METHODS

GENOMIC LIBRARY SCREENING BY PLAQUE HYBRIDIZATION

Preparation of bacterial lawn: E. coli strains K803 and NM539 were used as hosts for the genomic libraries constructed in λ-EMBL4 and λ-EMBL3 respectively. A fresh bacterial colony from an agar plate was inoculated into 50 ml of LB broth supplemented with 0.2% 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 were used for transduction and the remaining cells could be kept at 4°C upto one week for further use.

Estimation of titer of the genomic library: In order to determine the number of λ-phage particles, the phage lysate was serially diluted 10, 100, 1000 and 10000 fold in phage buffer and mixed with 0.2 ml of the above bacterial lawn. The mixture was incubated for 30 min at 37°C and plated with molten top agar (45°C - 50°C) onto LB plates and incubated at 37°C overnight. The number of plaques were counted and total plaque forming units
(pfu) were determined from the dilution factor into consideration.

**Plating of genomic library and transfer of plaques onto nitrocellulose for hybridization:** To screen a library of *Arabidopsis* (genome size 7x10^7 bp), a total of 1600 plaques should be examined for one genome representation. A total of 50,000 plaques of the *Arabidopsis* genomic library constructed in \(\lambda\)-EMBL-4 were plated using K803 as the host strain (since K803 is not a P2 lysogen, it does not discriminate between recombinant and non-recombinant phages) and 20,000 plaques of the second library of *Arabidopsis* constructed in \(\lambda\)-EMBL-3 vector, were plated using NM539 as the host (since NM539 is a P2 lysogen, it does not permit the growth of non-recombinant phages). The appropriate number of plaques (5000 pfu) (after determining the titer of the genomic library) in a volume of 50 ul or less were mixed with 0.2 ml plating bacteria and incubated at 37°C for 20 min. Three ml of top agarose (0.7% in LB) at 50°C was added and poured after mixing onto a 82 mm LB plate. The plates were incubated at 37°C until the plaques had grown enough but confluent lysis of the lawn was avoided. The plates were chilled at 4°C for an hr to allow the top agarose to harden.

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For transfer of the plaques, nitrocellulose circles were carefully placed onto the agar surface avoiding trapping of any air-bubbles. Three asymmetric puncture marks were made through the nitrocellulose using a sterile needle. The positions of the punctures were marked on the bottom side of the plates. After a min of laying the filter on the plaque surface the filter was removed carefully using a forcep and transferred to a Whatman 3 MM paper with their plaque surface side up. Immediately after the transfer of every filter was 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 layers of 3 MM paper soaked with neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0) and left for 5 min. The filters were then rinsed in 2 x SSC and placed on Whatman 3 MM paper to dry. They were baked at 80°C for 2 hrs or at 68°C for 4 hrs and stored under vacuum till use.

Hybridization of nitrocellulose filters containing replicas of bacteriophage plaques: The baked filters were floated on 6 x SSC until they became
thoroughly wet from beneath and then submerged for 5 min. The filters were transferred only by one to a circular glass crystallizing dish and stacked one over the other. About 50 ml of prewashing solution (50 mM Tris-HCl, 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/nitrocellulose circle) (50% formamide, 5 x Denhardt's solutions, 5 x 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-labeled DNA probe was denatured by heating at 100°C for 5 min to and added to the prehybridizing solution covering the filters 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 loss of fluid by evaporation.

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 and inverting the filters. The filters were then washed twice in 0.4 x SSC and 0.1% SDS for 1-
1.5 hrs at 50°C for moderate stringency (when the genomic library was screened with a heterologous probe) or in 0.2 x SSC, 0.1% SDS at 68°C for 2 hr with one change of the washing solution for a high stringent condition (when the genomic library was screened with a homologous probe). 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 X-ray films at -70°C with an intensifying screen. When Indu X-ray films were used, it took two days for the plaque signals to appear. After development, the films were aligned with the filters using the marks left by the radioactive ink on the saran wrap. The positions of the asymmetric punctures were marked on the X-ray film. By aligning these dots on the X-ray film with the 3 asymmetric dots on the plates the areas of positive plaques were identified and several plaques picked-up for each positive signal after the first screening 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 hr for the phages to diffuse out. The phages from these suspensions were replated and screened again by hybridization. Approximately 500 plaques per plate were
plated and the positive clones after the second round were separated by the same manner as described above. Usually more than one plaque was 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 and its homogeneity was confirmed by re-plating fourth time and hybridizing with the probe. The positive recombinant phage clones were picked and DNA from the clones were isolated for further analysis.

MEDIUM SCALE ISOLATION OF λ-PHAGE DNA

Preparation of phage stock: About $10^5$ pfu of bacteriophage (or 1/20 th of a re-suspended plaque) were mixed with 0.1 ml of plating bacteria (K803 or NM538 prepared as described under genomic library screening) and incubated at 37°C for 20 min. After this 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 had solidified, the plates were inverted and incubated for 8-12 hrs. When the lysis was almost confluent, 5 ml of SM was added and the plate was left at 4°C for several hrs with intermittent gentle shaking.
The *E. coli* strain, NM 538 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$^\circ$C with vigorous shaking for 3-5 hr or until visible lysis occurred. One ml of chloroform was added and shaken at 37$^\circ$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$^{10}$ and 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$^\circ$C for 30 min. Six gram of solid sodium chloride 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 at least 2 hrs. The phage pellet was collected by centrifuging at 5000 rpm for 20 min and resuspended in 10 ml of a solution containing 100 mM NaCl, 50 mM Tris-HCl, pH 7.5 and 10 mM MgSO$_4$. 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$^\circ$C in Sorvall SS34 rotor to pellet 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:

