of air bubbles between the gel and the filter. All four edges of the nitrocellulose filter were covered by thin strips of plastic sheet (or Saran wrap) in order to prevent short circuiting of fluid between the gel and stack of the blotting paper. Two pieces of 3 MM paper cut to the size of the gel were placed over the membrane filter after wetting them in 2X SSC. A stack of blotting papers of 1" to 2" height was placed over the 3 MM papers and the whole pyramid was kept pressed by keeping a heavy glass plate on the paper stacks. The transfer of DNA was allowed to proceed for about 15 hrs. The paper stack, when became wet, was replaced. At the end of the transfer, the paper towels and the 3 MM papers were removed, the positions of the gel slots on the nitrocellulose filter were marked with a marking pencil and the membrane filter was peeled off from the gel using a forcep, and transferred to 6X SSC. After 5 min, the filter was taken out, excess fluid allowed to drain off and dried at room temperature on a sheet of 3 MM paper. Then the nitrocellulose was baked for 2 hrs at 80°C. The baked filters were stored under vacuum at room temperature till use. Alternatively, the nitrocellulose or nylon filter was crosslinked by Stratagene UV
crosslinker at the optimal condition of 120,000 micro joules/cm². For this one or two sheets of absorbent paper soaked in 10X SSC were kept on the floor of the UV crosslinker. Slightly dempended filters were placed into the UV crosslinker, with the nucleic acid side up. After the UV exposure, the filters were dried at room temperature on a sheet of 3 MM paper.

**LABELLING THE DNA PROBES**

The DNA probes used for the hybridization experiments were labelled either by nick translation (Rigby et al., 1977) using *E. coli* DNA polymerase I or by the random primer labelling method (Feinberg and Vogelstein, 1983) using the USB random primer kit. The nick translation reaction was set up by aliquoting 2.5 μl of 10X nick translation buffer (0.5 M tris-HCl, pH 7.2, 0.1 M MgSO₄, 10 mM dithiothreitol and 500 μg/ml BSA), 2.5 μl of 0.5 mM unlabeled dNTP mix (containing the dNTPs other than the labeled dNTP), 0.1 to 0.5 μg of DNA to be labeled, 30 μCi of 3000 Ci/mmol alpha-³²P dATP or alpha-³²P dCTP, 0.5 μl DNase I (diluted 1000 times from a stock of 10 mg/ml), 5 U DNA polymerase I (BRL) and sterile water was added to make 25 μl of the final volume. The contents were mixed well and incubated at 15°C for 60-90 min. The reaction was stopped with the addition of 2 μl of 0.5 M EDTA. The nick-translated DNA was separated from unincorporated dNTPs by chromatography on a Sephadex G-50 column packed in a sterile Pasteur pipette plugged with sterile glass wool. The column was
washed with several volumes of TE. The nick translated reaction mixture was applied onto the column in a volume of 100 μl. A reservoir of TE was connected to the column and the flow rate was adjusted manually using a clamp connected to the tubing, such that the flow rate was equivalent to that of the eluate. The eluate was collected as 0.5 ml fractions (up to 10 fractions) into microfuge tubes. The radioactivity in each of the tubes was measured by dispensing 1 μl of the fraction onto small blotting papers and counting them in 5 ml of scintillation fluid (0.4 g PPO and 0.025 g POPOP dissolved in 1 litre of toluene) in a liquid scintillation counter. The DNA excluded from the Sephadex gel was collected by pooling the fractions that represented the leading peak of the radioactivity. The purified labeled DNA probe was stored at -20°C till use. Just before its use for hybridization, the DNA probe was denatured by boiling at 100°C for 5 min and then chilling it on ice. The DNA by this method was usually labeled to a specific activity of 5 x 10^8 to 2 x 10^9 cpm/μg of DNA.

