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2.1 Materials:

2.1.1 Chemicals: 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. Yeastolate and Lactalbumin hydrolysate were obtained from Difco,(USA). Neutral red and fetal calf serum were purchased from Flow Laboratory, (UK). Acrylamide, bis and TEMED were purchased from LKB (Sweden) and ammonium persulphate from BioRad,(USA). Lambda DNA and ΦXDNA were purchased from New England Biolabs,(USA). Agarose was from Boehringer Mannheim (FRG) and low gelling temperature agarose was from BRL (USA). Nylon membranes (Genescreen plus) for immobilization of DNA or RNA was purchased from DuPont, USA. Nitrocellulose membranes were from Amersham (UK). Sepharose 4B was purchased from Pharmacia, USA. Rainbow coloured protein molecular weight markers and anti-mouse IgG-HRP conjugate were from Amersham (UK). Antibodies to the subunits of hCG were available from the NII Reagent Bank. Luciferase and luciferin were purchased from Sigma Chemical Co. (USA) or Boehringer Mannheim (FRG). All other fine chemicals were of the highest purity available locally. Millipore filtered or double distilled autoclaved water was used at all times. Plastic ware used in tissue culture was purchased from Greiner (Germany).

2.1.2 Enzymes: All restriction enzymes, T4 DNA ligase, DNA polymerase (Klenow fragment), and RNase A were purchased from New England Biolabs (USA) and/or Amersham (UK) and/or Boehringer Mannheim (FRG).
Endoglycosidases were obtained from Boehringer Mannheim (FRG).

2.1.3 Kits: Multiprime DNA labelling system was purchased from Amersham (UK) and Nick Translation Kit was from DuPont (USA). Sialic acid detection Kit was purchased from Boehringer Mannheim (FRG).

2.1.4 Radioisotopes: $^{32}$P-αdCTP and carrier free $^{125}$I were purchased from Amersham (UK). Hyperfilm, cassettes and intensifying screens were from Amersham (UK). Kodak OG film for luc assay was from Kodak (USA).

2.1.5 Bacterial strains: *E. coli* strains DH5α and TG1 were from our laboratory stock which were originally obtained from the American Type Culture Collection, USA.

2.1.6 Cell line: *Spodoptera frugiperda* (Sf9) (American Type Culture Collection, Rockville, Accession no. CRL 1711)

2.1.7 Larvae: Larvae of *Spodoptera* sp. were obtained from natural collections.

2.2 Methods:
2.2.1 DNA modifications
2.2.1.1 Plasmid DNA isolation: 3 ml of LB containing 50 μg/ml of ampicillin was inoculated with a single bacterial colony and grown overnight at 37°C with vigorous shaking. The cells were pelleted down at full speed for 5 min and dissolved in 100 μl of ice cold TEG. 10 μl of lysozyme (30 mg/ml) was added and incubated at RT for 5 min. 200 μl of freshly made lysis buffer
was added and after vigorous shaking, was incubated on ice for 5 min. 150 µl of ice cold potassium acetate was added and vortexed for 15 sec. This was incubated on ice for 7 min and chromosomal DNA was pelleted down at full speed for 5 min. The aqueous phase was phenol extracted and plasmid DNA precipitated with two vol of EtOH at RT for 2 min. Plasmid DNA was pelleted at full speed for 5 min and the pellet washed with 70% alcohol. The pellet was dried in the Speed vac before proceeding for digestion.