1.45 g/ml CsCl 1.5 ml
1.50 g/ml CsCl 1.5 ml
1.70 g/ml CsCl 2.0 ml

The phage suspension was loaded on the top of the gradient and centrifuged at 25,000 rpm at 15°C for 2 hrs. The phages that banded at the interphase of the 1.45 g/ml and the 1.5 g/ml solutions of CsCl were collected by aspirating from the top and dialysed against 1000 fold volume of 10 mM NaCl 50 mM Tris-HCl, pH 8.0 and 10 mM MgCl₂ for 2 hrs changing the buffer once after one hr. 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 with an equal volume of chloroform twice. The aqueous phase was adjusted to 0.3 M Na acetate and the DNA was precipitated with two vol. of cold ethanol. The fibrous DNA precipitate was spooled out using a sealed capillary tube and washed with 70% alcohol. After drying briefly (not to complete dryness), the DNA was dissolved in TE (10 mM Tris-HCl, pH 8 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 BACTERIOPHAGE λ DNA

Growth of bacteria and infection with the phage were as described for the medium scale isolation of DNA. Five ml of the lyset 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 centrifugation at 10,000 x g for 20 min at 4°C in 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 by shaking with equal volume of phenol for 20 min each time and centrifuging for 2 min to separate the phases. The aqueous phase was extracted once with an equal volume of chloroform. To the aqueous phase 30 ul of 3 M Na acetate and 600 ul of chilled ethanol were added and mixed well. The DNA precipitate
was collected by centrifugation for 2 min, washed with 70% ethanol, dried briefly under vacuum and dissolved in 50 ul TE buffer. Five ul was used for restriction enzyme digestion.

LARGE SCALE ISOLATION OF PLASMID DNA

The alkaline lysis method of Birnboim and Doly (1979) was followed for the isolation of plasmid DNA. Growth of bacteria and amplification of the plasmid were as described by Maniatis et al. 1981.

Ten ml of LB medium containing appropriate antibiotics was inoculated with a single colony of the bacteria containing the plasmid of interest and was grown overnight at 37°C with vigorous shaking. This overnight culture was used to inoculate 500 ml of LB medium with the antibiotic to get a starting OD₆₀₀ of 0.05 and then allowed to grow till OD₆₀₀ was approximately 0.4. For amplification of the plasmid, 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 x g
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-HCl, pH 7.8 and 1 mM EDTA).

**Lysis of the bacterial cells:** The cell pellet was resuspended in 10 ml of solution I (50 mM glucose, 25 mM Tris-HCl, pH 8, 10 mM EDTA), 5 mg/ml lysozyme was added to it and 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 mixed the contents 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 on a Sorvall SS 34 rotor. The supernatant was collected by filtering it 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 was washed once with 70% cold ethanol. The pellet was dried briefly under vacuum and

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dissolved in 8 ml of TE buffer (10 mM Tris-HCl, pH 8 and 1 mM EDTA).

**CsCl-density gradient centrifugation:** For every ml of the DNA solution one g 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 should be 1.55 g/ml (γ = 1.3860). The cesium chloride-DNA solution was transferred to TV865 rotor tubes and the tubes were filled to capacity with liquid paraffin oil. Centrifugation was carried out in Sorvall OTD-75B Ultracentrifuge for 7 hrs at 58,000 rpm or overnight (16 hrs) at 50,000 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 by adding two volumes of water and 6 volumes of ethanol. The precipitate was collected by centrifugation, washed once with 70% ethanol, dried in vacuo and dissolved in TE.
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. Bacteria were grown to saturation density 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, 1 mM EDTA and 20 ug/ml of RNase A. After 30 min of incubation at 37°C the sample was extracted with phenol, phenol:chloroform isoamyl/ alcohol (50:48:2) and chloroform successively. To the aqueous phase, two volumes of ethanol was added, mixed and incubated at -20°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 μl of TE. Four μl was used for restriction enzyme digestion.

ISOLATION OF GENOMIC DNA FROM ARABIDOPSIS THALIANA SEEDLINGS

Plant material: Arabidopsis seeds were surface sterilized in 80% ethanol followed by sterilization in 5% sodium hypochlorite for 10-15' and after thorough rinsing in water the seeds were dispersed into flasks containing 0.8% agar in half strength MS medium (Murashige and Skoog, 1961). 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 week old plants.

The procedure used for the DNA isolation from Arabidopsis seedlings was adapted from the protocols of Ausubel et al., (1987) and 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 containing 100 mM Tris-HCl, pH 8, 100 mM EDTA, 250 mM NaCl and 100 μg/ml of proteinase K 2 ml per/g of tissue and it was swirled to suspend the tissue thoroughly. Then 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 7500 x g at 4°C. Isopropanol (0.6 volume) was added to the supernatant and mixed gently to precipitate the nucleic acids. The nucleic acids were pelleted down at 7500 x g for 15 min at 4°C, and dissolved directly in TE (10 ml/5 g and 5 ml/5 g). 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% CTAB, mixed well and incubated at 65°C for 10 min. The extract was emulsified by gentle inversion with an equal volume of chloroform/isoamyl alcohol (24:1) and after centrifugation (13000 x g, 10 min), the aqueous phase was removed with a large bore pipette. One tenth volume of 10% CTAB containing 0.7 M NaCl was added and the chloroform/isoamyl alcohol extraction was repeated. The final aqueous phase was clear. A CTAB-nucleic acid precipitate was formed when the NaCl concentration was reduced from 0.7 to 0.35 M by the addition of one volume of 1% CTAB in 50 mM Tris-HCl, pH 8 and 10 mM EDTA. After keeping for 30 min at room temperature the precipitate was recovered by low speed
centrifugation (2000 x g, 5 min). The CTAB-nucleic acid pellet was dissolved in 2 ml of 1.0 M CsCl (density = 1.12 g/ml) in 50 mM Tris-HCl, pH 8.0, 10 mM EDTA and 20 ug/ml ethidium bromide. The solution was briefly warmed to 60°C to speed dissolution of CTAB-nucleic acid pellet and transferred to an ultracentrifuge tube, 2.4 ml of 6.6 M CsCl (density = 1.82 g/ml) in 50 mM Tris HCl pH 8, 10 mM EDTA, and 0.1% Sarkosyl was added allowing considerable mixing to preform a crude density gradient. After centrifugation (3 hr, 58,000 rpm in a TV865 rotor) the DNA was visualized under long wave UV-illumination, and the DNA band was carefully aspirated out from the top. Ethidium bromide was removed by n-Butanol saturated with TE. The DNA was precipitated by the addition of two volumes of H$_2$O and 7.5 volumes of chilled ethanol. A white fibrous DNA precipitate was formed upon mixing and it was transferred to a fresh tube containing 70% ethanol by hooking the precipitate onto the tip of a pasteur pipette. It was pelleted down by centrifugation and dried very briefly but not completely. The DNA dissolved in TE and its concentration was determined spectrophotometrically by meaning the adsorption 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 by gel electrophoresis. The fragments of interest for sub-cloning were electroeluted onto DEAE cellulose paper and DNA was recovered by centrifugation.