The USB random primer labelling kit was also used to label the DNA fragments. DNA was denatured by heating for 10 min at 95°C in a volume of at least 10 μl and then chilled on ice. In a separate microfuge tube 3 μl of three dNTP mixture (excluding the labelled deoxynucleotide triphosphate) in a ratio of 1:1:1 was mixed with 2 μl of the reaction mixture (random hexanucleotides in 10X reaction buffer) and 5 μl alpha-32P deoxynucleotide (50 uCi, 3000 Ci/nmol) and after mixing it thoroughly it was
transferred to the vial containing denatured DNA. To this 1 ul Klenow enzyme (2 units) was added and reaction was continued at 37°C, for 30 min to 2 hrs. Reaction was terminated by adding 2 ul of 0.2 M EDTA, pH 8.0 or by heating to 65°C for 10 min. By this method, the DNA was usually labelled to a specific activity of $1.8 \times 10^9$ cpm/ug of DNA. The labeled DNA obtained by this method was used directly without removing the non-incorporated dNTPs.

HYBRIDIZATION OF SOUTHERN FILTERS

The baked or irradiated filter was floated on to 6x SSC till it became wet and then soaked in the same solution for 2 min. It was then transferred to a plastic box or hybridization tube supplied by Robbins Scientific containing the prehybridizing solution warmed to 42°C. Two to ten ml of prehybridizing solution was used for each square centimeter of the filter. Prehybridization was carried out for 2-4 hr. The prehybridization solution contained the following: 5X SSPE, 0.5% SDS, 5% Denhardt's solution, 50% Formamide and 100 ug/ml denatured salmon sperm DNA. For hybridization $^{32}$P-labeled denatured DNA probe was added directly to the prehybridization solution and the incubation continued. When more than one filters were being hybridized, the incubation was carried out with gentle agitation. Hybridization was carried out for 4-8 hr in case analysis of cloned DNA and for 16-24 hr in the case of Southern blots of
genomic DNA. After carrying out the hybridization for sufficient length of time, the filter was removed and transferred quickly to a solution of 2X SSC and 0.5% SDS and agitated at room temperature for 5 min. The filter was then transferred to a fresh tray with 2X SSC and 0.1% SDS and agitated for 15 min. It was repeated once more. After washing at the room temperature, the filter was washed at high stringent conditions when homologous DNA probe was used or at moderate and low stringencies when heterologous probe was used. For high stringency washes, the filters were incubated in 0.1x SSC and 0.1% SDS at 65°C for 1 to 2 hr with gentle agitation. The washing solution was changed once in the middle. For moderate stringency, the filters were washed in 0.4x SSC and 0.1% SDS at 50°C for 1 hr and for the low stringency, the washings were done in 2x SSC and 0.1% SDS at 60°C for 1 hr.

After washing, the hybridized filters were wrapped in Saran wrap and exposed to X-ray film with an intensifying screen at -70°C. Radioactive ink was applied in an asymmetric manner on the outer surface of the Saran wrap to facilitate the alignment of DNA lanes on the filters with autoradiographic image.

CLONING OF DNA FRAGMENT INTO M13 VECTOR AND SEQUENCING

Cloning of the DNA fragments into M13mp vectors for sequencing by the dideoxy chain termination method: Single stranded DNA templates were generated after cloning into the M13mp vectors and were used for sequencing (Carlson and Messing, 1984). The
presence of convenient restriction sites in the CaM gene facilitated the cloning of small fragments in both the orientations of M13mp18 and M13mp19 vectors. This allowed sequencing of both the strands. In cases, where the fragments were flanked by the same restriction sites, the opposite strands were identified by one track analysis.

For the construction of clones of the various fragments into a M13 vector for sequencing, the target DNA was cut with various restriction enzymes and the digests were incubated with M13 DNA cleaved with the enzymes meant for only sub-cloning single fragments. The target and M13mp RF (replicative form) DNAs were digested with two sets of enzymes and ligated for bidirectional cloning of the sub-fragments of the target DNA for sequencing.

**Transfection:** Small volume of ligated mixture (1/10th the original volume) was used to transfect the M13 host cells, JM101 or JM107 as described under transformation of *E. coli* with plasmid DNA. Upon plating in the presence of X-gal and IPTG both blue and colorless plaques were appeared. Colorless plaques containing recombinant phages were used for the isolatin of single stranded DNA.

