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2.1 MATERIALS

2.1.1 Chemicals: All fine Chemicals were of the highest purity available locally. Proteinase K, X-Gal, IPTG, Triton X-100 were purchased from Boehringer Mannheim, FRG. Amphotericin B, Gentamycin, Trypan blue, Lysozyme, BSA (fraction V), Ficoll, PVP, Trizma base, EtBr, SDS, Salmon sperm DNA, PEG 6000, and Sephadex G-50 were obtained from Sigma Chemical Co., USA. Grace's medium was purchased from Hazelton Biologics, USA. TC yeastolate and Lactalbumin hydrolysate were procured from Difco Co. USA. Neutral red and FCS were purchased from Flow Laboratory, UK. Acrylamide, Bis, TEMED and APS were purchased from BioRad, USA. Redistilled phenol was saturated with Tris base according to Sambrook et al. (1989).

HindIII digested bacteriophage lambda DNA, HaeIII digested OX174 DNA (for use as DNA size markers), and oligonucleotide linkers, were purchased from New England Biolabs, USA.

Unless otherwise stated, Millipore filtered, double distilled, autoclaved H_2O was used at all times.

All the tissue culture disposable plastic wares were procured from Corning, USA and Nunclon, Nunc, Denmark.

2.1.2 Enzymes: T4 DNA ligase, T4 DNA polymerase, DNase I, RNase A, other DNA modifying enzymes and restriction enzymes were purchased from New England Biolabs, USA.

2.1.3 Radioisotopes: ^32P-<omega>CTP, ^35S-<omega>ATP, ^35S-methionine and carrier free ^125Iodine were purchased from NEN Research Products (DUPONT), USA.
2.1.4 Kits: Nick translation kit was purchased from Amersham, USA. Sequenase Version-2 DNA sequencing kit was from US Biochemicals, USA.

2.1.5 Bacterial strains: E.coli strains JM109, DH5α and TG1 were available in the laboratory, originally obtained from the American Type Culture Collection, USA.

2.2 METHODS

2.2.1 Insect cell culture: Spodoptera frugiperda (Sf9) (American Type Culture Collection, Rockville, Accession Number CRL 1711) cells having a doubling time of 18-24 h were subcultured 3x a week at 27°C in TNMFH media+10%FCS containing gentamycin and amphotericin B. Sf9 cells were maintained as a monolayer or as suspension culture.

TNMFH: To make TNMFH a 1 l pouch of powdered Grace's media was dissolved in 800 ml H₂O, to which was added 0.35g NaHCO₃. After adjusting the pH to 6.05 with 10M KOH, 3.3g TC yeastolate and 3.3g Lactalbumin hydrolysate was added and the vol was adjusted to 1 l. The medium was sterilized by passing through 0.22 μm millipore filter and tested for sterility by incubating at 37°C for four days. Complete media was prepared by adding 50 μg/ml Gentamycin, 2.5 μg/ml Amphotericin B and 10% FCS as and when needed.

2.2.1.1 Monolayer cultures: Subculturing in flasks were done as follows:

The cells were gently resuspended from a nearly confluent culture
by rapidly pipetting the medium across the monolayer with a pasteur pipet avoiding foaming. 0.5-1 ml of the culture (2-2.5x10^6 cells) was transferred to a new 25 cm² flask containing 4 ml of complete medium and incubated at 27°C. Cell viability was checked by examining the cells under low magnification (10x) in LM after the addition of 0.1 ml of Trypan blue (0.4% stock solution made up in buffered isotonic salt solution, pH 7.2-7.3) to 1 ml of cells (only non-viable cells take up Trypan blue). Cells with 98-99% viability were used for experimental purposes.

2.2.1.2 Suspension cultures: Fresh cells (> 98% viable) were seeded in an autoclaved conical flask (with a magnetic bar) at about 0.5-1.0x10^6 cells/ml. The flask was then incubated at 27°C with continuous shaking at 50-60 rpm. Cells were subcultured, after their density reached 2-2.5x10^6 cells/ml, by removing 80% of the suspension and replacing it with fresh complete medium. Fresh seeding was done at a cell density of 0.5x10^6 cells/ml. After every few passages, cells were concentrated by low speed centrifugation before reseeding. This was necessary to prevent accumulation of byproducts and potential contaminants in the culture. Sf9 cells are not anchorage dependent and therefore were easily transferred between monolayer and suspension cultures repeatedly without noticeable loss of viability or effect on growth.

2.2.2 Infection of insect cells with virus
2.2.2.1 Monolayer cultures: Cells were counted and seeded into flasks or plates at an appropriate density (as per Table 2.1) and
allowed to attach for 15 min. The media was removed then and appropriate amount of virus inoculum was added. Unless specific multiplicity of infection (moi) was desired, the minimum vol of virus inoculum which could cover the cell surface was added. Cells were incubated for 1 h at 27°C after which the virus inoculum was removed and fresh medium added. The cells were further incubated for 2-4 days and checked daily under microscope for cytopathic effects (CPE) and general signs of infection. To collect extra-cellular virus (ECV) for future inoculum, protein purification etc, the infected cell medium was transferred to centrifuge tubes and the cells were pelleted at low speed (500xg) for 15 min. The supernatant, containing ECV, was stored at 4°C. Cell pellet was saved for DNA or RNA purification, SDS polyacrylamide gel electrophoresis of infected cell proteins, purifications of non-secreted proteins etc.