Purification of the DNA fragments by electrophoresis onto DEAE-cellulose paper: Strips of DEAE cellulose paper (DE-81) were soaked in 2.5 M NaCl for several hours and washed several times in water. The fragments of 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 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

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taken out carefully, washed in sterile water and transferred to an Eppendorf vial containing 0.5 ml of the DEAE elution buffer (1.5 M NaCl, 1 mM EDTA and 20 mM 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. The loose DEAE cellulose pellet 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.

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

The ligation reaction was set up as follows:

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

In a total volume of 10 ul
The concentration of the total target and vector DNA in the ligation reaction was kept at 20-50 ng/μl 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 genetic marker present in the vector.

**Transformation of E. coli with plasmid DNA**

For the pUC plasmids, *E. coli* strain MC1029 or JM101, for pBR 325 plasmid DNA, *E. coli* strain HB101 and for M13 RF DNA, *E. coli* strain JM101 were used for transformation. The LB plates used for transformation contained 100 μg/ml of ampicillin, 20 μg/ml of X-gal and 20 μg/ml of IPTG, or 15 μg/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 calcium chloride treatment (Mandel and Higa, 1970) with minor modifications. Bacterial cells were grown to saturation density at 37°C overnight. Flasks containing
100 ml of LB medium was inoculated with one ml of the overnight culture and grown with vigorous shaking at 37°C till \( \text{OD}_{600} \) was 0.3 - 0.4. The culture was chilled on ice for 15-20 min, transferred to 50 ml Sorvall centrifuge tubes 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 MgSO<sub>4</sub> and recentrifuged at 4°C. The pellet was gently suspended in half of original volume of ice cold 50 mM CaCl<sub>2</sub>, 10 mM Tris-HCl (pH 8.0), by gently swirling the tube and incubated on ice for 30 min. The cells were then collected by centrifugation at 5000 rpm for 5 min at 4°C and the pellet was resuspended in 1/10th the original volume of ice cold 50 mM CaCl<sub>2</sub>, 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 upto 2 days after the preparation of the cells.

**Transformation protocol:** Sterile glass test tubes (10 ml volume) 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 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 or pBR plasmids
were being used for transformation, 1 ml of LB medium was subsequently to each tube added 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 the bacterial colonies containing the plasmids to appear.

Top agar plating protocol for JM101 cells transformed with M13 RF DNA: To the JM cells (plus M13 RF DNA) after the heat-shock treatment as described above, 3 ml of melted top agar (0.8% agar in LB) at 42°C, 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 top agar, the plates were top agar 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. 1981. 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 this aids 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 1/2" was placed in a dish and 20 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 20 x SSC were placed on the sponge.

The gel, after neutralization, was placed upside down on these 3 MM papers. A piece of nitrocellulose filter paper cut into desired size was floated on a solution of 2 x SSC until it became wet completely. The wet nitrocellulose paper was then placed carefully on top of the gel, such that one end extends 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 2 x 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, were replaced. At the end of the transfer, the paper towels and the 3 MM layers 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 6 x 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 or for 4 hrs at 68°C. The baked filters were stored under vacuum at room temperature till use.

Bidirectional transfer of DNA from agarose gel onto two nitrocellulose filters:

DNA was transferred from a single gel to two nitrocellulose filters essentially as described by Smith and Summers (1980). After electrophoresis the gel was
soaked in 10 x SSC for 30 min. Two pieces of nitrocellulose filters were cut to the size of the gel and wet first in water and then in 10 x SSC. One corner from each filter was cut. One piece of nitrocellulose filter laid on a dampened 3 MM paper cut to the size of the gel. The gel was laid on to it carefully. Without delay the gel was covered with a second piece of nitrocellulose paper without trapping of any air bubbles followed by another piece of dampened 3 MM paper. The entire sandwich of 3 MM papers, nitrocellulose filters and gel was transferred onto a stack of blotting papers cut to the size of the gel, and a second stack of blotting papers was laid onto the sandwich and the whole set-up was kept pressed by a heavy glass plate. After about two hours the blotting paper stacks were removed and after removing the 3 MM papers the positions of the slots were marked on the nitrocellulose filter and the nitrocellulose filters removed and transferred to 6 x SSC and processed for hybridization as described earlier.

