MATERIALS AND METHODS
2. MATERIALS AND METHODS

(1) Chemicals used were of highest purity available. All solutions were made in double distilled water.

(2) Wherever indicated, solutions were sterilized, either by autoclaving at 15 pounds/sq. inch for 20 min or by filter sterilization.

2.1 Common buffers and media

(1) Endo R (6X): 30% Ficoll 400; 60mM EDTA, pH 8.0; 0.6% SDS; 0.06% Bromophenol blue.

(2) Laemmli's sample buffer (Laemmli, 1979): 0.625M Tris-Cl, pH 6.8; 2% SDS; 5% mercaptoethanol, 15% sucrose and 0.002% bromophenol blue.

(3) PBS: 137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.4mM KH₂PO₄.

(4) TBS: 10mM Tris Cl, pH 8.0, 150mM NaCl.

(5) TBST: TBS containing 0.05% Tween 20.

(6) TAE: 40mM Tris-acetate, 2mM EDTA.

(7) TE: 10mM Tris Cl, pH 8.0, 1mM EDTA.

(8) L-Broth (LB)

```
Bactotryptone   10g
Bactoyeast extract  5g
NaCl           10g
Water   to 1 litre
pH adjusted to 7
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(9) L-agar: LB containing 1.5% Agar (Difco)

(10) LB-amp: LB containing 50μg/ml ampicillin.

(11) Top agar: LB containing 0.7% Agar (Difco)
2.2 Plant material

Seeds of *Amaranthus hypochondriacus* were obtained from National Bureau of Plant Genetic Resources, Simla, India. Seeds were ground to a fine powder in a mortar and pestle and defatted by extraction with cold acetone. Seeds as well as the defatted meal were stored at 4°C under desiccation.

2.3 Protein extraction

Seed meal (3g) was homogenized for 1h in 30 ml extraction buffer (25mM Tris-Cl, pH 8.5; 0.5M NaCl, 2mM PMSF). The pellet obtained on centrifugation was reextracted for another hour in the same buffer. Supernatants of both the extractions were pooled and used as crude extract. Seeds were also directly homogenized in Laemmli's sample buffer to extract total protein.

2.4 Protein estimation

Protein was estimated by Bradford's method (Bradford, 1976) using bovine gamma globulin as a standard.

2.5 Electrophoresis

2.5.1 SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the discontinuous buffer system of Laemmli (1970). A 30% Acrylamide containing 0.8%
Bis-acrylamide stock was treated with mixed bed ion exchange resin (Sigma) for 1 h with shaking to remove ionic impurities (Maniatis et al. 1982). The separating gel consisted of 12% acrylamide, 0.375M Tris-Cl, pH 8.8, 0.01% TEMED and 0.2% ammonium persulfate (APS). Stacking gel had 4% acrylamide and 0.125M Tris-Cl, pH 6.8, with same amounts of TEMED and APS. Electrophoresis was routinely performed in mighty small apparatus (Hoefer) at constant voltage of 150 Volts. Samples containing sample buffer (Laemmli, 1970) were denatured in boiling water bath for 5 min prior to loading. Standard protein mixture (Sigma/Pharmacia) was run simultaneously to calculate the protein size.

2.5.2 2-Dimensional gel electrophoresis

2D gel electrophoresis was performed by the procedure of O'Farrell et al. (1975, 1977).

First dimension : Non equilibrium pH gradient gel electrophoresis (NEPHGE)

(1) Glass tubes (3mm internal diameter) were washed with chromic acid and then placed in alcoholic-KOH for about 12 h. They were rinsed thoroughly with distilled water and air dried.
(2) The separating gel contained 9.5M urea, 4% acrylamide, 2% Triton X-100 and 2% v/v ampholines pH 3-10.

(3) Samples to be analysed were dialysed against water, lyophilized and dissolved in IEF loading buffer (9.5M urea, 2% Triton X-100, 2% ampholine pH 3-10, 5% mercaptoethanol)

(4) Samples were directly loaded onto the gel and overlayed with 20 ul of sample overlay solution (9.5M urea, 2% ampholines pH 3-10). Electrophoresis was performed using 10mM phosphoric acid in the upper tank and 20mM NaOH in the lower tank at 450 V for 4 h.

(5) At the end of the run, gels were removed from the tubes, using a syringe and incubated in Laemmli buffer for 30 min and kept frozen until second dimension was performed.

Second dimension

Gels were analysed on SDS-PAGE in the second dimension as described earlier (Section 2.5.1).

2.5.3 Staining and destaining of gels

Gels were routinely stained in 0.25% (w/v) Coomassie blue R 250 in water: methanol: acetic acid (in 5:5:2 ratio) for 2 h to 12 h. Gels were destained in a solution containing 25% methanol and 10% acetic acid.
2.6 Sucrose density gradient centrifugation

Sucrose density gradient centrifugation was performed to determine the 'S' value of different protein fractions in the seed (Barker et al., 1976).

(1) Seed meal (0.5g) was homogenised in 5 ml extraction buffer and centrifuged to remove the debris.

(2) Crude extract (0.2 ml) containing 0.5-1 mg protein was layered on a 12 ml linear, 5-20% sucrose gradient made in the extraction buffer.

(3) It was centrifuged at 25,000 rpm for 24 h in SW 41Ti rotor (Beckman). 1 ml fractions were collected and analysed on SDS-PAGE.

(4) 'S' value was calculated by simultaneously running protein standards of known 'S' value, -amylase (8.9 S) and carbonic anhydrase (2.8S) and then calculating by the formula

\[
\frac{S_1}{L_1} = \frac{S_2}{L_2}
\]

Where \(S_1\) and \(L_1\) are the sedimentation coefficient and distance travelled from top of the gradient respectively of known protein and \(S_2\) and \(L_2\) are those of the unknown samples.
2.7 Purification of 35 kDa albumin protein

2.7.1 Removal of globulins from the crude extract
Crude extract from 1 g seed meal was extensively dialysed against 25mM Tris-acetate, pH 8.5 at 4°C. Insoluble globulins were removed by centrifugation at 15,000 rpm (Sorvall, SS34 rotor) for 15 min at 4°C. Supernatant obtained was the source of albumin protein.

2.7.2 Chromatofocussing
Albumin fraction, containing about 15mg protein, was loaded onto a DEAE-Sepharose column (1x25cm; 25ml/h) which had previously been equilibrated with 25mM Tris acetate, pH 8.5. Column was washed with the same buffer and the bound protein was eluted with a multicomponent buffer system as suggested by Prestidge and Hearn (1979) with some modifications. The various buffers used are given in Table I. Each buffer was used at a final concentration of 2mM and the pH of the buffer mix was adjusted to 5.0 with acetic acid. A natural pH gradient was generated in the column and the proteins were eluted on the basis of their isoelectric points. Fractions (1.6 ml) were collected and pH was checked. They were also analysed on SDS-PAGE (Laemmli, 1970). Protein corresponding to a molecular weight of 35 kDa which eluted at pH 7.4 was concentrated using centricon-30 (Amicon) to a final volume of 1 ml.