Cesium chloride purification of plasmid DNA: 250 ml of LB containing 50 µg/ml of ampicillin was inoculated with a single bacterial colony and grown overnight at 37°C with vigorous shaking. The cells were pelleted down at 2500 rpm for 10 min. The cell pellet was dissolved in 5 ml of TEG and lysozyme was added to a final concentration of 5 mg/ml. It was incubated on ice for 10 min and 10 ml of freshly made lysis buffer was added. This was incubated on ice for a further 10 min. 7.5 ml of ice cold sodium acetate was added and incubated on ice for 20 min. Chromosomal DNA was precipitated at 18K rpm and the supernatant was treated with 0.6 vol of isopropanol to precipitate the plasmid DNA. Plasmid DNA was pelleted at 12,000 rpm and the pellet dried in the speed vac. The pellet was dissolved in 10 ml TE. 10 g cesium chloride was dissolved in the DNA solution and 800 µl of 10 mg/ml ethidium bromide was added. This was mixed and transferred into quick seal tubes, air bubbles removed by filling to the brim with paraffin oil and ultracentrifuged at 45K rpm at 15°C in a TV865.1 Sorvall rotor. The plasmid band was visualized under a transilluminator and collected by puncturing the tube immediately under the band with a 21g needle and drawing out the DNA solution (Sambrook et al., 1989). The band was extracted with water saturated butanol till all the ethidium bromide was extracted out and then precipitated at -70°C with two
vol of EtOH for 30 min. It was then given a 70% EtOH wash and dissolved in TE.

2.2.1.2 Digestion and ligation reactions: For the purpose of cloning, the vector or insert DNA were digested with restriction enzymes (1 unit per μg DNA) in the presence of 1X buffer. Digestion vol was made up with water and the reaction incubated at the appropriate temperature for the appropriate length of time. The reaction terminated by heating the reaction at 65°C for 5 min.

Ligations were carried out in appropriate conditions and buffer as recommended by the manufacturer at 15°C for 6 h or at 4°C for o/n using T4 DNA ligase in a 20-25 μl vol. Every experimental ligation was monitored using restriction enzyme digested lambda DNA as control. A shift in mobility of the ligated lambda DNA was indicative of ligation.

2.2.1.3 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.

<table>
<thead>
<tr>
<th>TBE</th>
<th>per 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>108 g</td>
</tr>
<tr>
<td>Boric acid</td>
<td>55 g</td>
</tr>
<tr>
<td>EDTA (0.5M)</td>
<td>40 ml</td>
</tr>
<tr>
<td>pH</td>
<td>8.0</td>
</tr>
</tbody>
</table>

2.2.1.4 Preparation and transformation of competent cells: 5 ml LB was inoculated with DH5α or TG1 strain of E.coli. A primary bacterial culture was
grown overnight and a secondary culture of 100 ml was started from the primary culture. When the OD_{660} reached 0.3, the cells were placed on ice. All subsequent steps were performed in the cold. Cells were pelleted at 2500 rpm for 10 min. The cell pellet was gently dissolved by swirling and pipetting in 50 ml of sterile 0.1M CaCl$_2$. This was incubated on ice for 20 min and then the cells were pelleted down at 800 rpm at 4°C. The pellet was very gently dissolved by swirling and pipetting in 8.3 ml of sterile 0.1M CaCl$_2$. Glycerol was added to a final concentration of 15%, and the cells were frozen at -70°C in aliquots of 400 μl each.

**Transformation of competent cells:** Competent cells were thawed quickly by placing in between fingers, put on ice and 100 μl added to plasmid DNA or to the ligation mix. The mixture was incubated on ice for 20 min. The cells were given a heat shock of 37°C for 5 min or 42°C for 2 min. 500 μl of LB was added, the cells allowed to grow at 37°C for 30 min and then pelleted at full speed for 30 sec before plating out on selective medium. For pUC derived plasmids, an additional selection using XGal (25μl, 4% w/v in dimethyl formamide) and IPTG (25 μl, 100mM in water) was used.

**2.2.1.5 Purification of DNA from agarose:** DNA fragments to be used for cloning or labelling were purified from either low melting point or normal agarose. The DNA was fractionated on a 1% low melting point agarose gel. The band of interest was cut out from the gel weighed and warmed at 65°C for 5 min in equal volume of LMP extraction buffer (400mM NaCl, 20mM Tris pH 7.9, 2 mM EDTA pH 8.0). The DNA was extracted with 0.4 vol of phenol saturated with Tris as follows:

- 1/3 to the molten agarose followed by vortexing to give a clear suspension
-1/3 to this clear suspension followed by vortexing to give a cloudy suspension
-1/3 finally to precipitate the agarose.