LABELLING THE DNA PROBES BY NICK-TRANSLATION

The DNA probes used for the hybridization experiments were labelled by nick translation (Rigby et al. 1977) using E.coli DNA polymerase I. The nick-
translation reaction was set up by aliquoting 2.5 ul of 10 x nick translation buffer (0.5 M Tris-HCl, pH 7.2, 0.1 M MgSO₄, 10 mM dithiothreitol and 500 ug/ml BSA), 2.5 ul of 0.5 mM unlabeled dNTP mix (containing the dNTPs other than the labeled dNTP), 0.1 to 0.5 ug of DNA to be labeled, 30 uCi of 3000 Ci/mmol (³²P) ATP or (³²P) CTP, 0.5 ul DNase I (diluted 10⁵ times from a stock of 10 mg/ml), 5 U E. coli DNA polymerase I (BRL) and water. The contents were mixed well and incubated at 15°C for 60-90 min. The reaction was stopped by the addition of 2 ul of 0.5 M EDTA. The nick translated DNA was separated from unincorporated NTPs by chromatography on a Sephadex G-50 column packed in a sterile Pasteur pipette plugged with sterile glass wool was washed with several volumes of TE. The nick translated reaction mixture was applied onto the column in a volume of 100 ul. 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 the flow rate of the elute. The elute was collected as 0.5 ml fractions (upto 10 fractions) into Eppendorf tubes. The radioactivity in each of the tubes was measured by dispensing 1 ul of the fraction onto small blotting papers and counting them in 5 ml scintillation fluid (0.4 g PPO and 0.025 g POPOP
dissolved in 1 litre 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 $5 \times 10^7$ to $2 \times 10^8$ cpm/ug of DNA.

**HYBRIDIZATION OF SOUTHERN FILTERS**

The baked filter was floated on to 6 x SSC till it became wet and then soaked in the same solution for 2 min. It was then transferred to a plastic box containing the prehybridizing solution warmed to 42°C. Two-tenth ml of prehybridizing solution was used for each square centimeter of the filter. Prehybridization was carried out for 2-4 hrs.

Prehybridization solution contained the following:

- 5x SSPE
- 0.5% SDS
- 5% Denhardt's solution
- 50% Formamide
- 100 ug/ml denatured salmon sperm DNA
For hybridization of the filter, $^{32}\text{P}$-labeled, denatured DNA probe was added directly to the prehybridization solution and the incubation continued. When more than one filter were being hybridized the incubations were carried out with gentle agitation. Hybridization was carried out for 4-8 hrs when fragments of cloned DNA were being analysed and for 16 - 24 hrs when the Southern blot of genomic DNA was being hybridized. After carrying out the hybridization for sufficient length of time, the filter was removed and transferred quickly to a solution of 2 x SSC and 0.5% SDS and agitated at room temperature for 5 min. The filter was then transferred to a fresh tray with 2 x 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 0.1 x SSC and 0.1% SDS at 65°C for 2 hrs with gentle agitation. The washing solution was changed once in the middle.

For moderate stringency the filter were washed in 0.4 x SSC and 0.1% SDS at 50°C for 2 hrs and for the low
fig. 2.

SANDWICH HYBRIDIZATION:

Insert in M13 ss DNA → hybridize → target

target on Southern blot

M13 RF → nick translation

single strand M13

sandwich

denature, hybridize

target
stringency, the washings were done in 2x SSC and 0.1% SDS at 60°C for 2 hrs.

After washing the hybridized nitrocellulose filters were wrapped in saran wrap and exposed to X-ray film with an intensifying screen at -70°C. Radioactive ink was applied as dots in an asymmetric manner on the saran wrap containing the filter, outside the area of the filter to facilitate the alignment of the DNA lanes on the filter with the autoradiographic image.

**Hybridization using SS DNA probes**

Apart from the conventional way of hybridizing with the labelled probes directly, an indirect way of hybridization strategy called "Sandwich hybridization" was also followed. This strategy involves two stages of hybridization. In the first stage, the target DNA is hybridized with unlabeled single stranded recombinant phage DNA containing the probe of the interest as an insert; and in the second stage, labeled RF M13 phage DNA is hybridized to the vector tails which protrude from the hybridized target sequences (Fig. 2).

After prehybridization, about 0.5 - 1 ug of the single stranded recombinant M13 phage DNA containing the insert to be used as the probe was added to the
prehybridization solution containing the nitrocellulose filter and this "cold" hybridization was carried out at 42°C for 4-8 hrs. Just before the second stage the filter was rinsed in 5 x SSPE twice to wash to eliminate the unhybridized single stranded phage DNA and the filter was transferred to the fresh prehybridization solution. In the second stage nick translated, denatured, RF of M13 phage DNA was added and allowed to hybridize for 2-4 hrs. After this "second hybridization" filter was washed as described earlier.

M13 CLONING AND SEQUENCING OF DNA

Cloning of the DNA fragments into M13 mp vectors for sequencing by the dideoxy chain termination method: Dideoxy sequence analysis using M13 mp vectors has been designed for simple and efficient generation of single stranded DNA templates (Carlson and Messing, 1984). The presence of convenient restriction sites in the PAL gene allowed various fragments to be cloned in both the orientations into the polylinker region of mp18 as well as mp19. This allowed sequencing of both the strands. In cases, where the fragments were flanked by the restriction enzyme sites (eg. 0.5 kb HindIII
fragment) the opposite strands were identified by so-called one track analysis (described under the footnote of sequencing protocols). Construction of nested deletions was undertaken in order to sequence large fragments which did not have convenient restriction enzyme sites for cloning into M13 vectors.

For the construction of sub-clones of the various fragments into a M13 vector for sequencing, the target DNA was cut with various restriction enzymes and the digest were incubated with M13 DNA cleaved with the enzymes meant only for sub-cloning a single fragment. The target and M13 mp RF DNAs were digested with two sets of enzymes and ligated for directional cloning of the sub-fragments of the target DNA for sequencing.