### TABLE 1: Multicomponent buffer system for chromatofocusing

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pKa at 25°C</th>
</tr>
</thead>
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<tr>
<td>MES</td>
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<tr>
<td>MOPS</td>
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<td>TES</td>
<td>7.4</td>
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</tr>
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<tr>
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</tr>
<tr>
<td>ETHANOLAMINE</td>
<td>9.5</td>
</tr>
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</table>
2.7.3 Gel filtration

Sephadex G-75 column was poured (1.6x22 cm) and equilibrated with 25 mM Tris acetate, pH 8.5 containing 0.1M NaCl. 1 ml of the concentrated sample, obtained after chromatofocussing, was loaded onto the column and elution was continued in the equilibration buffer at a flow rate of 10ml/h. Appropriate fractions obtained after analysis on SDS-PAGE were pooled and concentrated using centricon.

2.8 Purification of 54 kDa globulin protein

(1) Globulin proteins (2 mg) were separated on a preparative (150x160x1.5mm) 12% SDS-PAGE according to the method of Laemmli (1970). Protein standards were simultaneously run. Proteins were visualized by soaking the gel in cold 0.25M KCl until the bands appeared (Hager and Burgess, 1980). 54 kDa band was cut and equilibrated in three changes of water and stored in water at 4°C until elution.

(2) Protein was electroeluted from the gel in 50 mM ammonium bicarbonate containing 0.1% SDS using electrophoretic concentrator (ISCO, Inc., USA) set at 3 watts for 2 h.
(3) Eluted protein was precipitated with five volumes of cold acetone at -20°C for 1 h. Precipitate obtained was dissolved in water. Residual SDS was removed by incubating the protein solution with 50 mM KCl in ice water bath for 30 min. SDS-KCl complex formed was removed by centrifugation as suggested by Suzuki and Terada (1988).

2.9 Molecular weight determination

Native molecular weight of albumin protein was determined on Sephadex G-200 column (2.5x30 cm) equilibrated with 20 mM Tris-HCl pH 8.0 containing 0.1M NaCl. Column was calibrated with standard proteins: aldolase (158,000 kDa), bovine serum albumin (67,000 kDa), carbonic anhydrase (29,000 kDa) and Cytochrome C (12,400 kDa). Void volume was calculated by passing blue dextran through the column. Calibration curve for molecular weights was drawn by plotting $K_{av}$ values against molecular weights. $K_{av}$ was calculated using the formula

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where,

$V_e$ = elution volume of the protein

$V_o$ = elution volume of Blue dextran (Void volume)

$V_t$ = total bed volume.
2.10 Aminoacid analysis

Amino acid analysis of purified proteins was determined by Dr. R. Prusti, Seattle, Washington and Dr. R. Nagaraj, CCMB, Hyderabad.

2.11 Antisera preparation

Purified 35 kDa albumin protein (150ug) was emulsified with Freund's complete adjuvant and injected subcutaneously at multiple sites on the back of a New Zealand white rabbit. A booster injection of 100ug in incomplete Freund's adjuvant was given after 21 days. Rabbit was bled 20 days later and serum separated. Presence of antibodies was checked by Ouchterlony immunodiffusion technique (Garvey et al, 1977). Antibodies to the 54 kDa polypeptide were obtained similarly, except that the rabbit was injected with crushed gel slice containing protein. Gel slice containing the 54 kDa protein (approximately 100 ug) homogenised in 1 ml PBS was emulsified in Freund's adjuvant and injected as per the immunization schedule described for the 35 kDa protein.

2.12 Western blot analysis of proteins

2.12.1 Transfer of proteins to nitrocellulose

Proteins were transferred from polyacrylamide gel to nitrocellulose membrane in Towbin's transfer buffer (25 mM
Tris base, 192 mM glycine, 20% v/v methanol) (Towbin et al, 1979) using a transblot apparatus (Hoefer Scientific Instruments). Transfer was carried out at 0.3 A for 4h. After transfer, the blot was stained for 5 min with 0.5% Ponceau S in 1% acetic acid and destained in 1% acetic acid (Salinovich and Monteharo, 1986) to reduce the background. Standard proteins were marked and the blot was then fully destained in water.

2.12.2 Detection of Immunoreactive polypeptides

(1) Blots were treated with 5% non fat dry milk (Hi-Media) in TBST for 2 h to overnight to block the non specific binding sites.

(2) They were rinsed in TBST twice and incubated in 1:5000 dilution of primary antibodies in TBST for 2h at room temperature.

(3) To remove the unbound antibodies, blots were washed three times in TBST, 10 min each wash.

(4) Blots were next incubated with Anti rabbit IgG-alkaline phosphatase conjugated antibodies (Promega) at a dilution of 1:7500 in TBST, as recommended by the suppliers, for 2 h at room temperature.
(5) Blots were washed as described earlier and immunoreactive peptides were visualized by incubating the blot in color development solution of NBT/BCIP (Promega) in alkaline phosphatase buffer (100 mM Tris-Cl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂) till the bands appeared.

(6) Color development was stopped by transferring the blots to water.

2.13 Affinity purification of Antibodies

Antibodies were purified by a modified procedure of Elledge and Davis (1987).

(1) Purified protein (20 ug) was run on a 12% SDS-PAGE and blotted onto nitrocellulose membrane.

(2) Protein band was visualized by Ponceau S staining and was neatly cut into 1 cm long pieces.

(3) After destaining the filters with water, they were incubated for 1 h in PBS containing 20% FCS (Foetal Calf Serum) to block the non specific binding sites.

(4) Filters were next washed three times with PBS and incubated with antibodies at 4°C for 2 h. A control nitrocellulose filter was simultaneously put for incubation.
(5) Filters were washed with PBS containing 0.1% Tween-20. Control filter was separated from the experimental filter. Bound antibodies were eluted separately with 0.2 ml of 0.2 M glycine-HCl (pH 2.5) and the eluent was immediately neutralized by the addition of 0.1 ml potassium phosphate buffer, pH 9.0 containing 5% FCS. Filters were re-eluted.

(6) Eluted antibodies were made to 1 ml by addition of 0.4 ml of PBS containing 5% FCS, mixed, aliquoted and stored at -70°C.

2.14 Glycoprotein staining

PAS (Periodic acid, Schiff’s reagent) was used for glycoprotein staining as described by Johnstone and Thorpe (1982).