**Size selection of subclones:** Colorless plaques which were picked separately and used for infecting 2 ml of LB medium containing 1:50 dilution of overnight culture of JM101. The infected culture was grown with vigorous shaking at 37°C for 5
hr. The size selection of the clones was done by direct gel electrophoresis of single stranded DNA isolated from a number of clones. A small volume (200 ul) of the culture was centrifuged to pellet the cells. Two microlitre of SDS solution (2% SDS, 10 mM EDTA, 10 mM Tris-cl, pH 7.4 and 50% glycerol) was added to 20 ul of the supernatent, mixed and incubated at 65°C for 5 min and was loaded on a 0.7% agarose gel. The approximate size of the insert in the clone was determined by running ss DNAs containing insert of known size as a marker. The gel was stained with ethidium bromide (0.5 µg/ml) after the electrophoresis and photographed. The DNA was isolated from the desire culture by the method as described under "Isolation of single stranded DNA for sequencing". The sequencing of the insert in the clones was performed using the dideoxy chain termination method (Sanger et al., 1977).

Isolation of M13 RF DNA: A single colony of E.coli, JM101 or JM107 cell grown on M9 plate was inoculated in 100 ml LB and incubated with shaking at 37°C till OD600 reached 1.0. To another 100 ml LB medium, an agar plug containing a blue M13mp plaque was transferred with the help of a Pasteur pipette and grown overnight at 37°C to obtain the phage stock. To 900 ml of 2X YT medium, the M13 host cells in 100 ml LB were added and grown to OD 600 of 1.5. The 100 ml phage stock was added to it and shaken at 37°C for 15-30 min to facilitate infection. Chloramphenicol was added to a final concentration of 15 µg/ml from a stock solution of 30 mg/ml in ethanol and shaken at 37°C for 2 hr to
allow accumulation of RF DNA. The closed circular M13 RF DNA from the cells was isolated by the alkaline lysis method used for the plasmid DNA preparation and purified over cesium chloride density gradient as described under large scale isolation of plasmid DNA.

**Isolation of single stranded DNA template for sequencing:**

Transformation of JM101 cells with M13mp DNA containing an insert produce white (colourless) plaques upon growth on indicator plates. The non-recombinant phages produce blue plaques. Templates for sequencing were prepared by growing the phages from white plaques and the single stranded DNA was isolated from the viral particles.

Two ml of YT medium was inoculated with a single colony of JM101 grown on M9 minimal medium plates and grown overnight at 37°C with shaking. The overnight culture was diluted 1:50 times in YT medium to get desired volume of cells. Five ml of the diluted cells were dispensed into sterile tubes and inoculated with a well-separated white plaque. All the tubes were incubated at 37°C with vigorous shaking for 4.5-5.5 hr. The culture (~1.5 ml) was transferred to a microfuge tube and centrifuged in a microcentrifuge for 5 min at room temperature. The supernatant (~1.2 ml) was collected without disturbing the cell pellet and the pellet was saved for the isolation of RF or as stock for the clone. Three hundred ul of 20% PEG/2.5 M NaCl was added to the supernatant, briefly mixed well by vortexing and left at room temperature for 15 min. It was centrifuged for 10 min to pellet
down the phage and the supernatant was aspirated out without disturbing the pellet. The pellet was suspended in 100 ul TES buffer (20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 0.1 mM EDTA) and extracted with 50 ul of phenol saturated with TE by vortexing for 15-20 seconds and phases were separated by centrifugation for 2 min. The aqueous phase was extracted with chloroform and after brief centrifugation, the aqueous phase (~80 ul) was removed and the ssDNA was precipitated with 200 ul of ethanol after adding 9 ul of 3 M Na-acetate, pH 5.0 and left at -70°C for 30 min. The DNA was recovered by centrifugation for 10 minutes at 4°C and washed with 70% ethanol. The DNA pellet was briefly dried under vacuum and resuspended in 10-20 ul TE.

**DIDEOXY SEQUENCING REACTION**

The reactions were performed using the large fragment of DNA polymerase I (Klenow) as well as chemically modified T7 DNA polymerase (Sequenase version 2.0).