A 4.8 kb EcoRI fragment containing the complete PAL gene and the 5'upstream region of the gene (Fig.17), was cleaved with PstI and the resultant 2 kb EcoRI-PstI and 2.8 kb EcoRI-PstI fragments were cloned into M13 mpl8 DNA digested with EcoRI and PstI in a single ligation reaction and the inserts were identified by screening the ss DNAs from the recombinant plaques on a 0.7% agarose gel. The same constructs were used for generation of deletions as described in the following section.
Generation of deletions of sub-clones for sequencing by partial digestion with restriction endonucleases

Principle: In this novel method (Lamperti and Villa-Komaroff, 1990) deletions are created by partial digestion of an M13 bacteriophage construct with frequent cutting restriction endonucleases. Both the M13 vector DNA and the insert are partially digested. When the digestion products are repaired, religated and transfected into the M13 host, plaques are formed by the surviving intact M13 DNA and possibly containing the variable portions of the insert. By this means a series of deletions can be generated in a single tube. The different deletion sub-clones are sorted out by sizing of the single stranded DNA on agarose gels. Two and 2.8 kb EcoRI-PstI fragments were cloned into M13 mpl8 DNA for the construction of deletions.

Linearization: Fifteen ug of the RF forms of DNA from the above two clones as linearized with PstI in a volume of 300 ul and the completion of the digestion was checked by electrophoresis on 0.7% agarose.

Setting up of partial digestions with the enzyme TaqI: The linearized DNA was ethanol-precipitated and dissolved in 150 ul of sterile water. Ten ul of the
linearized DNA was aliquoted into each of 6 tubes. Five ul of 10 x TaqI restriction enzyme buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.4, 6 mM 2-mercaptoethanol) and 100 ug/ml BSA were made upto 50 ul with sterile H₂O. To all the six tubes numbered I to VI, 0.15, 0.15, 0.5, 0.5, 0.5 and 4 U of the enzyme were added and incubated for 5, 12, 5, 10, 15 and 5 min respectively at 65°C. An aliquot was removed from the tube No.VI containing 4 U of the enzyme and kept for complete digestion for 1 hr. The digestions were stopped after appropriate timings by quickly adding 50 ul of phenol and mixing well. The phases were separated by centrifugation and the aqueous phase was extracted with chloroform. The DNA from the final aqueous phase was precipitated by adding equal volume of 4 M ammonium acetate and 4 volumes of ethanol and kept at -70°C for 30 min. The DNA was collected by centrifugation, washed with 70% ethanol and dissolved in 10-20 ul 1/10 TE (10 mM Tris-HCl, pH 8, 1 mM EDTA). One-tenth of this was electrophoresed on a 0.7% agarose gel and sized against the linearized insert and vector DNAs. The fractions which gave most of the bands within the linearized vector and the linearized DNA used for the construction of deletions, were pooled.

Repair ligation and subsequent digestion: The pooled fractions were repaired by DNA polymerase I large
fragment (Klenow) in a total volume of 50 ul containing 20 mM Tris-HCl (7.6), 10 mM MgCl₂, 200 μM of each of the four dNTPs, 1-2 U Klenow and kept for 15 min at room temperature and the reaction was stopped by heating at 65°C for 15 min.

The repaired DNA (50 ng) was ligated in a volume of 100 ul in the same buffer used for repair but with 10 mM DTT, 1 mM ATP and 1-2 units T₄ DNA ligase for 4 hrs at room temperature. In order to eliminate the background produced by the DNA molecules remaining undigested during the deletion procedure, a post-ligation digestion with the restriction enzyme used for linearizing the initial DNA molecules was performed.

**Transfection:** After the digestion of the ligated DNA, 1/10th was used to transfect JM host cells as described under transformation of *E. coli* with plasmid DNA. Upon plating in the presence of X-gal and IPTG no blue plaques were seen (as expected from the location of TaqI sites in M13 mp vector DNA) and there were variable sized, colourless 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 vigorously shaking at 37°C for 5 hrs. The size selection of the subclones was done by
direct gel electrophoresis of the single stranded DNA isolated from a number of plaques. A small volume (100 ul) of the culture was centrifuged to pellet cells and to 20 ul of the supernatant, 2 ul of SDS stop solution (2% SDS, 10 mM EDTA, 10 mM Tris-HCl, pH 7.4 and 50% glycerol) was added, mixed and incubated at 65°C for 5 min and loaded on a 0.7% agarose gel.

The approximate size of the insert in the clones was determined by running ss DNAs containing inserts of known size as size markers. The gel was stained with ethidium bromide (0.5 ug/ml) after the electrophoresis and photographed.

A priming test, which is required to check the presence of primer in the cases where the restriction enzyme (frequent cutter) has sites on the vector DNA between the gene II and polylinker region leading to the formation of plaques inspite of the absence of a short stretch of the vector DNA along with the primer, was not necessary when TaqI was used as it does not have sites on the vector DNA between gene II of the vector and the polylinker region. The DNA was isolated from desired cultures by the method as described under "Preparation of single stranded M13 DNA for sequencing".
The sequencing of the inserts in the clones was performed by dideoxy chain termination method as described under dideoxy sequencing protocols (Sanger et al., 1977).