(1) Purified albumin protein (50 ug) was run on a 12% SDS-PAGE along with protein standards.

(2) Gel was equilibrated in 100 ml of 7% acetic acid for 1 h at 4°C.

(3) Gel was next incubated with 100 ml of freshly prepared 1% (w/v) periodic acid in 7% acetic acid for 1 h at 4°C, in dark.

(4) It was washed at 4°C for 24 h with several changes of 7% acetic acid.
The color was developed by incubating the gel in cold Schiff's reagent until the bands appeared.

2.15 Haemagglutination assay

Haemagglutination assay was performed as described by Lis and Sharon (1972). Briefly, erythrocytes were collected from blood by centrifugation and suspended in PBS, taking 5 ml of PBS for each ml of packed erythrocytes. It was trypsinized and treated with different concentrations of purified protein. Concanavalin A was used as a positive control.

2.16 RNA isolation

RNA was isolated by the procedure of Ausubel et al. (1987) with some modifications.

2.16.1 Precautions taken during RNA isolation:

a. All the glassware was acid washed.

b. Centrifuge tubes, kept only for RNA work, were used.

c. Fresh microfuge tubes and tips were used.

d. Fresh double distilled water was used to prepare solutions and all solutions and glassware were autoclaved.
2.16.2 Equilibration of phenol

Redistilled phenol containing 0.1% hydroxyquinoline was equilibrated with an equal volume of TLE (0.2M Tris, 0.1M LiCl, 5 mM EDTA, pH adjusted to 8.2 with HCl) containing 30 mM NaOH. Phenol phase was taken and extracted two more times with TLE alone. Equilibrated phenol was stored at 4°C in a dark bottle.

2.16.3 Isolation procedure

(1) Seeds (10g) were ground to a fine powder in mortar and pestle using liquid nitrogen.

(2) Seed meal was immediately transferred to a mixture consisting of 80 ml TLES (TLE containing 1% SDS) and 40 ml phenol. It was vigorously shaken for 5 min, so that no clumps remained. Chloroform (40 ml) was then added to it, mixed vigorously and the slurry was incubated at 50°C for 30 min with occasional mixing.

(3) It was centrifuged at 15,000 rpm (Sorvall, SS34 rotor) for 30 min at 25°C.

(4) The aqueous phase was taken and extracted three more times at 4°C with 80 ml of phenol/chloroform and once with chloroform alone.
(5) RNA was selectively precipitated from the aqueous phase by the addition of Lithium chloride (LiCl) to a final concentration of 2M. Precipitation was allowed to go on for 16 h at 4°C.

(6) RNA pellet obtained was washed with 2M LiCl and was suspended in 4 ml water. It was reprecipitated with LiCl for 2-3 h at 4°C.

(7) Pellet obtained was again washed with 2M LiCl and dissolved in 2ml sterile water. To this, 200ml of 3M sodium acetate pH 5.2 and 5.5 ml of cold ethanol were added and RNA was precipitated overnight at -20°C.

(8) RNA was pelleted at 10,000 rpm, for 15 min at 4°C. Pellet was washed with 70% ethanol, dried under vacuum and dissolved in 1 ml sterile water. It was heated to 65°C to aid solubilization and whatever remained undissolved was discarded.

(9) RNA concentration was estimated by measuring absorbance at 260 nm. 1 OD at A_{260} is equivalent to 40 ug RNA (Maniatis et al., 1982).

2.17 Isolation of Poly(A)^+ RNA

Poly(A)^+ mRNA was separated from the total RNA by the procedure of Okayama et al. (1987) using oligo (dT)-cellulose chromatography.
2.17.1 Column preparation

Oligo (dT)-cellulose (Pharmacia) was suspended in TE (10mM Tris-Cl, pH 7.5; 1 mM EDTA) and the fines were removed by decantation. If the column was previously used, it was washed with 0.1M NaOH and rinsed several times with sterile water before use. Two columns, one of 1.5 cm and the other of 0.5 cm in height were made in autoclaved Econocolumns (0.6 cm diameter) (Bio-Rad) and washed with several column volumes of TE until the pH of the column reached that of TE. The columns were then equilibrated in TE containing 0.5M NaCl.

2.17.2 Poly(A)+ mRNA separation

RNA (5mg) in sterile water was heated to 65°C for 10 min and chilled on ice. Equal volume of 2X binding buffer containing 1M NaCl, 20mM Tris-Cl pH 7.5 and 2mM EDTA was added to it and was applied to the 1.5 cm column. The flow through was applied four times more and the column was washed with 25 ml of 1X binding buffer. The bound RNA was eluted with TE. 0.2 ml fractions were collected and analysed by spot assay as described later. Fractions containing RNA were pooled and rechromatographed on the second oligo (dT)-cellulose column in the same manner as above. Poly(A)+ RNA containing fractions were precipitated
by the addition of 0.2 volumes of 2 M NaCl and 3 volumes of ethanol at -20°C, overnight. RNA pellet was obtained by centrifugation in a microcentrifuge for 15 min at 4°C. Pellet was washed with 70% ethanol, vacuum dried and suspended in 50-100 ul of sterile water. RNA was quantitated by spot assay as well as spectrophotometrically.

2.17.3 Spot assay (Okayama et al., 1987)

Sample (1 ul) from each fraction was mixed with 10 ul of 1 ug/ml ethidium bromide (freshly prepared from a 10mg/ml stock solution) and spotted on a piece of Saran Wrap. Yeast tRNA of known concentration was diluted to get a doubling dilution series ranging from 10 ng to 1 ug. It was spotted in a similar manner, and was used as a standard for Poly(A)+ mRNA quantitation.

2.18 In vitro translation

In vitro translation was carried out in the nuclease treated rabbit reticulocyte lysate system (Promega). Total RNA (5ug) or Poly(A)+RNA (0.5ug) was translated in a total volume of 20 ul containing 20 uCi of [35S] methionine [>800Ci/mmol] (BARC). The reaction was carried out at 30°C for 2h. Incorporated counts were determined as described below and the translated product was either directly checked on SDS-PAGE or after immunoprecipitation (Section 2.19).
2.18.1 Determination of incorporated counts

(1) Incorporated counts were estimated by spotting 2 ul aliquot on small squares of Whatman # 1 which had previously been saturated with 5% TCA containing 5 mM cold methionine and dried.

(2) Filters were washed with ice-cold 5% TCA (10 ml per filter) in a beaker for 15 min and then in fresh 5% TCA at 90°C for 10 min.

(3) $H_2O_2$ to a final concentration of 3% was added to the filters and incubated for another 10 min.