**Sequencing with Klenow:** The BRL M13 sequencing kit was used. An aliquot of each of the four dNTPs was diluted to a concentration of 0.5 mM from the stock of 10 mM and diluted dNTPs were mixed as follows:

<table>
<thead>
<tr>
<th></th>
<th>A^0</th>
<th>C^0</th>
<th>G^0</th>
<th>T^0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mM dCTP</td>
<td>20</td>
<td>1</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>0.5 mM dGTP</td>
<td>20</td>
<td>20</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>0.5 mM dTTP</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>10X Polymerase reaction buffer</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>
The dideoxy nucleotides were diluted to the following concentrations from a stock of 10 mM.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Concentration</th>
<th>Dilution Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddATP</td>
<td>0.1 mM</td>
<td>(1:100 dilution of 10 mM stock)</td>
</tr>
<tr>
<td>ddCTP</td>
<td>0.3 mM</td>
<td>(1:33 dilution of 10 mM stock)</td>
</tr>
<tr>
<td>ddGTP</td>
<td>0.5 mM</td>
<td>(1:20 dilution of 10 mM stock)</td>
</tr>
<tr>
<td>ddTTP</td>
<td>1.0 mM</td>
<td>(1:10 dilution of 10 mM stock)</td>
</tr>
</tbody>
</table>

Annealing of primer to the template: To a 0.5 ml microfuge vial, the following were aliquoted:

- 3-5 ul ss template DNA (0.5-1.0 ug)
- 1 ul M13 17mer sequencing primer (3 ng)
- 1 ml 10x polymerase reaction buffer
- x ul H₂O

Total: 12.4 ul

The contents were mixed and centrifuged briefly. The vials were heated for 5 minutes at 90-100°C and then left at room temperature to facilitate the annealing for 30-40 minutes.

During the annealing reaction, the following were aliquoted to four tubes labelled as A, C, G and T. Tube A contained 1 ul A₀ mix and 1 ul diluted ddATP, tube C contained 1 ul C₀ mix and 1 ul diluted ddCTP, tube G contained 1 ul G₀ mix and 1 ul diluted ddGTP and tube T contained 1 ul T₀ mix and 1 ul ddTTP.

When the temperature of the primer-template tube came down to below 35°C, the followings were added: 3 ul of 35S- dATP (1000
Ci/nmole), 1 ul of 0.1 M DTT and 1 ul (1.5 units) of diluted large fragment of DNA polymerase and contents were mixed thoroughly. Three to four ul of this was aliquoted to the four vials labelled A, C, G and T which contained dideoxy nucleotides and were mixed. The reaction tubes were incubated at 30°C. After twenty min, 1 ul of 0.5 mM dATP was added to each of the four tubes and after mixing it was incubated further for 15 min at 30°C (chase reaction). The reactions were stopped by adding 5 ul of dideoxy stop buffer (95% formamide, 0.05% bromo-phenol blue, 0.05% xylene-cyanol and 20 mM EDTA).

**Reaction using Sequenase Version 2.0:** The Sequenase Version 2.0 (USB) kit was used to perform the reaction.

**Annealing of primer with template:** For DNA sequencing, one ug for single stranded or 4-5 ug of double stranded DNA templates were used in the volume of 7 ul. Two ul of 5X annealing buffer and 3 ng of primer in the volume of 1 ul was used. The contents were mixed and heated for 2 min at 65°C (annealing of double stranded template can be also done by keeping the mixture at 37°C for 30 min) and then allowing it to cool slowly to room temperature over a period of 30 min. When the temperature came down to 35°C, 1 ul of 0.1 M DTT, 2 ul diluted labelling mixture (1:5 in sterile water), 0.25 ul of ^35^S- dATP (5 uCi to 10 uCi) and 2 ul (2 units) of diluted (1:8 in Sequenase dilution buffer) Sequenase Version 2.0 were added. The contents were mixed thoroughly and incubated for 2-5 min at 15-20°C.
Termination reaction: Before starting the reaction, four tubes labelled as A, C, G and T were pre-warmed to 37°C and 2.5 ul of appropriate dideoxy termination mixture were aliquoted into each of them. Then, 3.5 ul of labelled reaction mix was added to each of the four termination tubes. After thorough mixing chain termination reaction was continued for 10-15 min at 37°C. The reactions were stopped by adding 4 ul of stop solution.

DOUBLE STRANDED DNA SEQUENCING

The supercoiled plasmid DNA for double stranded sequencing was isolated using the method of Kreig and Melton (Promega Protocol, 2nd edition) as described under the heading "Large scale plasmid isolation".