Isolation of M13 RF (replicative form) DNA: A 100 ml of LB medium was inoculated with a single colony of the M13 host E. coli cells grown on M9 minimal medium plate and incubated with shaking at 37°C till OD\_600 reached 1.0. To another 100 ml LB medium one blue plaque containing agar plug from the mp vector plate was transferred with the aid of a Pasteur pipette and grown overnight at 37°C to get a phage stock. To 900 ml of 2x YT medium, the M13 host cells in the 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 ug/ml from a stock solution (30 mg/ml in ethanol) was added 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 plasmid DNA isolation and purified by cesium chloride density gradient as described under large scale isolation of plasmid DNA.
Preparation of single stranded DNA template for sequencing: Transformation of the *E. coli* host with M13 mp DNA containing an insert produce white (colorless) plaques upon plating and growth on indicator plates. Non-recombinant phage produce blue plaques. Templates for sequencing were prepared by growing the phages from the 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 growth was diluted 1:50 times in YT medium to get desired volume of cells. Five ml of 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 microfuge tubes and centrifuged in a microcentrifuge for 5 min at room temperature. The supernatant (1.2 ml) was collected without disturbing the cell pellet. The bacterial cell pellet was saved for RF DNA or as a stock for the clone. Three hundred ul of 20% PEG/2.5 M NaCl was added to the supernatant, mixed well by briefly vortexing and left at room temperature for 15 min. It was centrifuged for 10
min to pellet the phages and the supernatant was aspirated out completely without disturbing the pellet. The pellet was resuspended in 100 ul TES buffer (20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 0.1 mM EDTA) and extracted with 50 ul phenol saturated with TE by vortexing vigorously for 15-20 sec and centrifuging to separate the phases for 2 min. The aqueous phase was extracted with chloroform in the same manner and after centrifugation 80 ul of aqueous phase was removed and the ss DNA precipitated with 200 ul of ethanol after adding 9 ul of 3 M sodium acetate (pH 5.0) and left at -70°C for 30 min. The DNA was pelleted by centrifuging for 10 min at 4°C and washed with 80% ethanol. The DNA pellets after aspirating out the washings were briefly dried under vacuum and resuspended in 10-20 ul TE. The presence of template DNA was checked by loading 1 ul of the preparations on to a 0.7% agarose gel (ss viral DNA with an insert of 300 bp or greater will have reduced mobility in comparison to ss M13 mp DNA). Three to five ul (0.5 - 1.0 ug) of the DNA was used as template in dideoxy sequencing reactions.
DIDEOXY SEQUENCING REACTIONS

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

Dideoxy sequencing using Klenow: BRL's M13 sequencing kit was used. The kit contained Klenow fragment, 10 x polymerase reaction buffer (70 mM Tris-HCl, pH 7.5, 70 mM MgCl₂, 500 mM NaCl), polymerase dilution buffer (100 mM KPO₄, pH 7.5, 50% (v/v) glycerol), all the four deoxynucleotides at a concentration of 10 mM and dideoxynucleotides as 10 mM solutions and the 17-mer oligonucleotide primer.

Protocol for dideoxy sequencing reactions: An aliquot of each of the four dNTPs was diluted to a concentration of 0.5 mM. The four individual dNTP mixes were prepared from the 0.5 mM dNTP solutions as follows:

<table>
<thead>
<tr>
<th></th>
<th>A₀</th>
<th>C₀</th>
<th>G₀</th>
<th>T₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>ul</td>
<td>ul</td>
<td>ul</td>
<td>ul</td>
<td>ul</td>
</tr>
<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.

- ddATP 0.1 mM (1:100 dilution of 10 mM stock)
- ddCTP 0.3 mM (1:33 dilution of 10 mM stock)
- ddGTP 0.5 mM (1:20 dilution of 10 mM stock)
- ddTTP 1.0 mM (1:10 dilution of 10 mM stock)

Annealing of the primer to the template

To a 0.5 ml Eppendorf tube the following were aliquoted

- 3-5 ul of ss template DNA (0.5 - 1.0 ug)
- 1 ul M13 17mer sequencing primer (4 ng)
- 1 ml 10x polymerase reaction buffer
- x ul H₂O.

12.4 ul total

The contents were mixed and centrifuged briefly. The vials were placed then heated for 5 min by 5 ml test tube filled with H₂O at 90° - 100°C. Then the template-primer mixture was allowed to equilibrate to room temperature for 30-45 min for annealing.
During the annealing reactions the following were aliquoted to 4 tubes A, C, G and T.

- Tube A - 1 ul $A^O$ mix and 1 ul diluted dd ATP
- Tube C - 1 ul $C^O$ mix and 1 ul diluted dd CTP
- Tube G - 1 ul $G^O$ mix and 1 ul diluted dd GTP
- Tube T - 1 ul $T^O$ mix and 1 ul diluted dd TTP

After the temperature of the primer-template tube had come down to below 35°C, the following were added directly to the tube containing hybridized template-primer.

- 3 ul $^{35}$S dATP (1000 Ci/mmol 1 m Ci/100 ul)
- 1 ul 0.1 M DDT
- 1 ul diluted large fragment (Klenow) of DNA polymerase I (1.5 U)

The entire contents were mixed by gently pipetting in and out of the Eppendorf tip several times.

Three to five ul of this was aliquoted to the four reaction tubes A, C, G and T which contain the nucleotides. The contents were mixed and centrifuged briefly to collect at the bottom. The reaction tubes were incubated at 30°C. After 20 min, 1 ul of 0.5 mM
dATP (1:20 dilution of 10 mM stock) was added to each of the 4 tubes, mixed and incubated at 30°C for a further 15 min to extend the polynucleotide chains those might have prematurley been terminated due to low dATP concentrations.

The reactions were stopped by adding 5-10 ul of dideoxy stop buffer (95% formamide, 0.05% Bromophenol blue, 0.05% Xylene cyanol and 20 mM EDTA) and denatured by heating at 90°-100°C for 3-5 min and were quickly transferred to ice. One to two ul of the each reaction per lane was loaded onto the sequencing gel. The samples were denatured every time just before loading.

**Dideoxy sequencing reactions using modified T₇-DNA polymerase (Sequenase)**

The USB Corporation's sequenase kit was used to perform the reactions. The kit contained in addition to the enzyme, annealing buffer (400 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, and 50 mM NaCl), 0.1 M dithiothreitol (DTT) labeling nucleotide mixtures (for use with radio-labeled dATP), termination nucleotide mixtures (one for each of the dideoxynucleotide containing 80 uM dGTP, 80 uM dATP, 80 uM dTTP, 80 uM dCTP) and 50 mM NaCl. In addition the 'G'-mixture contained 8 uM ddGTP, the "A" mix 8 uM
ddATP, the "T", 8 uM ddTTP and the "C" 8 uM ddCTP), and the stop solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol blue and 0.05% Xylene cyanol.