2.2.2.2 Suspension cultures: The cells were counted and checked for viability. For high virus titres and optimal expression of recombinant proteins, more than 99% viable cells were selected. Desired number of cells were obtained after concentrating suspension culture by centrifugation. The amount of virus inoculum required was calculated by the equation:

\[
1\text{ml of inoculum} = \text{moi(PFU/cell)} \times \text{number of cells/ml of virus (PFU/ml)}
\]

The cell pellet was resuspended in the desired vol of virus inoculum and complete medium was added to achieve an initial density of \(1 \times 10^7\) cells/ml, and incubated at 27°C for 1 h at RT. Cells were resuspended by the addition of fresh complete medium.
to a density of \(5 \times 10^6\) cells/ml and the suspension was transferred to a spinner flask. The culture was incubated at 27°C with constant stirring for 2-4 days. Infection was regularly checked by examining the infected cells under a microscope. Cell pellet and ECV was collected as described earlier for monolayer infection.

Table 2.1

<table>
<thead>
<tr>
<th>Type of vessel</th>
<th>Cell density</th>
<th>Min. virus inoculum</th>
<th>Incubate in final volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 wells plate</td>
<td>2x10^6/well</td>
<td>10 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>24 &quot;</td>
<td>3x10^5/well</td>
<td>200 μl</td>
<td>500 μl</td>
</tr>
<tr>
<td>T-25 flask</td>
<td>3x10^6/flask</td>
<td>1 ml</td>
<td>10 ml</td>
</tr>
<tr>
<td>35 mm pet. dish</td>
<td>1.8x10^6/dish</td>
<td>100 μl</td>
<td>2 ml</td>
</tr>
<tr>
<td>Spinner flask</td>
<td>2x10^6/ml</td>
<td>4 ml</td>
<td>20 ml</td>
</tr>
</tbody>
</table>

2.2.3 Microscopic observation for CPE of infected cells:
Cytopathic effects were observed 24 h pi under light microscope. Typical CPEs are enlarged cells, enlarged nuclei, tendency to detach from the monolayer, irregular shapes and finally lysis.

2.2.4 Co-transfection: 1.5 ml of freshly grown cells (> 99% viable) were seeded in 35 mm petridishes at a density of 1x10^6 cells/ml, carefully avoiding cell debris or floating cells. Transfection mixture was made by adding the constituents in the following order:

- 2x Hepes buffer pH 7.1 475 μl
- 100 mM glucose 95 μl

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Wild type AcNPV DNA \( x \mu l \) (1 \( \mu g \))

Recombinant transfer vector DNA (CsCl purified) \( y \mu l \) (25 \( \mu g \))

Sterile distilled water \( z \mu l \)

Total volume \( 950 \mu l \)

Wild type viral DNA was added very carefully as it is very susceptible to shearing. 50 \( \mu l \) of CaCl\(_2\) (2.5 M) was slowly added in a drop wise fashion to the above and incubated at RT for 30 min. After removing the medium from petri dishes the transfection mix was very gently added on top of the cells and incubated at RT for 60-90 min. The transfection mix was replaced with 2 ml of complete medium and incubated for 3-4 days in a box containing wet paper towels to provide humidity. The cells were observed periodically under light microscope for CPE. The transfection supernatant was used for detecting the presence of recombinant virus after infecting cells in a 96 well plate followed by dot blot hybridization.

### 2.2.5 Dot blot hybridization:

Sf9 cells (99.9% viable) were seeded in 96 well plate at an appropriate density for 15-20 min. The cell supernatant was withdrawn from the co-transfected petri dishes and was used to make serial dilutions of \( 10^{-1} \) to \( 10^{-5} \) in complete medium. For each dilution, cells were seeded in duplicate plates. Once the cells were attached, the medium was withdrawn carefully and 25 \( \mu l \) of \( 10^{-4} \) and \( 10^{-5} \) dilutions was added to each well. Cells were incubated at RT for 60-90 min. 100 \( \mu l \) of complete medium was added to each well and incubation was
further continued at 27°C in a humid environment. The cell supernatant was carefully removed at 72 h pi and stored at 4°C. This served as a stock of ECV which was to be referred back to once recombinant virus was detected in dot hybridization. To the cells, 200 µl of 0.5 N NaOH was added and mixed by pipeting. The alkali was neutralized with the addition of 20 µl of 10M ammonium acetate and thoroughly mixed as this solution tends to settle down. The entire lysate was transferred and blotted to nitrocellulose using a manifold blot apparatus. The wells in the manifold were washed and mixed with 1M ammonium acetate and 0.02 N NaOH. The membrane was then rinsed in 4x SSC for 2 min, air dried and DNA immobilized by baking at 80°C for 2 h, before proceeding for prehybridization and hybridization.

Conditions for prehybridization and hybridization:
Prehybridization was carried out at 55°C for 3-4 h in excess of prehybridization buffer.

Prehybridization buffer:

\[
\begin{array}{ll}
\text{SSC} & 6x \\
\text{Denhardt's} & 5x \\
\text{ssDNA} & 200 \mu g/ml \\
\text{SDS} & 0.5\%
\end{array}
\]

The probe DNA (50-200 ng @ $10^8$ cpm/µg) was added after it was denatured by boiling for 10 min and rapid cooling on ice for 15 min. Hybridization was carried out at 55°C for 15 h in the same buffer. The membranes were washed as follows with gentle shaking:
a. 2.0x SSC with 0.5% SDS, 2 X 10 min at RT.
b. 1.0x SSC with 0.2% SDS, 2 X 20 min at 55°C.
c. 0.5x SSC with 0.1% SDS, 2 X 15 min at 65°C.

Each membrane was rinsed in 3mM Tris-HCl (pH 8.0), damp dried and exposed to x-ray film at -70°C with two intensifying screens. The film was developed manually using KODAK chemicals.