(4) Filters were rinsed with cold 5% TCA for 5 min and then with ethanol for 5 min. They were dried and counted in Toluene based Scintillation fluid (Spectrochem).

2.19 Immunoprecipitation of the translated product

2.19.1 Preparation of Protein A-Sepharose bound to Antibodies

(1) Protein A-Sepharose beads (50 mg; 5mg/reaction) (Pharmacia) were swollen in 0.5 M sodium phosphate buffer, pH 8.0 for 1-2 h. It was washed twice with 0.1M Sodium phosphate buffer, pH 8.0 and suspended in 1 ml of the same buffer (Ey et al., 1978).
(2) Beads were divided into two parts. To one part, 50 ul of immune serum and to the other part, 50 ul of preimmune serum (for 10 reactions) was added and incubated with end over end shaking for 3h at room temperature.

(3) After the binding of IgGs to Protein A-Sepharose, the beads were collected by centrifugation and washed twice with 0.1M sodium phosphate buffer to remove unbound serum components and stored at 4°C until use.

2.19.2 Immunoprecipitation

Immunoprecipitation was done by the procedure of Anderson and Blobel (1983).

(1) To 15 ul of the translation mix, 6 ul of 10% SDS was added and boiled for 5 min in a boiling water bath.

(2) It was diluted with 21 ul of water and 170 ul of dilution buffer [ 2.5% Triton X-100, 190 mM NaCl, 6 mM EDTA, 50 mM Tris-Cl, pH 7.5, 0.5% Aprotinin (Sigma)] and incubated with Protein A beads loaded with preimmune IgG with shaking at room temperature for 2-3 h.

(3) Beads were removed by centrifugation and the supernatant was treated with immune IgG bound Protein A beads for 3 h.
(4) Beads were taken after discarding the supernatant and were washed three times with wash buffer (0.1% Triton X-100, 0.02% SDS, 150 mM NaCl, 50 mM Tris Cl, pH 7.4, 5 mM EDTA. 0.5% Aprotinin) and once with water.

(5) Immunoprecipitated antigen was eluted by boiling the beads in Laemmli buffer and electrophoresed on SDS-polyacrylamide gel. The gel was stained, destained and fluorography was done using sodium salicylate (Chamberlain, 1979). The gel was then dried and exposed to Kodak, X-Omat AR film at -70°C.

2.20 cDNA synthesis

cDNA synthesis involves the conversion of mRNA into double stranded cDNA. The method used here is based on the procedure of Gubler and Hoffman (1983). Oligo (dT) was used as a primer for first strand cDNA synthesis.

Glassware and solutions

Only fresh, siliconised microcentrifuge tubes and micropipet tips were used throughout. Siliconization was done as described by Maniatis et al., 1982.

2.20.1 First strand cDNA synthesis
cDNA was synthesized using Amersham’s cDNA synthesis kit.
(1) mRNA (3 ug) was denatured by heating at 65°C for 5 min followed by rapid chilling on ice.

(2) To the denatured mRNA on ice, 3 ul of dNTP mix (10 mM d(A,G,T)TP and 5 mM d(CTP), 2.5 ug of oligo (dT) primer and 5 uCi of $^{32}\text{P}$ dCTP (>3000 Ci/mmole) were added. Sodium pyrophosphate and RNase inhibitor were also used during cDNA synthesis to enhance the yield of full length cDNA.

(3) All the components were gently mixed and 60 units of reverse transcriptase was next added to the reaction mixture in a final volume of 30 ul. First strand synthesis was carried out at 42°C for 45 min. The reaction was stopped by chilling on ice.

(4) An aliquot (2 ul) mixed with 20 ul of 0.25 M EDTA was kept aside for analysis.

2.20.2 Second strand cDNA synthesis

(1) To the first strand cDNA synthesis mix kept on ice, 50 uCi of $^{32}\text{P}$ dCTP ( >3000 Ci/mmol), 2.5 units of E.coli RNase H, and 66 units of DNA polymerase I were added. It was mixed gently and then incubated sequentially at 12°C for 1 h and 22°C for 1 h. RNase H nicks the mRNA template and the cDNA synthesis is brought about by DNA polymerase I.
(2) The reaction was stopped by incubating at 70°C for 10 min. To make ds cDNA blunt ended, 6 units of T4 DNA polymerase was added and the reaction was carried out at 37°C for 10 min.

(3) Reaction was stopped by the addition of 6 ul of 0.25 M EDTA. 2 ul sample in 20 ul of 0.25M EDTA was kept aside for analysis.

2.20.3 Quantitation of cDNA synthesized

(1) Amount of cDNA synthesized was calculated by checking for the incorporated counts. 2 ul aliquot of the sample diluted in 20 ul EDTA was spotted on Whatman DE81 paper. It was dried and counted without any scintillation fluid.

(2) The filter was washed five times in 0.5M Na₂HPO₄, twice in water, twice in ethanol, dried and counted again. Percent incorporation was then calculated.

a) Percent incorporation in the first strand synthesis was 5%.

b) Percent incorporation in the second strand synthesis was 7%

c) Weight of ds cDNA synthesized was about 2.7 ug.
2.20.4 Purification of double-stranded cDNA

(1) To 150 ul of ds cDNA, equal volume of Phenol (equilibrated in TE)/Chloroform (1:1; v/v) was added and vortexed briefly.

(2) Phases were separated by centrifugation for 1 min and the upper aqueous phase was collected without taking the interface.

(3) Phenol phase was reextracted with 75 ul of TE and two aqueous phases were pooled.

(4) Combined aqueous phase was reextracted once with phenol/chloroform and once with chloroform alone.

(5) To the aqueous phase equal volume of 4 M ammonium acetate (filter sterilized) and twice the combined volume of cold ethanol were added and incubated over night at -20°C to precipitate cDNA.

(6) Ethanol precipitate was warmed to room temperature with gentle shaking and centrifuged for 15 min in a microcentrifuge.

(7) Pellet was washed once with 50 ul of 2M ammonium acetate and 100 ul ethanol at room temperature and once with 200 ul of cold ethanol. It was dried and dissolved in 10 ul TE.
2.20.5 Alkaline agarose gel electrophoresis

2.20.5.1 Sample preparation

(1) First and second strands of cDNA having approximately 10,000 cpm were used for gel analysis.

(2) Samples were mixed with 2 ug of carrier DNA (Salmon Sperm DNA) and denatured by the addition of 1/3 of their combined volume of 1 M NaOH at 45°C for 30 min.

(3) 1 M HCl and 1 M Tris, pH 8.0 (volume equal to that of 1 M NaOH) were sequentially added to the reaction tube.

(4) The resulting mixture was extracted with an equal volume of phenol/chloroform and precipitated with ethanol as described earlier (Section 2.16).