Sequencing reaction protocol using Sequenase

Annealing of the primer: In a 0.5 ml Eppendorf vial the following were aliquoted.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer</td>
<td>0.5 pmol of 4 ng</td>
</tr>
<tr>
<td>ss template DNA</td>
<td>0.5-1.0 pmol (lug of template)</td>
</tr>
<tr>
<td>5 X Annealing buffer</td>
<td>2 ul</td>
</tr>
<tr>
<td>H₂O</td>
<td>X ul</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10 ul</strong></td>
</tr>
</tbody>
</table>

The contents were mixed and the tube was kept in a 65°C water bath for 2 min and then allowed to cool slowly to room temperature over a period of about 30 min.

Labeling reaction: The following were added to the annealed primer-template.

- DTT: 1 ul
- Labeling nucleotide mix: 2 ul
-\(^{35}\)S-dATP 5 uCi - 10 uCi
Sequenase 3 units

Total volume was made upto 15 ul.

The contents were mixed thoroughly and incubated for 2-5 min at 15-20\(^{\circ}\)C.

Termination reactions: Before starting the reaction, the tubes were prewarmed to 37\(^{\circ}\)C and to each of the four tubes labeled A, C, G and T, 2.5 ul of the appropriate dideoxy termination mixture was aliquoted. Then 3.5 ul of the labeled reaction mixes were added to each of the four termination tubes. After thorough mixing the chain-termination reaction was continued for 2-5 min at 37\(^{\circ}\)C. The termination reactions were stopped by the addition of 4 ul of stop solution. Just before loading, the samples were heated to 75 - 80\(^{\circ}\)C for 2 min and 1-2 ul of the samples were loaded in each lane.

Sequencing close to the primer: Decreasing the concentrations of nucleotides present during the labeling reaction will limit the lengths of chain extensions. This results in emphasizing sequences very close to the primer but the distant sequence (after 300) are not readable. By increasing the concentrations of
nucleotides in the labeling reactions, longer chain extensions are allowed during labeling and longer sequences could be read but not the sequence close to the primer. Thus labeling reactions were carried out using various dilutions of the labeling mix (from 3 to 15 pmol of each of the dNTPs i.e. 5 times dilution to no dilution of the labeling mix) respectively in order to read the sequence from the primer till the maximum capacity.

Labels used for Sequencing: Use of \(^{35}\)S label resulted in sharp bands but the exposure time was prolonged. \(^{32}\)P alone was not preferred as the resolution of the bands on the autoradiogram was very poor when \(^{32}\)P was used as the label. In order to shorten the exposure time and get good resolution a mixture of \(\alpha^{32}\)P dATP and \(\alpha^{35}\)S-dATP was tried and gave good results. One ul of \(\alpha^{32}\)P-dATP (3000 Ci/mmol: 1 mCi/100 ul) in its first half life period, was mixed with 5 ul of \(\alpha^{35}\)S-dATP (1000 Ci/mmol, 1 mCi) and 3 ul of this mixture for sequencing with Klenow and 1 ul was used when Sequenase was employed.

One lane analysis, was done to identify DNA templates containing opposite strands (in order to sequence both the strands) when the insert was cloned.
into a single site of the polylinker region of M13 mp vector. ss DNA from several recombinant plaques was purified and sequencing reactions for only one were done. The same lane analysis should be done for all the templates. Upon electrophoresis on a sequencing gel, the lanes of templates containing opposite strands display different patterns while the lanes of templates containing same strand exhibit similar pattern.

PROTOCOL FOR POURING AND ELECTROPHORESIS OF SEQUENCING GELS

The plates were cleaned thoroughly using detergent, without causing any scratches and rinsed with double distilled water. The right sides (gel side) of the plates were rinsed once with ethanol. The right side of one plate was siliconized. Another plate was treated with $\gamma$-silane ($\gamma$-methacryloxy-propyl-trimethoxy silane) solution. The $\gamma$-silane treatment was done thrice and left for 10 min each time. Treating the plate with $\gamma$-silane results in covalent binding of the gel to the plate and hence named anti-siliconization. The siliconized and antisiliconized plates were rinsed with acetone and left to dry. The plates were assembled with the treated sides inside and the spacers on the sides. The plates and the spacers on
both sides were held together using clamps. The bottom side was sealed with a Scotch tape. No other sealing was done as the clamps and the spacers gave enough protection on the sides. While the plates were getting ready, 6% acrylamide/urea solution was prepared by mixing 4.5 ml of 40% acrylamide (acrylamide : bisacrylamide 19:1) 15 g urea, 3 ml 10x TBE and making up the volume to 30 ml. After the urea dissolved, the solution was filtered through Whatmann No. 1 filter paper and degassed under vacuum. Ammonium persulfate (300 ul) and 4 ul of TEMED were added, mixed well and poured immediately. The assembled plates were held at a 45° angle and the prepared acrylamide solution was poured from one corner of the plates tilting this side slightly downwards, thus allowing smooth flow along one side of the plates. When it became full the plates were brought to almost horizontal position (eg. by resting it on a pipette so that the open end is slightly up) and the flat surface of the BRL's sharkstooth comb was inserted between the plates to a depth of 2-3 mm below the short plate and held in position between the plates with clamps at the sides and at the centre from above. Care was taken to avoid entrapping air bubbles when the comb was inserted. The gel was left in the same position or in horizontal position for polymerization.
Sequencing gels were run after at least 2 hrs of pouring.