<table>
<thead>
<tr>
<th>SSC (20x):</th>
<th>g per l</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>175.3 g</td>
</tr>
<tr>
<td>Sodium citrate, pH 7.0</td>
<td>88.2 g</td>
</tr>
</tbody>
</table>

Denhardt's solution (50x):  
- Ficoll 10.0 g  
- PVP 10.0 g  
- BSA (Fraction V) 10.0 g

2.2.6 Plaque Assay: 1.5 ml of fresh cells (>99% cell viability) were seeded at a density of 1.2 X 10^6 cell/ml in 35 mm petri dishes for 10-15 min. A serial dilution of virus ranging from 10^-1 to 10^-9 was made in 200 µl of complete medium. The medium was removed from seeded cells by gently tilting the dish to one side. 100 µl of viral dilution was added drop by drop to each petridish and an incubation at RT for 60-90 min was carried out. The viral dilution was then removed and 2 ml of 3% LMP agarose (which was diluted in equal vol of complete medium) was overlaid. After the overlay polymerized, 1 ml of complete medium was added to each plate and the plates incubated at 27°C with a
few layers of soaked paper towels. CPE was observed at daily intervals. 3-5 days later the liquid medium was removed from top of the agarose overlay and the petridishes were dried by placing them in an inverted position on a paper towel. 1 ml of neutral red staining solution (1.2 ml of neutral red in 20 ml of plaque assay buffer) was added to each petri dish and incubated at RT for 1 h. The stain was then drained off and the plates were left inverted overnight at 27°C which enabled the plaques to get well formed. The recombinant plaques were scored on the basis of differential refraction (due to occ- phenotype) by placing on top of an illuminator (white light) box. The plaques were further confirmed under light microscope. Occ- plaques appeared clear along with some cell debris, while occ+ plaques showed polyhedrin.

Agarose overlay: 3% agarose (BRL, LMP) was prepared in double distilled water and autoclaved. It was then melted and reconstituted with equal vol of complete medium before overlaying.

Plaque assay buffer:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.2 g</td>
</tr>
<tr>
<td>KCl</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1.14 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>6.0 g</td>
</tr>
</tbody>
</table>

The above were dissolved in 800 ml of H₂O and pH adjusted to 7.3. After adjusting the final vol to 1 l, it was filter sterilized and stored at 4°C.
2.2.7 Determination of virus titre by plaque assay: Titre of the virus was determined using procedures adopted from (Volkman and Summers, 1975) 10 fold dilutions ranging from $10^{-1}$ to $10^{-9}$ of the virus were made in complete medium. Duplicate plates were set up for each dilution and a plaque assay was carried out as described earlier. The plaques were counted, the optimal number being 50-100 per plate. Virus titre was calculated in terms of PFU/ml as follows:

$$\text{PFU/ml} = \frac{1}{\text{dilution}} \times \text{number of plaques} \times \frac{1}{(\text{ml inoculum/plate})}$$

2.2.8 Virus amplification: 100 ml spinner flask cultures of Sf9 cells were set up containing $10^5$ cell/ml at a cell viability of $99\%$. Cultures were grown at 27°C for 2 days till they reached a density of $5 \times 10^3$ cells/ml. Cells were concentrated by centrifugation at 500 rpm for 15 min, medium was removed and the cell pellet was infected with the virus at an moi of 0.1 PFU per cell for 1 h at 27°C. Fresh medium was added and the cells were grown for 4-5 days till 80-90% of the cells were infected (as evident from CPE). Infected cells were pelleted at low speed (1000 rpm for 10 min) and medium collected. Virus particles in the medium were pelleted by centrifugation at 24 k rpm for 1 h (Beckman SW41 rotor) at 4°C. The virus pellet was soaked o/n in a small vol (1 ml) of TE(pH 7.8) and vortexed at full speed to obtain a clear suspension. The viral suspension was layered onto a discontinuous sucrose gradient (made in TE). The gradient consisted of a layer of 10% sucrose on top of a 50% sucrose cushion. After centrifugation at 24 k rpm for 1 h at 4°C, the virus band at the interphase of the sucrose layer was
harvested, diluted in TE (pH 7.8) and was repelletted at 24 k rpm for 1 h at 4°C. The pellet was soaked overnight in 2 ml TE and then resuspended in TE and stored at 4°C.

TE:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl, pH 8.0</td>
<td>25 mM</td>
</tr>
<tr>
<td>EDTA, pH 8.0</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

2.2.9 Viral DNA Isolation: 600 µl of the viral suspension in TE was incubated at 50°C for 100 min after addition of 20 µl Proteinase K (1 µg/µl). 200 µl of 10% SDS, 10 mM EDTA (made fresh by dissolving 1 g N-lauryl sarcosinate sodium salt in 200 µl of 0.5 M EDTA) was added before adjusting the final vol to 1 ml with TE and incubated at 60°C for 50 min. The lysate was then loaded on 54% CsCl gradient (14 g CsCl + 11.92 ml TE + 200 µl EtBr) and centrifuged at 45 k rpm for 20 h at 21°C. DNA band was removed by puncturing the tube with a 19G needle. The DNA was then extracted gently by H₂O saturated butanol and dialysed against 1x TE o/n with 2-3 changes. The concentration of DNA was checked by measuring the absorbance at 260 nm.