(5) The pellet, after drying, was dissolved in 20 ul of alkaline loading buffer (50 mM NaOH, 1 mM EDTA, 2.5% Ficoll, 0.025% bromocresol green).

2.20.5.2 Electrophoresis

(1) A 1.4% agarose gel in 50 mM NaCl and 1 mM EDTA was poured in a horizontal gel electrophoresis apparatus.

(2) Once the gel was set, alkaline electrophoresis buffer (30 mM NaOH, 1 mM EDTA) was added to it, so as to cover the gel to a depth of 3-5 mm.
(3) Buffer was allowed to soak into the gel for 30 min and then the buffer was removed.

(4) DNA samples along with end-labeled lambda Hind III digest were loaded and electrophoresed at 70 volts, until the dye migrated 1/3 the length of the gel.

(5) The gel was removed from the apparatus and incubated for 30 min in 7% trichloroacetic acid at room temperature (two changes).

(6) The gel was placed on a saran wrap and mounted onto a glass plate and dried for several hours under many layers of filter paper.

(7) Dried gel was exposed to an X-ray film for autoradiography.

2.21 Construction of cDNA library

cDNA library was constructed in the expression vector lambda gt11. Fusion protein formed between galactosidase and the foreign protein encoded by cDNA insert is detected by immunoscreening (Young and Davis, 1983).

2.21.1 EcoR I methylation

Double stranded cDNA (1 ug) was methylated at EcoR I sites using S-adenosyl-L-methionine in the presence of 20 units of
EcoRI methylase at 37°C for 1 h. To check for methylase reaction, an aliquot of methylated sample was analysed by EcoR I digestion.

2.21.2 Ligation of EcoR I linkers to cDNA

Phosphorylated EcoR I linkers (1 ug) were next ligated to cDNA in the presence of 5 units of T4 DNA ligase at 15°C for 16 h. Linkered cDNA was then digested with EcoR I to generate cohesive ends. All the internal EcoR I sites were protected due to EcoR I methylation.

2.21.3 Separation of linkered cDNA

Linkered cDNA was separated from the excess linkers by running the reaction mixture through a small gel filtration column. Since the cDNA was labeled, fractions (200 ul) were monitored by Cerenkov counting. Two fractions with the highest counts were pooled (400 ul) and precipitated with 40 ul of 3 M sodium acetate, pH 5.5 and 1 ml ethanol, overnight at -20°C. cDNA was pelleted in a microcentrifuge at 4°C for 15 min, washed in cold absolute ethanol and air dried. cDNA pellet was counted by Cerenkov and dissolved in STE (100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA) at a concentration of 50 ng/ul.
2.21.4 **Ligation of EcoR I linkered cDNA to lambda gt 11 DNA and *in vitro* packaging**

EcoR I linkered cDNA (25 ng) was ligated to 1 ug of lambda gt 11 vector DNA using 2.5 units of T4 DNA ligase for 16 h at 15°C. It was next packaged *in vitro* as per the instructions of the supplier (Amersham).

2.21.5 **Preparation of phage plating cells**

(1) A single colony of E.coli, Y 1090, was picked up from a freshly streaked plate and inoculated into 5 ml of growth medium (LB-amp containing 0.4% filter sterilized maltose). It was grown overnight (12 to 13 h) at 37°C with shaking.

(2) 0.5 ml of the overnight culture was added to 25 ml of fresh growth medium and was allowed to grow to an OD_{600} of 0.35 - 0.4.

(3) Culture was cooled on ice to arrest cell growth and cells were pelleted at 5000 rpm for 5 min at 4°C.

(4) Cell pellet was suspended in 7 ml of ice cold, sterile 10 mM MgSO₄ and mixed thoroughly. It was stored at 4°C and used upto one week.
2.21.6 Titration of lambda gt11 recombinant phages

(1) A 5 ul aliquot was taken from the final packaged phage mixture and diluted in SM buffer (100 mM NaCl, 8 mM MgSO₄, 50 mM Tris-Cl pH 7.5, 0.01% gelatin) to get a series of dilutions ranging from $10^2$ to $10^5$.

(2) 100 ul of plating cell suspension (Section 2.21.5) was mixed with 100 ul of different phage dilution and was incubated at 37°C for 15 min.

(3) Top agar was melted and maintained at 45°C. To this, ampicillin to a final concentration of 50 ug/ml, and 10 ul per ml each of 100 mM IPTG and 2% X-gal was added.

(4) To each plating mix, 4 ml of liquid top agar was added, mixed quickly and poured onto a 90 mm dry LB-amp plate. The plate was equilibrated to 37°C before use.

(5) Once the top agar had set completely plates were incubated upside down at 43°C, for 3-4 h until plaques appeared.

(6) Number of plaques obtained were counted. The phage titre and relative number of blue and white plaques on each plate were estimated.

(7) Number of recombinants obtained were 33,000. Cloning efficiency per ug cDNA
\[
\text{no. of recombinants} = \frac{\text{ng cDNA used}}{1000} \times 1000 = 1.3 \times 10^6 \text{ pfu/ug cDNA}
\]

2.22 Immunoscreening of the library

(1) About 34000 recombinant phages were absorbed to 0.25 ml of E.coli Y 1090 plating cells and plated in the same manner as described for the titration protocol except that plates did not contain any IPTG and X-gal. The plates were incubated at 43°C till the plaques appeared.

(2) While the plates were being incubated, nitrocellulose filters (Schleicher and Schuell) of the same size as the petridish, were soaked in 10 mM IPTG and air dried.

(3) One IPTG soaked nitrocellulose filter, previously numbered, was carefully placed on each plate so that no bubbles were trapped. Plates were incubated upside down for 4 hr at 37°C.

(4) Each filter was marked asymmetrically by pushing a needle through the filter into the agar, at four asymmetric points.

(5) Filters were removed and washed in TBS. A duplicate filter was prepared by placing a second filter on each
plate, in the same manner as before. Plates were stored sealed in plastic bags at 4°C till immunoscreening was over.

(6) Nitrocellulose filters were screened for the presence of positive clones as described for Western blot analysis (Section 2.13.2).

(7) Positive plaques were picked up using a cut tip and placed in 500 ul of SM buffer with a drop of chloroform.

(8) Recombinant positive plaques were purified by repeated screenings.

2.23 Amplification of clones

(1) All the positive lambda clones were titrated and plated at a density of $10^5$ pfu per 90 mm plate as described earlier (Section 2.21.6).

(2) Plates were incubated at 43°C until the plaques were just confluent.

(3) 5 ml of SM buffer was added to each plate and left overnight at 4°C.