Alkali denaturation of supercoiled plasmid DNA: To hybridize the primer efficiently, double stranded plasmid must be converted to a single stranded form prior to sequencing. This was accomplished by alkali denaturation of DNA. Supercoiled plasmid DNA (3-5 ug) was taken in a microfuge vial in a volume of 20 ul and to it 2 ul of 2 M NaOH and 2 mM EDTA solution were added and incubated for 30 min at 37°C. The mixture was neutralized by adding 0.1 volume of 3 M sodium acetate (pH 4.5-5.5) and DNA was precipitated with 2-4 volumes of ethanol by keeping it at -70°C for 15 min. The DNA was recovered by centrifugation at room temperature and was washed with 70% ethanol. The DNA pellet was dissolved in 7 ul of
distilled water. Sequencing reaction were performed as described for single stranded DNA sequencing.

**PREPARATION AND RUNNING OF SEQUENCING GELS**

Plates were cleaned thoroughly using detergent and rinsed finally with double distilled water. The notched plate was siliconized while the other plate was anti-siliconized with silane (Gamma-methacryloxy-propyl-trimethoxy silane) solution. The silane treatment was done thrice and left for 10 min each time. The siliconized and the anti-siliconized plates were rinsed with acetone and left to dry in an incubator. The plates were assembled with the treated side facing each other and the spacers were placed on the sides. The plates and the spacer in between were held by clamps, while the bottom side was sealed with a tape. Six percent acrylamide-urea, INSTA-GEL was prepared as follows:

<table>
<thead>
<tr>
<th>INSTA-GEL (500 ml)</th>
<th>40% Acrylamide</th>
<th>Urea</th>
<th>10X TBE</th>
<th>H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock (19:1; ml)</td>
<td>(g)</td>
<td>(ml)</td>
<td>(ml)</td>
<td>(ml)</td>
</tr>
<tr>
<td>75</td>
<td>250</td>
<td>50</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Ninety ml of Insta-gel was used for making a single gel. Seven hundred ul of 10% ammonium persulfate was added and the gel solution was degassed under vacuum. It was filtered through Whatman No.1 filter paper and 4 ul of TEMED was added. After mixing well it was poured immediately. The assembled plates were
held at 45° degree angle and acrylamide solution was poured from one corner of the plate using a 100 ml syringe containing fine needle. When the acrylamide solution reached the top of the paired glass plates, the flat surface of the sharks tooth comb was inserted in between the plates to a depth of 2-3 mm below the notched plate and it was held in between the plates with the help of clamps. The gel was left in the horizontal position for polymerization. Atleast 2 hr time was allowed for the gel to polymerize fully before running.

**Electrophoresis of sequencing gel:** The comb was carefully taken out and the top of the gel was rinsed with distilled water. The comb was washed and placed between the plates with the teeth down towards the gel. The comb was inserted down till the teeth make slight indentation in the gel surface. After this the assembly was fixed in OWL electrophoresis apparatus and both chambers (upper and lower) were filled with 1X TBE buffer. Gel was pre-electrophoresed for 30-45 min. before loading the sample. The electrophoresis was carried out at 80 Watts so that a constant temperature (approx. 50°C) of the plates was maintained.

**Sample application and Autoradiography of sequencing gel:** The DNA samples were heated at 90-100°C for 3-5 min just prior to loading. After the pre-run the wells were washed with 1X TBE using a needle and syringe to remove urea. The samples (1-2 ul) were applied using a Pipettman directly from the heating block.
Second and the third loading of the sample sets were done in a single gel at different times, so that, sequence upto a maximum possible readable point in a single auto-radiogram can be determined. In a 6% gel bromophenol blue migrates along with position of 26-mer and xylene-cyanol migrates at a position of 106-mer oligonucleotide. After the electrophoresis, the gel sandwich was pried open with a scalpel and the gel containing glass plate was transferred to a tray containing fixer (10% acetic-acid and 10% methanol) and treated for 15 min to remove urea. Plate was dried in an oven at 80°C for 3-4 hr and exposed to X-ray film at room temperature for 3-4 days.

**DNA-sequence analysis:** The nucleotide sequences were analysed using the Hitachi software package DNASIS.