2.2.10 Total cellular DNA isolation: Sf9 cells, seeded at an appropriate density, were infected with virus and at ~72 h pi the culture medium (supernatant) was removed and stored at 4°C. Lysis buffer was added (500 µl for each of the wells in a 24 well plate) to help the cells to detach from the plate. The cells were then transferred to eppendorf tubes and incubated on ice for 5 min. During this time the tubes were vortexed 3-5 times at 15
s intervals. The nuclei were pelleted by centrifugation at 2 k rpm for 3 min and the supernatant (cytoplasmic fraction) was discarded. The nuclei pellet was washed with 1x cold PBS and repelleted at 2 k rpm for 3 min and suspended in 450 μl of extraction buffer. After addition of 2 μg of proteinase K, the nuclear suspension was incubated at 50°C for 1 h. 50 μl of 10% sarcosyl was then added and incubation continued at 50°C for another 2 h. The lysate was extracted twice with phenol:chloroform isoamyl alcohol (CIA). Each time the organic phases were mixed rapidly by inverting the tubes and spun at 2 k rpm for 3 min to separate the two phases. After the addition of 1 ml EtOH to the aqueous phase, a cotton like precipitate was formed. Residual nucleic acid was further precipitated by incubating at -80°C for 10 min followed by centrifugation at 2.5 k rpm for 20 min. The resulting DNA pellet was washed with 1 ml of 90% EtOH, dried in a speed vac, suspended in TE and processed for restriction digestion.

Lysis buffer:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl, pH 7.5</td>
<td>0.03 M</td>
</tr>
<tr>
<td>Mg Acetate.4H₂O</td>
<td>0.01 M</td>
</tr>
<tr>
<td>Nonidet P-40 (NP-40)</td>
<td>1%</td>
</tr>
</tbody>
</table>

Extraction buffer:

<table>
<thead>
<tr>
<th>Ingredient</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl, pH 7.5</td>
<td>12.1 g</td>
</tr>
<tr>
<td>Na₂EDTA.2H₂O</td>
<td>33.6 g</td>
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<tr>
<td>KCl</td>
<td>14.9 g</td>
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</tbody>
</table>
PBS 1x

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per l</th>
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<tbody>
<tr>
<td>NaCl</td>
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</tr>
<tr>
<td>KCl</td>
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<tr>
<td>Na₂HPO₄</td>
<td>1.15 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

2.2.11 Purification of total RNA from infected cells: Sf9 cells were seeded at an appropriate density in T-25 flasks and were infected with the virus. At 72 h pi the culture medium was removed and stored at 4°C to be used later. Cells were washed thoroughly in complete medium and lysed by vigorous vortexing in a 1:1 mixture of lysis buffer. The lysate was extracted with phenol-chloroform, aqueous phase was adjusted to 0.1% SDS, 20 mM Na₂EDTA. The phenol phases were re-extracted with lysis buffer and the aqueous phases were pooled together, extracted once again with phenol-CIA and RNA was precipitated at -70°C for 20 min using 100% EtOH. The RNA pellet was dried in a speed vac. RNA was denatured using sample buffer which contained denaturant and was run on a 1.4% agarose gel at 120 V for 1 h.

Lysis buffer:

- Tris-HCl, pH9.0: 30mM
- NaCl: 300mM
- DTT: 0.075%
- Mg Acetate.4H₂O: 10mM

RNA gel Running buffer:

- 1x MOPS (20 mM MOPS, 5mM
- Sodium acetate, 1mM EDTA, pH 7.0)
RNA gel for 100 ml:

1.4 g agarose was dissolved in 10 ml of 10x MOPS and 88 ml H₂O by heating and 1.6 ml of formaldehyde and 7.7 µl EtBr was added and the vol was adjusted to 100 ml.

RNA sample buffer:

4 µl Blue juice (500 µl glycerol, 450 µl H₂O and 50 µl saturated bromophenol blue)
4 µl 10x MOPS
6.5 µl Formaldehyde
18.0 µl Formamide
32.0 µl

2.2.11.1 Northern blot analysis: After electrophoresis the gel was washed twice, 15 min each, in 10x SSC and capillary transferred to nitrocellulose filter paper using 10x SSC, o/n. The filter was baked at 80°C for 2 h and prepared for prehybridization and hybridization.

2.2.12 SDS polyacrylamide gel electrophoresis of ³⁵S-methionine labeled total cell proteins: Sf9 cells were infected with the recombinant virus at desired moi and at 52 h pi the medium was removed from the dish, cells rinsed carefully with 1 ml of methionine free medium and 1-2 ml of fresh methionine-free medium was added. Incubation at 27°C for 1 h was carried out to deplete intracellular methionine, after which this medium was replaced with fresh 200 µl of methionine-free medium containing 15 µCi ³⁵S methionine. In-vivo labeling of total cellular proteins was
performed by incubating the cells for 3 h at 27°C. The medium was analyzed if the protein of interest was secretory. For non-secretory proteins, the cell pellet was washed with 1 ml of plaque assay buffer. The supernatant was discarded and the cell pellet was suspended in 150 μl of RIPA buffer to lyse the cells and solubilize the proteins.

RIPA buffer:

- Tris-HCl, pH 7.4 50 mM
- NaCl 150 mM
- SDS 0.1 %
- Sodium deoxycholate 0.1 %
- Triton-X 100 0.1 %

The proteins in the supernatant as well as the cell pellet were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). The separating gel was polymerized in a Hoeffer mini gel apparatus for 30 to 45 min and the stacking gel for 15 to 20 min. The thickness of the gel was 1.5 mm. Samples to be analyzed were mixed with an equal vol of sample buffer and incubated in a boiling water bath for 2 to 5 min. These were loaded into the sample wells and the electrophoresis carried out at a constant current of 20 to 30 mA.