(4) Phages that had diffused into SM were removed and stored over chloroform.

(5) Phage titre for each clone was estimated.
2.24 Fusion protein analysis

(1) Phages were induced for fusion protein synthesis as described in the screening protocol (Section 2.28).

(2) Plaques, after induction with IPTG, were picked up using a cut tip and lysed in Laemmli buffer. They were electrophoresed on SDS polyacrylamide gel and analysed by Western blot (Section 2.13.2).

2.25 DNA isolation from phages

2.25.1 Preparation of liquid lysate

Liquid lysates were prepared as described by Miller (1987).

(1) LB-amp (25 ml) containing 5 mM CaCl₂ was inoculated with an overnight Y 1090 cell culture and the cells were allowed to grow at 37°C with shaking till OD₅₅₀ reached 0.2.

(2) Phages were next added to the culture at an moi (multiplicity of infection) of 0.01 (10⁹ phages/litre).

(3) The flasks were shaken vigorously at 37°C for 6-7 h until lysis occurred. To each flask, 1 ml chloroform was added and shaking was continued for another 30 min. Flasks were left overnight in cold.

(4) Phage titre was estimated as described in Section 2.21.6, using LB-amp plates without IPTG and X-gal. Phage titre was about 10¹⁰ - 10¹¹ pfu/ml for different clones.
2.25.2 Isolation of DNA from phage lysate

DNA was isolated by the CTAB (Cetyltrimethylammonium Bromide) procedure of Manfioletti and Schneider (1988) with some modifications. The phages were concentrated using PEG (Miller, 1987) before DNA isolation.

(1) Phage lysates were brought to room temperature and to each, DNase I to a final concentration of 1 μg/ml, was added and incubated at room temperature for 30 min.

(2) Lysates (25 ml) were transferred to SS34 (Sorvall) tubes and, to each tube, 1.5 g of solid NaCl was added and mixed well. They were incubated on ice for 1 h.

(3) Debris was removed by centrifugation at 12000 rpm for 10 min at 4°C.

(4) 2.5 g of solid PEG 8000 (final concentration of 10% w/v) was next added to the supernatant, mixed and left on ice for 1 h to precipitate the phage particles.

(5) Phages were collected by centrifugation and the supernatant was completely drained off by briefly leaving the tubes in a tilted position.

(6) Bacteriophage pellet was suspended in 1 ml TSM (10 mM Tris-Cl pH 7.5, 10 mM MgCl₂, 20 mM NaCl) and gelatin was
added to a final concentration of 50 mg/ml (from a 0.3% stock).

(7) Equal volume of an 80% slurry of DE52 (Whatman), equilibrated in TSM, was added to it and mixed.

(8) Resin was spun down and the supernatant (about 1.5 ml) was transferred to SM24 tubes (Sorvall).

(9) To each tube, 20 mM EDTA, 100 mM Tris, pH 8.0 and 50 ug/ml proteinase K (final concentrations) was added and incubated at 45°C for 15 min.

(10) CTAB was next added to each tube to a final concentration of 0.5% (from a 5% w/v stock in 0.5 M NaCl). Tubes were heated for 3 min at 68°C, cooled on ice for 3-5 min and spun for 10 min.

(11) Pellet obtained was redissolved in 2.5 ml of 1.2 M NaCl. Ethanol (2.5 volumes) was added and the precipitate was seen immediately.

(12) Precipitate was briefly spun to get the pellet. Pellet was washed in 70% ethanol, air dried and dissolved in 200 ul TE.

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2.26 Precipitation of DNA using spermine tetrahydrochloride

Spermine tetrahydrochloride (Sigma) was used to precipitate DNA from dilute solutions ranging from 0.1 to 100 ug DNA/ml by the procedure of Hoopes and McClure (1981).

(1) Spermine to a final concentration of 0.1 to 1.0 mM (from a 0.1 M stock solution) was added to the DNA sample and was incubated on ice for 30 min.

(2) The precipitate was collected by centrifugation and was directly dissolved in Endo R (1X) and electrophoresed.

(3) When DNA amount was more (Amaranthus chromosomal DNA, digested with different enzymes) spermine was extracted in extraction buffer (75% ethanol, 0.3M sodium acetate, 10 mM magnesium acetate) by leaving it on ice for 1h with intermittent vortexing, prior to electrophoresis.

2.27 Agarose gel electrophoresis

Agarose gel electrophoresis was performed as described by Maniatis et al.(1982). TAE, containing ethidium bromide to a final concentration of 0.5 ug/ml, was used during electrophoresis.

2.28 Analysis of the cloned DNA

(1) Size of the insert DNA was checked by digesting the cloned lambda DNA (0.25-0.5 ug) either with EcoR I (BRL) or
with a combination of Kpn I-Sac I (Pharmacia) for 6 to 12 h in appropriate buffers, as recommended by the suppliers.

(2) EcoR I digested DNA was end labeled by klenow labeling reaction using 1 uCi of $[^32P]dATP$ and 1 unit of DNA polymerase I klenow fragment (USBC) at room temperature for 30 min.

(3) Restriction fragments of cloned DNA along with the standards, prepared by digesting lambda DNA with HindIII and pUC19 DNA with DdaI, (end labeled for EcoR I digest) were analysed on agarose gel.

(4) For EcoR I digests, the gel was fixed in 7 % (w/v) TCA, dried and autoradiographed. Kpn I-Sac I double digests were analysed by UV fluorescence after ethidium bromide staining.

2.29 Subcloning

In order to get high DNA yield, the EcoR I digested insert from lambda-cDNA clone was subcloned into the EcoR I site in the plasmid vector pTZ18U (United States Biochemical Corporation). Plasmids are useful subcloning vectors because they can be easily transfected into cells, amplified and purified to yield large quantities of DNA. Subcloning was done by the shotgun cloning protocol as described by Davis et al. (1986).
2.29.1 Ligation of insert to the vector

(1) Purified plasmid pTZ18U (5 ug) and lambda-AmAl clone (1ug) were digested with 2.5 units of EcoRI at 37°C for 6 h.

(2) After checking for digestion on agarose gel, the 5' phosphate of the vector DNA was hydrolysed using 0.5 units of calf intestinal alkaline phosphatase for 30 min at 37°C, so as to get the linearized DNA.

(3) Linearised plasmid vector was extracted with phenol/chloroform (1:1) and then with chloroform alone. DNA was ethanol precipitated and dissolved in 10 ul TE.

(4) Digested cloned DNA was directly added to the reaction mixture containing 0.1 ug of dephosphorylated vector DNA and 100 units of T4 DNA ligase in a final volume of 50 ul. Ligation was carried out at 15°C for 16 h. The large lambda gt11 arms will not be taken up by the plasmid for insertion, nor will the plasmid self-circularize due to dephosphorylation. The entire reaction mixture was then taken for transformation.