**Electrophoresis of sequencing gels:** Just before starting the electrophoresis, the tapes and clamps were removed from the gel-sandwich. The comb was carefully slid out and the top of the gel was rinsed with distilled water to remove any unpolymerized acrylamide. The comb was rinsed and placed between the plates with the teeth down toward the gel. The comb was inserted down till it made contact with the surface of the gel allowing only slight indentation of the gel surface by the teeth and not piercing the gel. The gel sandwich was clamped onto the upper tank of the electrophoretic apparatus. An 2 mm thick aluminium plate of the dimensions of the glass plate was also clamped along with the plates in the front side to distribute the heat generated during electrophoresis uniformly in order to avoid the smiling effect on the bands. The upper and lower reservoirs were filled with 1 x TBE buffer. Gels were pre-electrophoresed for 15-30 min before loading the sample. The electrophoresis was carried out at a constant voltage of 40 W so that constant temperature (approximately 50°C) of the plates was maintained.
Sample application: The DNA samples were heated at 90°-100°C for 3-5 min just prior to loading and chilled on ice. After the pre-electrophoresis, the wells were washed with 1 x TBE using a needle and syringe to remove the urea that would have diffused out of the gel into the well, so that the sample settles as narrow bands in the wells allowing fine resolution of the bands upon separation. The samples were applied using a Pipetman. Usually 1-2 ul of the samples were loaded and after loading every two sets of samples it was electrophoresed for 5 min and then loading of the other sets were continued.

Second and third loadings of the same sample sets were done in a single gel at different times so that reading the sequence upto a maximum readable point in a single autoradiogram was possible. The migration of the dyes bromophenol blue and xylene cyanol in relation to the migration of the oligonucleotides is constant for a given percentage of the gel. In the 6% gel bromophenol blue migrates along with the position of 26mer and xylene-cyanol migrates at a position of 106 bases oligonucleotide.
AUTORADIOGRAPHY OF SEQUENCING GELS

After the electrophoresis, the gel sandwich was pried open with a scalpel and the plate onto which the gel is bound was transferred to a tray containing fixer (10% acetic acid and 10% methanol) and treated for 15 min to remove the urea which would otherwise cause quenching. The plate was removed, the fluid sticking to the plate was drained, dried in an oven at 70°C for 1-2 hrs, carried with Saran wrap and exposed to X-ray film at room temperature.

MEDIA, BUFFERS AND SOLUTIONS.

LB Medium:

Per liter:

- Bacto-tryptone 10 g
- Bacto-yeast extract 5 g
- NaCl 10 g

pH adjusted to 7.5 with sodium hydroxide

YT medium:

Per liter

- 8 g bactotryptone
- 5 g bacto-yeast extract
- 5 g NaCl

Autoclaved to sterilize.
M9 agar plates.

10 x M9 salts Per liter
60 g Na₂HPO₄
30 g KH₂PO₄
5 g NaCl
10 g NH₄Cl

20 ml of 10x M9 salts and 3 g of agar in 175 ml H₂O were autoclaved. Cooled to 60°C and then added 0.2 ml 1 M MgSO₄ (filter sterilized), 2 ml 10 mM CaCl₂ (filter sterilized), 2 ml 20% (w/v) glucose (filter sterilized) and 0.2 ml 10 mg/ml thiamine. The volume was adjusted to 200 ml with sterile water and poured onto plates.

BUFFERS:

10 x TBE:

121.1 g Tris-base
55 g boric acid
7.5 g Na₂ EDTA

Dissolved in H₂O and volume adjusted to 1 litre.

TE Buffer:

10 mM Tris HCl pH 8.
1 mM EDTA
Phage buffer:

5.8 g NaCl
2 g MgSO₄·7H₂O
50 ml 1 M Tris Cl pH 7.5
5 ml 2% gelatin

made upto 1 litre and sterilized by autoclaving

10 X ligation buffer:

0.66 M Tris HCl, pH 7.5
50 mM MgCl₂
50 mM dithiothreitol.

SOLUTIONS:

40% Acrylamide: 38 g acrylamide and 2 g bisacrylamide were dissolved in H₂O and made upto 100 ml. Deionized by stirring with 2 g of Amberlite-MB-1 resin, filtered through Whatman 3 MM paper and stored at 4°C in dark.

20 x SSC: 175.3 g of NaCl and 88.2 g of sodium citrate were dissolved in 800 ml of H₂O. pH was adjusted to 7.0 and the volume was made upto 1 litre and sterilized by autoclaving.

20 x SSPE: 174 g of NaCl, 27.6 g of NaH₂PO₄·H₂ and, 7.4 g of EDTA were dissolved in 800 ml of H₂O. pH was
adjusted to 7.4 and volume made upto 1 litre and sterilized by autoclaving.

50 x Denhardt's solution

5 g Ficoll
5 g Polyvinylpyrrolidone
5 g BSA (Pentax Fraction V)

Volume made upto 500 ml with \( \text{H}_2\text{O} \).

\text{Xgal} 2% solution in DMSO or DMF. (5-bromo-4-chloro-3-indolyl (3-D-galactoside))

\text{IPTG} 100 mM solution (23.8 mg/ml) (isopropyl -\( \beta \)-D-thiogalactopyranoside)

\text{ATP} 0.1 M

60 mg of ATP dissolved in 0.8 ml \( \text{H}_2\text{O} \) and the pH adjusted to 7 by adding few crystals of Tris base and the volume made upto 1 ml with \( \text{H}_2\text{O} \). Dispensed into small aliquots and stored at -70\(^{\circ}\)C.

\text{ANTIBIOTICS:}

\text{Tetracycline:}

15 mg/ml in 50% (v/v) ethanol stored in light proof containers at -20\(^{\circ}\)C.
Ampicillin:

100 mg/ml in water sterilized by filtration and stored at -20°C.

Chloramphenicol:

34 mg/ml in ethanol stored at -20°C.

**ENZYME**

**Proteinase K**

20 mg/ml in H₂O stored at -20°C

**DNase I**

10 mg/ml (50% w/v glycerol, 20 mM Tris-HCl pH 7.5, 1 mM MgCl₂) stored at -20°C.

**RNase A**

(parcreatic RNase)

10 mg/ml (10 mM Tris-HCl, pH 7.5 and 15 mM NaCl).

Heated to 100°C for 15 min to inactivate DNases and allowed to cool slowly to room temperature and stored at -20°C.
Fig.3: SCHEMATIC DIAGRAM OF pPAL5, PHASEOLUS cDNA IN pAT 153 PLASMID.

H - Hind III
P - Pst I
T - Taq I