Staining and destaining of the gels:
The gels were stained with Coomassie blue for 2 to 3 h and destained with the destaining solution (40% methanol and 10% acetic acid in water) over a period of 24 h with several changes.
The solutions were as following:

- **Acrylamide**: 30 %
- **Bis-acrylamide**: 1 %
- **Tris-HCl, pH 8.7**: 1.5 M
- **Tris-HCl, pH 6.8**: 0.5 M
- **Sodium dodecyl sulphate (SDS)**: 10 %
- **Ammonium persulphate (APS)**: 10 %
  
  (prepared just before use)

**Electrode buffer:** 0.025 M Tris base, 0.192 M glycine and 0.1 % SDS; pH 8.3.

**Sample buffer**: 0.16 M Tris base, 2.3% SDS, 10% glycerol, 5% mercaptoethanol and 0.1% bromophenol blue; pH 6.8.

Coomassie blue stain (0.25%) was prepared in 40% methanol and 10% acetic acid in water.

The recipes for the preparation of separating gel is as folowing:

- **Final concentration of Acrylamide**: 12.5 %
  
  - **Acrylamide**: 6.25 ml
  - **Bis-acrylamide**: 1.55 ml
  - **Tris-HCl(1.5 M)**: 3.75 ml
  - **Distilled water**: 3.25 ml
  - **SDS**: 0.15 ml
  - **APS**: 0.05 ml
  - **TEMED**: 0.005 ml

The stacking gel was prepared as follows:

- **Acrylamide**: 0.5 ml
- **Bis-acrylamide**: 0.5 ml
- **Tris-HCl(0.5 M)**: 1.25 ml
- **Distilled water**: 2.675 ml
2.2.12.1 Western blot analysis: After the proteins were electrophoresed on a 12.5% SDS-PAGE, they were transferred to nitrocellulose membrane in an electroblot (Hoeffer) apparatus at constant current of 300 mA in transfer buffer, for 2 h at RT as described by Towbin et al., (1979). The membrane was then blocked with a 2% BSA solution (in 50mM PBS) and washed with 50mM PBS containing 0.05% Tween 20. It was then treated with the first antibody which was diluted in 50mM PBS (with 1%BSA) and incubated for 2 h at RT with continuous shaking. The membrane was washed thoroughly and incubated with the second antibody, which was diluted in 50mM PBS (with 1%BSA), 0.05% Tween-20, at RT for 1 h followed by washing, and the colour was developed with 4 α chloro naphthol (3.3 mg of 4 α chloro-naphthol was dissolved in 1 ml of methanol and the vol was made up to 5 ml with 50 mM PBS and then 5 μl of H₂O₂ was added). The reaction was stopped by washing the membrane in distilled H₂O.

2.2.13 Radioimmunoassay: Culture supernatant were analysed for the presence of α and β subunit of hCG by radioimmunoassay using a monoclonal antibody specific to the subunit of hCG. Briefly, an antibody dilution giving 30-40% binding of ¹²⁵I-hCG (40-60 μCi/μg, 10,000 cpm) in the absence of competing hormone, was incubated with 0, 1.56, 3.125, 6.25, 12.5, 25, 50 or 100 ng/ml of αhCG standard or 0, 1.25, 2.5, 5, 10, 20, 40 ng/ml of...
βhCG (or culture supernatant dilutions), 40% normal horse serum and tracer. Incubations were carried out for 16 h at 4°C and immune complexes were precipitated using 66.6% v/v double distilled alcohol and 6.6% w/v ammonium acetate. The biological activity of hCG produced by Sf9 cells was determined by employing a rat testicular receptor binding assay and mouse Leydig cell bioassay.

2.2.13.1 Rat testicular receptor binding assay: Medium containing specific concentration of one of the hCG subunits was incubated with 10 μg of a standard preparation of the other subunit (prepared from commercial hCG of biological activity 10,000 IU/mg) for 16 h at 27°C. Formation of the αβ dimer was analyzed by a rat testicular homogenate radioreceptor assay. Rat testicular homogenate was prepared by a modification of an earlier method. Briefly, testes from adult Wistar rats were decapsulated and homogenized in 50 mM Tris HCl buffer pH 7.4, containing 5mM MgCl₂, 0.1% BSA and 0.1% sodium azide. The homogenate was centrifuged at 500 x g at 4°C and pellet resuspended in 8 ml of Tris buffer per pair of testes. 50 μl of the homogenate was incubated with 100 μl of different amounts of standard hCG or medium containing the dimer and 50 μl of ¹²⁵I-hCG (100,000 cpm, 40-60 μCi/μg) at 37°C for 2 h. The assay was terminated by the addition of 1 ml of cold Tris buffer. After centrifugation (2000 x g) the pellet was washed once in Tris buffer, and counted in a gamma counter. Dimer concentration was calculated by comparison with the inhibitory response of standard hCG. The capacity of the dimer to stimulate testosterone
production was analyzed by a mouse Leydig cell bioassay.

2.2.13.2 Mouse Leydig cell bioassay: Adult NMRI male mice were sacrificed by cervical dislocation, their testes were removed and minced in cold DMEM containing 0.38% NaHCO₃ + 2% FCS. The homogenate was gently stirred on a magnetic stirrer to help liberate individual cells. The cell suspension was passed through a nylon mesh and incubated at 34°C for 1 h under a 95% air + 5% CO₂ atmosphere. Cells were then centrifuged at 400 x g for 10 min at 4°C. The supernatant was discarded and 10 ml of fresh medium was added per pair of testes. 200 µl of this cell suspension was added into tubes containing 0, 1.56, 3.12, 6.25, 12.5, 25, 50 and 100 pg hCG or dilution of medium containing the dimer. Incubations were carried out for 3 h under conditions described above. Cells were killed by heating the tubes at 70°C for 15 min. RIA was carried out for testosterone, using WHO matched reagents, as described in the Methods Manual.