2.29.2 Transformation of E.coli

(1) E.coli JM101 competent cells were prepared in the same way as described for Y 1090 cells (section 2.21.5) except that the cells were suspended in 50 mM CaCl₂.
(2) In a sterile tube, 0.1 ug of ligated plasmid DNA was mixed with 200 ul of competent cells and was incubated on ice for 30 min.

(3) Cells were given a heat shock in a 42°C water bath for 3 min.

(4) After adding 1 ml of LB, cells were grown for 1 h at 37°C with shaking to allow expression of the antibiotic resistance gene.

(5) Transformed cells (200 ul) from each tube were spread on LB-amp plates with the help of a glass spreader.

(6) Untransformed cells and cells transformed with undigested vector were also plated, which served as controls.

(7) Plates were incubated upside down at 37°C for 12-13 h.

(8) Transformants were picked up and patched on LB-amp plates containing X-gal and IPTG. On freshly prepared LB-amp plates, 40 ul of X-gal (20 mg/ml stock in dimethyl formamide) and 4 ul of IPTG (200 mg/ml) was spread with the help of a spreader. Plates were left at room temperature for 1 h before use (Maniatis et al., 1982).

(9) Plates were incubated at 37°C overnight and white colonies were picked up for further analysis.
2.30 Plasmid isolation

Plasmid DNA was isolated by the procedure described in 'Protocols and Applications' (Promega).

(1) A single colony from an LB-amp plate was used to inoculate 100 ml of Terrific broth (Sambrook et al., 1989). It was made by adding 1.2g of bactotryptone, 2.4g of bacto yeast extract and 0.4 ml of glycerol to 100 ml water. It was autoclaved and then 10 ml of a sterile solution of 0.17M KH$_2$PO$_4$ and 0.72 M K$_2$HPO$_4$ was added to it.

(2) Cells were pelleted at 5000 rpm for 5 min in capped SS34 tubes (Sorvall) and suspended in 5 ml of cold freshly prepared lysis buffer (25 mM Tris-Cl, pH 8.0, 10 mM EDTA, 50 mM glucose and 10 mg of lysozyme). It was incubated in ice water for 10 min.

(3) Freshly prepared, 10 ml solution of 0.2N NaOH and 1% SDS was added to the culture at room temperature, mixed and incubated on ice for 15 min to denature DNA.

(4) The above solution was neutralized with 3 M sodium acetate, pH 4.6 and incubated on ice for 20 min. This causes the plasmid DNA to renature. A thick precipitate was immediately seen.
(5) Precipitate was removed by centrifugation at 12,000 rpm for 15 min. Plasmid DNA, obtained in the supernatant, was treated with 50 μl of DNase-free RNase A (1 mg/ml stock) at 37°C for 30 min. DNase-free RNase was prepared as described by Maniatis et al., 1982.

(6) It was extracted twice with one volume of phenol/chloroform (1:1, equilibrated in TE), once with chloroform and then precipitated with two volumes of ethanol at -20°C for 30 min.

(7) Ethanol was completely drained off and the pellet was dried and dissolved in 1.6 ml water. NaCl was added to a final concentration of 0.4 M and DNA was reprecipitated with 2 ml of 13% PEG for 1 h in ice water bath.

(8) DNA precipitate was washed with 5 ml of 70% ethanol, air dried and dissolved in 1 ml TE.

(9) DNA was quantitated spectrophotometrically by measuring absorbance at 260 nm. Assuming OD 1 at 260 nm to be 50 μg, total DNA concentration was estimated (Maniatis et al., 1982).

(10) To check for the insert size, 0.5 μg DNA was digested overnight with EcoR I and the product was checked on 1.5% agarose gel.
2.31 Restriction site analysis

Cloned plasmid DNA carrying the insert, was digested with various enzymes, to identify internal restriction sites. The digested samples were resolved on 1.2% agarose gel containing ethidium bromide.

2.32 Purification of the insert

Insert was purified using DE81 paper (Whatman) as described by Ausubel et al (1987).

(1) Plasmid DNA (10 μg) was digested with 20 units of EcoR I for 6 h and electrophoresed on 1.5% agarose gel containing ethidium bromide.

(2) A strip of DEAE cellulose paper, DE81 (Whatman), was soaked in 2.5M NaCl for 3-4 h and washed several times in water. It was equilibrated in TAE for 20 min.

(3) When the insert band was well separated, a slice of agarose was removed from below the band. DE81 paper was then placed next to the band and agarose slice was inserted back, without trapping any bubbles.

(4) Electrophoresis was continued till the entire insert band was transferred to the paper, which was monitored by UV fluorescence.
(5) Paper was washed in cold water and bound DNA was eluted in 500 ul of elution buffer (20 mM Tris-Cl, pH 7.5, 1 mM EDTA, 1.5 M NaCl) by incubating it at 37°C for 2 h with occasional vortexing.

(6) Supernatant, collected by repeated spins in the microfuge, was phenol extracted and the DNA was ethanol precipitated.

2.3.3 Isolation of genomic DNA

Genomic DNA was isolated by the procedure of Rogers and Bendich (1988).

(1) Seeds (30g) were ground to a fine powder in a blender using liquid nitrogen.

(2) Seed meal was transferred to a conical flask to which 30 ml of 2X CTAB buffer (2% CTAB; 100 mM Tris-Cl pH 8.0, 20 mM EDTA, 1.4 M NaCl) maintained at 65°C was added. It was mixed well and then 120 ml of 1XCTAB buffer (at 65°C) was added and incubated at 65°C for 20 min with occasional swirling.

(3) One volume (150 ml) of chloroform/isoamyl alcohol (24:1) was gently mixed with it so as to get an emulsion, which was centrifuged in SS34 tubes (Sorvall) to separate the phases.
(4) To the aqueous phase, 15 ml of 10% CTAB solution (10% CTAB in 0.7 M NaCl) was added, mixed, and extracted once with chloroform/isoamyl alcohol.

(5) DNA was then precipitated by the addition of equal volume of precipitation buffer (1% CTAB, 50 mM Tris-Cl, pH 8.0, 10 mM EDTA). Precipitate was collected by a brief spin.

(6) DNA pellet was dissolved in 5 ml of 1 M NaCl at 65°C for 10 min and ethanol precipitated to remove CTAB.

(7) Ethanol precipitate was dissolved in 10 ml TE. Contaminating RNA was precipitated by adding 5 ml of 7.5 M Ammonium acetate and incubating on ice for 30 min. RNA pellet was removed by centrifugation.