2.2.14 Luc assay: vAcluc or vAcβhCG-luc infected Sf9 cells were used at different time pi for detecting the presence of luc activity. 16µM luciferin (final concentration) was added to the infected cells in a 96 well plate which was exposed to KODAK, OG-1 x-ray film for a suitable time period. Luc activity was registered as a fogging of the x-ray film. The extent of luc activity correlated well with the intensity of darkening of the film. In case of high titre virus Luc activity could be detected visually.
2.2.15 Isolation of plasmid DNA by rapid alkali lysis method:
About 2 ml of LB containing ampicillin (50 µg/ml) was inoculated in an eppendorf tube with a single bacterial colony and incubated o/n at 37°C with continuous shaking. The cells were centrifuged for 1 min, supernatant discarded and cell pellet was resuspended by vortexing in 100 µl of ice cold TEG solution. After 5 min incubation at RT, with the cap of tube remaining open, 200 µl of freshly prepared lysis buffer was added, mixed manually and incubated for 5 min on ice. To this, 150 µl of ice cold 3M NaOAc was added and the suspension was vortexed for 10 s and left on ice for next 5 min. After a 5 min centrifugation in a microfuge the supernatant was carefully withdrawn, phenol extracted, DNA precipitated by the addition of two vol of EtOH and was incubated at RT for 2 min. The DNA pellet was collected by centrifugation for 10 min at RT and dissolved in TE after washing with 70% EtOH.

TEG solution:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl, pH 8.0</td>
<td>25 mM</td>
</tr>
<tr>
<td>EDTA, pH 8.0</td>
<td>10 mM</td>
</tr>
<tr>
<td>Glucose</td>
<td>50 mM</td>
</tr>
</tbody>
</table>

Lysis buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>1 %</td>
</tr>
<tr>
<td>NaOH</td>
<td>0.2 N</td>
</tr>
</tbody>
</table>

2.2.16 Large scale isolation and CsCl purification of plasmid:
A single bacterial colony was inoculated in 100 ml LB medium containing an appropriate antibiotic and grown o/n. Cells were pelleted at 2 k rpm for 15-20 min at 4°C and pellet suspended in 5 ml of TEG, to which was added 10 mg/ml lysozyme. After 5 min
incubation at RT, 10 ml of freshly made lysis buffer (2.2.15) was added, mixed by rapid inversion and cells were allowed to lyse for 10 min on ice. 7.5 ml of ice cold 5 M potassium acetate was added and mixed by vortexing for 10 s. After a further 20 min incubation on ice it was centrifuged for 9 min at 10 k rpm. The supernatant was carefully withdrawn and 0.6 vol of isopropanol was added and incubation continued for another 60-90 min at RT. The DNA was pelleted by centrifugation at 12 k rpm for 30 min, the pellet dried in a speed vac after a 70% EtOH wash and dissolved in 2 ml of TE. RNA was digested by RNase (20 mg/ml) treatment for 2 h at 37°C. (Alternatively, if CsCl purification was required, the DNA pellet was dissolved in 8 ml TE and processed for ultra centrifugation). The DNA was extracted with phenol followed by phenol-chloroform and chloroform-isoamyl alcohol. The aqueous phase was precipitated with EtOH at -70°C for 20 min. The DNA was pelleted at 12 k rpm for 15 min and washed with 70% EtOH. The DNA pellet was dried in a speed vac and dissolved in the least vol of TE. A typical yield was 3-4 µg DNA/ml of culture.

For CsCl purification, 8 gm of CsCl was added to 8 ml DNA followed by 200 µl of 10 mg/ml EtBr and transferred into quick seal tubes. The air bubbles in the quick seal tube were removed by filling the tubes with paraffin oil, heat sealed and centrifuged in an ultra centrifuge at 45 k rpm for 16-18 hrs at 20°C. After centrifugation, the plasmid DNA band was visible as a conspicuous band in ordinary light. The entire solution containing the plasmid DNA band was withdrawn by puncturing a hole just below the band, using a 21-G needle. EtBr was removed
from the DNA solution by extracting 3-4 times with water saturated butanol. Before precipitating the DNA by the addition of 2 vol of absolute EtOH at -70°C, CsCl was diluted 5 times with H₂O to prevent salt co-precipitation. The DNA was pelleted at 12 k rpm for 20 min at 4°C and washed with 70% EtOH. The pellet was dissolved in 1 ml of H₂O and re-precipitated as usual. The DNA was finally dissolved in required vol of TE.

2.2.17 Digestion and ligation reactions: For the purpose of cloning, vector or insert DNA were digested with restriction enzymes following the protocol given below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>X μl</td>
</tr>
<tr>
<td>Buffer</td>
<td>Y μl</td>
</tr>
<tr>
<td>Enzyme</td>
<td>1 unit/μg of DNA (never allowed to exceed 1/10 the reaction vol)</td>
</tr>
<tr>
<td>H₂O</td>
<td>Z μl</td>
</tr>
</tbody>
</table>

Incubation was carried out at 37°C for 4-6 h and the reaction was terminated after 3-4 h by heating at 65°C for 15 min.

Ligations were carried out in appropriate conditions and buffer as recommended by the manufacturer, at 15°C for 4-6 h or for o/n at 4°C using T4 DNA ligase in 20-25 μl vol. Conditions for blunt end ligations were same as for sticky end ligations, except that the total vol was kept at 10-15 μl. Every experimental ligation reaction was monitored using restriction enzyme digested lambda DNA. A shift in the mobility of ligated lambda DNA was indicative of ligation. For linker ligation, 1 μg of the linker was used.
2.2.18 Gel electrophoresis: Agarose gel was prepared in TBE. Electrophoresis was performed at 100 V for 2-3 h at RT depending on the size of the gel and the fragments to be separated. The gel was stained with EtBr (0.5 µg/ml) for 40 min and photographed using a Polaroid camera.