(8) Supernatant was ethanol precipitated and dissolved in TE.

(9) DNA was quantitated by running it on a 0.8% agarose gel along with a series of lambda dilutions of known concentration.

2.34 Probe preparation by Random Primer Extension (Feinberg and Vogelstein, 1984)

(1) Purified insert (25 ng), in a volume of 10 ul, was heat denatured in a boiling water bath for 5 min and chilled on ice.
(2) Probe preparation was carried out in a total volume of 25 ul containing 50 mM Tris Cl, pH 8.0, 5 mM MgCl₂, 10 mM B-ME, 0.08 mM each of GTP, TTP, dCTP, 200 mM HEPES pH 6.6, 0.3 mg/ml random hexanucleotide primer (Pharmacia), 0.4 mg/ml nuclease free BSA (Pharmacia), 25 uCi [³²P] dATP (>3000 Ci/m mole), 5 units of klenow polymerase (USBC). Reaction was carried out at room temperature for 16 h. Progress of the reaction was monitored by checking ³²P incorporation as described (Section 2.20.3).

(3) Reaction was stopped at 80% incorporation by the addition of 75 ul of TE and 2 ul of 0.5 M EDTA. Labeled probe was precipitated with Ammonium acetate and ethanol as described (Section 2.20.4) and dissolved in 100 ul TE. 10 ug of Salmon Sperm DNA was used as a carrier during precipitation.

2.35 Southern Hybridization

2.35.1 Restriction digestion of clones: All the lambda clones were digested with either Kpn I-Sac I or EcoRI in a 30 ul reaction volume for 12 h. Reaction was stopped by the addition of 5 ul of 6X EndoR and the samples were resolved on agarose gel. (Maniatis et al., 1982).

2.35.2 Restriction digestion of Genomic DNA: Genomic DNA (10 ug) was digested with 50 units each of EcoRI (NEB),
Hind III (NEB), Pst I (Boehr.), and Hae III (Pharmacia), in two separate reactions of 20 ul and 200 ul each. When the reaction volume was 200 ul, digested DNA was precipitated with spermine as described (Section 2.26). It was dissolved in 20 ul TE and resolved on 0.8% agarose gel.

2.35.3 Transfer of DNA to Gene Screen Plus membrane

Transfer of DNA to Gene Screen Plus membrane was performed as described in 'Gene Screen Plus Protocol' (NEN Research, Dupont).

(1) DNA was depurinated by incubating the gel in 0.25N HCl for 15 min at room temperature.

(2) Gel was incubated in 0.4N NaOH - 0.6M NaCl for 30 min and then neutralized in 1.5M NaCl - 0.5M Tris-Cl pH 7.5 for 30 min.

(3) DNA from the gel was then transferred to Gene Screen Plus membrane by capillary blotting using 10 X SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) for 24 h. Wells were marked and the membrane was then immersed in 0.4 N NaOH for 1 min for complete denaturation of immobilized DNA and neutralized in excess of 0.4M Tris-Cl, pH 7.5 in 2 X SSC.

(4) Membrane was air dried and stored within Whatman sheets at room temperature till hybridization.
2.35.4 Hybridization

(1) Filters were prehybridized in a solution containing 50% formamide (deionized), 1% SDS, 1M NaCl and 10% Dextran sulfate for 12 h at 42°C with slow shaking.

(2) Probe, having about $3 \times 10^7$ cpm along with 100 ug of Salmon Sperm DNA was heat denatured for 5 min in boiling water bath and immediately chilled on ice. It was added to the prehybridization solution and hybridization was continued for 24 h at 42°C.

(3) Filters were removed from the solution and washed in a beaker as follows: a) Two washes of 5 min each in 500 ml of 2XSSC at room temperature with agitation. b) Two washes in 500 ml of 2XSSC containing 1% SDS at 65°C for 30 min with agitation. c) Two washes of 500 ml each in 0.1% SSC at room temperature for 30 min.

(4) All the liquid was completely drained off. Filters were put inside plastic bags and exposed to X-ray film using intensifying screen.

2.36 Hybrid Selection of RNA

Hybrid selection was performed according to the procedure of Ricciardi et al. (1979).
2.36.1 Binding of Plasmids to NC

(1) Plasmid DNA (5 ug in 30 ul from each subclone of Amaranth cDNA) was denatured in a boiling water bath for 10 min and chilled on ice.

(2) It was incubated with 30 ul of 1 N NaOH at room temperature for 20 min and then neutralized with 240 ul of neutralization buffer (1M Tris-Cl, pH 7.5; 1.5 M NaCl).

(3) It was immediately bound to nitrocellulose membrane (Biorad) under gentle suction, which had previously been assembled in a slot blot apparatus. Each slot was loaded with 1.5 ug of DNA.

(4) The slots were rinsed with 500 ul of 2X SSC. The filter was removed from the apparatus, air dried, kept within aluminium foil and baked at 80°C for 2 h.

2.36.2 Removal of unbound DNA

(1) Filter was placed on a wet 3 MM Whatman paper (autoclaved) and the DNA bands were cut.

(2) Filters were washed in 1 ml water in boiling water bath for 1 min. They were rinsed in water once and blotted dry on 3 MM Whatman paper.
2.36.3 Hybridization and washing

(1) Membranes were prehybridized in 400 ul of prehybridization solution (65% deionized formamide, 0.4 M NaCl, 0.2% SDS, 20 mM Pipes, pH 6.4) at room temperature for 30 min.

(2) Prehybridization solution was aspirated out and 300 ul of preheated hybridization solution (Prehybridization solution containing 25mg total RNA, maintained at 70°C) was added to the filters. Hybridization was carried out for 3 h at 50°C.

(3) Hybridization solution was removed and diluted with 1.6 volumes of water. Unbound RNA was recovered by precipitation with 2.5 volumes of ethanol.

(4) Filters were washed ten times with wash buffer (10 mM Tris-Cl, pH 7.6, 0.15 M NaCl, 1 mM EDTA) containing 0.5% SDS, maintained at 65°C, and twice with wash buffer alone.

2.36.4 Elution of RNA

(1) Filters were separated out into separate microfuge tubes using a sterile needle.

(2) tRNA (10 ug) in 300 ul water was added to each tube. They were placed in boiling water bath for 1 min and snap frozen in liquid nitrogen.
(3) Samples were thawed at room temperature and filters were removed.

(4) Each sample was extracted with equal volume of phenol/chloroform and precipitated with ethanol. The pellet was directly dissolved in translation cocktail (Section 2.18) and checked for its translational activity. An aliquot was also analysed on SDS-PAGE.

(5) Rest of the translated product was immunoprecipitated (Section 2.19) and analysed on SDS-PAGE.