TBE (10x):

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>108.0 g</td>
</tr>
<tr>
<td>Boric acid</td>
<td>55.0 g</td>
</tr>
<tr>
<td>EDTA (0.5 M, pH 8.0)</td>
<td>40.0 ml</td>
</tr>
</tbody>
</table>

2.2.19 Preparation of competent E.coli cells for transformation: E.coli (DH5α, TG1 or JM109) cells of interest were grown o/n at 37°C in LB with continuous shaking to obtain a primary inoculum, 1.2 ml of which was used to inoculate 150 ml LB (pre warmed at 37°C). The culture was incubated with shaking at 37°C until A650 reached 0.2-0.3 (about 1-2 h later) and then chilled by placing on ice. Henceforth all operations were performed on ice (2-4°C). The cells were pelleted by centrifugation at 6 k rpm for 10 min and the pellet was resuspended in 42 ml of ice cold 0.1 M CaCl₂ by gentle swirling. Cell suspension was incubated on ice for 20 min and then centrifuged for 10 min at 6 k rpm. Cell pellet was gently resuspended in 20 ml of cold 0.1 M CaCl₂ and 9 ml of cold 80% sterile glycerol was added to it to achieve a final concentration of 15% glycerol. After thorough mixing, the cells were frozen at -70°C in 400 µl aliquots for future transformations.

LB culture medium:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-tryptone</td>
<td>10 g</td>
</tr>
</tbody>
</table>
Yeast extract ........................................... 5 g
NaCl ............................................................. 10 g

2.2.20 Transformation of *E. coli* cells with plasmid DNA: A vial of frozen competent cells was thawed by placing it in between fingers. 100 μl of thawed cells was added to 0.5 vol of ligation mixture (10-20 μl) and mixed by gentle tapping. After a 20 min incubation on ice it was heat shocked for 5 min at 37°C or 2 min at 42°C. The cells were incubated for 30 min at 37°C after the addition of 0.5 ml of LB and the transformed cells were plated (100-150 μl) on selection plates. For pUC derivatives, prior to plating of cells the plate was coated with a drop of x-gal (25 μl, 4% w/v in DMF) and IPTG (25 μl, 100 mM in H₂O). The plates were incubated at 37°C o/n.

2.2.21 Purification of DNA fragments from Low Melting Point (LMP) Agarose gel: Restriction enzyme digested DNA was resolved by electrophoresis in a low melting temperature agarose gel. The DNA band of interest was excised and the agarose gel fragment was weighed. Equal vol of extraction buffer was added and agarose was dissolved by heating at 65°C for 5 min. The DNA agarose solution was extracted with 0.4 vol of tris-saturated phenol (no chloroform) in the following manner:

a) 1/3 to the warm molten agarose followed by vortexing, giving rise to a clear suspension,

b) 1/3 more and vortexing (the suspension now became cloudy),

c) the remaining 1/3, resulting in the precipitation of
agarose.
This was then centrifuged for 10 min at 4°C in a microfuge. The aqueous supernatant was carefully removed to a fresh tube and extracted once with an equal vol of CIA (24:1 chloroform:isoamyl alcohol). The vol was reduced, if required, with butanol but not to less than half the original vol. The CIA extraction was repeated before proceeding for EtOH precipitation at -70°C (for 20 min) by the addition of 0.1 vol of 3M NaOAc (pH 5.2). The DNA pellet was washed with 70% EtOH.
Extraction buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>400 mM</td>
</tr>
<tr>
<td>Tris-HCl, pH 7.9</td>
<td>20 mM</td>
</tr>
<tr>
<td>EDTA, pH 8.0</td>
<td>2 mM</td>
</tr>
</tbody>
</table>

2.2.22 Nick translation, purification of labeled DNA, elution on Sephadex G-50 column: Nick translation of DNA was carried out using an Amersham kit to radiolabel the DNA fragment by aliquoting ~100 ng of purified DNA fragment and 5 µl of $^{32}$P-αdCTP (3000 Ci/mmol) at 12-15°C for 20-30 min depending on the length of the DNA to be labeled. The nick translated DNA was purified from unincorporated nucleotides using a spun column (Sambrook et al., 1989). For this, 1.0 ml of Sephadex G-50 resin pre-soaked in TE was packed in 1.0 ml syringe by centrifugation at 2 k rpm for 1.5 min. The nick translated reaction mixture (which was diluted in TE ~250 µl) was loaded on the column and spun at 2 k rpm for 1.5 min and the eluate was collected, 1 µl was withdrawn and counted in a Beckman Scintillation Counter under the Cherenkov channel. The specific activity of the probe was in the
range of $1-2 \times 10^8$ cpm/µg DNA. Once purified, the probe was boiled for 10 min at 100°C and cooled on ice for another 10 min and then added to the hybridization bag.

2.2.23 Southern analysis

2.2.23.1 Rapid alkali blot of DNA from gel to membrane:
The DNA which was electrophoresed and fractionated on the gel was immersed in 0.25 N HCl with gentle shaking for 15 minutes. The gel was then rinsed in H₂O and blotted onto a 'Gene Screen Plus' membrane using 0.4 N NaOH solution, for 2-3 h. The membrane was then neutralised in 6x SSC for 2-3 min, air dried and baked at 80°C for 15 min (Southern 1975).

2.2.23.2 Direct in-gel hybridization:
After the electrophoresis the gel was photographed, and placed on Whatman 3MM filter, covered with Saran Wrap and dried in a BioRad gel dryer for 1 h without heating and another 1 h at 75°C. The gel was soaked in water to remove the Whatman sheet adhering to the gel, incubated for 40 min in denaturation solution, followed by neutralization solution at RT for 40 min. The gel was equilibrated in 6x SSC for 5 min and hybridized to the radioactive probe ($1 \times 10^6$ cpm/ml hybridization buffer).

Denaturation Solution:

| NaOH | 0.5 N |
| NaCl | 0.15 M |

Neutralization Solution:

| Tris-HCl, pH 8.0 | 0.5 M |
| 50 |
Hybridization Buffer:

Hybridization Buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.1 M</td>
</tr>
<tr>
<td>SSPE</td>
<td>5.0x</td>
</tr>
<tr>
<td>Denhardt's</td>
<td>5.0x</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1%</td>
</tr>
<tr>
<td>salmon sperm DNA</td>
<td>10 µg/ml</td>
</tr>
</tbody>
</table>

SSPE (20x):

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>175.3 g</td>
</tr>
<tr>
<td>NaH₂PO₄•H₂O</td>
<td>27.6 g</td>
</tr>
<tr>
<td>EDTA</td>
<td>7.4 g</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
</tr>
</tbody>
</table>

2.2.23.3 SSC transfer:

The DNA was electrophoresed on the gel and then denatured using denaturation solution for 40 min with constant shaking. It was then neutralised using neutralization solution for 40 min and blotted overnight in 20x SSC solution. The membrane was air dried and then baked at 80°C for 2 h and proceeded for prehybridization and hybridization.

2.2.24 Colony hybridization: Bacterial colonies growing o/n in LB plate were transferred to a nylon membrane cut to the same size as the plate. The membrane was premarked for orientation purposes. The colonies on the membrane were lysed in-situ by placing on a Whatman 3MM paper presoaked in 10% SDS for 3 min. The membrane was similarly placed on Whatman 3MM paper presoaked...
in denaturation solution for 5 min to denature the DNA and then on neutralization solution for another 5 min. The membrane was then air dried and baked at 80°C for 2 h to irreversibly immobilize the DNA and proceeded for prehybridization and hybridization.

2.2.25 Prehybridization and hybridization: Prior to hybridization with the probe DNA, the membrane was prehybridized in the following solution for 4-6 h at 42°C:

- Deionized formamide: 100% 20 ml
- Denhardt's solution: 50x 5 ml
- SDS: 10% 5 ml
- NaCl: 5M 10 ml
- salmon sperm DNA: 10 mg/ml 150μl

Hybridization was carried out by adding radioactive labeled DNA probe (10⁶ cpm/ml) to the buffer. Hybridization was carried out for 12-18 h at appropriate temperature. The membrane was washed under following condition:

- 2x5 min, 2x SSC RT
- 2x30 min, 0.2x SSC 0.1% SDS 65°C

The membrane was rinsed in 0.3M Tris-HCl pH 8.0, damp dried, wrapped in Saran Wrap and exposed to x-ray film with double intensifying screen at -70°C or at RT without any screen. The film was developed manually using Kodak chemicals and accordingly
exposed for less or more time.

2.2.26 DNA sequencing: DNA was sequenced by Sanger's dideoxy chain termination method (Sanger et al., 1974) employing SEQUENASE VERSION 2.0 DNA sequencing kit using double stranded DNA, as per the following protocol:

**Denaturation:** The template DNA containing the insert to be sequenced was denatured in order to reduce it to a single stranded form amenable to enzymatic reaction. This was done by incubating 20 μl (≈5 μg) of the double stranded plasmid DNA template, with 5 μl of 2 M NaOH/1 mM EDTA for 5 min at RT. The single stranded DNA was purified by spin dialysis as described (2.2.22) and annealed to the primer.

**Annealing:** Denatured template DNA (8.5 μl), prepared above, was annealed to the sequencing primer (1 μl, 0.5 pmol/μl) in the presence of 10x annealing buffer (1 μl) for 15 min at 37°C. While annealing was going on, labeling mix was prepared for the labeling reaction.

**Labeling:** To 8 μl of dGTP labeling mix (diluted 1:5 with H₂O), was added 4 μl DTT, 7 μl TE, 2 μl ³⁵S-αdATP (20 μCi) and 1 μl SEQUENASE enzyme to prepare a labeling mix. Labeling reaction was started by the addition of 5.5 μl of labeling mix to 10 μl of the annealed primer, and allowed to continue for 10 min at RT.

**Termination:** While the labeling reaction was going on, 4 microfuge tubes were marked as A T G C and to these tubes were added 2.5 μl each of ddATP, ddTTP, ddGTP, ddCTP, respectively and placed on a 37°C block heater. Once the labeling reaction was over, 3.5 μl of the labeling mix was added to the side of each
tube, mixed by centrifugation for 1 s and incubated for 5-10 min at 37°C. The reaction was terminated by the addition of 4 μl stop solution. The sequencing reaction mix was stored at -20°C until ready for PAGE.

PAGE: The sequencing reaction products were resolved by electrophoresis on 6% polyacrylamide gel using a BioRad sequencing gel apparatus. Once the gel was polymerized, the sharkstooth comb was withdrawn, the broad well washed with gel running buffer (1x TBE) and electrophoresed at 50 W for 15-20 min. After the pre-run, 3 μl of sequencing reaction samples were prepared by heating for 2 min at 100°C, and loaded on to the wells made by the sharkstooth comb. Electrophoresis was carried out at 50 W for 3-4 h after which the glass plate was removed, gel carefully transferred to Whatman 3MM filter, dried at 80°C in a BioRad gel drier and exposed to KODAK XAR-5 film at RT for a few h to o/n.

Sequencing gel:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>25 g</td>
</tr>
<tr>
<td>40 % Acrylamide (38:2)</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>10x TBE</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>17.5 ml</td>
</tr>
</tbody>
</table>

After dissolving the above, the solution was filtered through Whatman 3MM filter.