This was pelleted at full speed for 5 min to remove the agarose. the aqueous
phase was extracted with chloroform-isoamyl alcohol and the volume reduced
if necessary with butanol and again extracted with chloroform-isoamyl alcohol.
This was then precipitated with 1/10 vol of sodium acetate and two vol of
EtOH.

DNA fragments were also purified from regular agarose gels as follows:
The DNA was fractionated on 8% agarose gel and the band of interest was cut
out. A 1.5 ml eppendorf tube was pierced with a 21G needle and packed with
glass wool. The cut out band was placed on the glass wool and the tube was
placed in a second 1.5 ml tube. This was spun at full speed for 7 min and the
eluate collected in the second tube was dried, washed with 70% EtOH and
dissolved in minimum vol of TE. This DNA was suitable for all molecular
biological reactions without any need for extraction with organic solvents or
further purification.

2.2.1.6 Labelling reactions: DNA fragments were labelled either by
random priming or by nick translation. 50-100 ng of fragment was used for
labelling in either case. For random priming reaction, the DNA was denatured
by boiling for 5 min and quenched on ice for 10 min. Primers, dNTP’s (-dCTP)
and 32P-αdCTP were added and the volume made up to 23 μl with water. The
reaction was started by the addition of the enzyme (Klenow fragment of DNA
polymerase) and incubated at 37°C for 30 min. In the case of nick translation,
the fragment DNA was treated with DNaseI. dNTP’s (-dCTP), 32P-αdCTP and
water was added to make up the volume to 23 μl. The reaction was started by
the addition of Klenow and the reaction carried out for 30 min at 15°C. The probe was purified either by using the spun column method or by gravity purification.

**Spun column:** A 1 ml syringe was packed up to the 1 ml mark with G-50 Sephadex, placed in a 1.5 ml eppendorf tube and spun exactly for 1 min at 1600 rpm to pack the column tightly. The labelling reaction volume was made up to 100 µl using TE and loaded on the column which was again spun for exactly the same time and at the same speed as before. The unincorporated nucleotides were retained in the column while the labelled probe eluted out.

**Gravity purification:** A 1 ml syringe was packed with G-50 Sephadex up to the brim and washed with 1 ml of TE. The labelling reaction was diluted to 100 ml volume and loaded on the column. Fractions of 2 drops per tube were collected and each fraction counted using the Geiger counter. Two distinct peaks of activity were obtained of which the first represented the labelled fragment and the second represented the unincorporated nucleotides. The probe fractions were pooled together.

**2.2.1.7 DNA hybridisations:**

**In-gel hybridisation:** DNA was fractionated on an agarose gel and photographed. The gel was dried under vacuum on a Whatman 3MM paper. The dried gel can be stored indefinitely at RT. Prehybridisation is not required for in-gel hybridisation and therefore the dried gel was processed directly for hybridisation. For hybridisation, the gel was denatured using 0.5N NaOH and 1.5M NaCl for 40 min with constant shaking at RT and then neutralized using 1.5M NaCl and 0.5M Tris pH 8.0 for 40 min at RT. The gel was then transferred to the hybridisation buffer (6X SSC, 0.1% SDS, 5X Denhardt’s and
1 µg/ml salmon sperm DNA) and hybridisation carried out by adding the denatured, quenched probe (~0.5X 10^5 cpm/cm^2) and incubating for 18-20 h at 55°C. Washing was done as described earlier.

**Rapid alkali blot:** DNA was fractionated on an agarose gel and photographed. The gel was first depurinated by treating with 0.15M HCl for 15 min with constant shaking (this step increases the efficiency of transfer of high molecular weight fragments). Nylon membrane (Genescreen Plus) cut to the exact dimensions of the gel was soaked in 0.4N NaOH for 10 min. The gel was placed on a platform with a wick of Whatman 3MM paper dipping into a tank of transfer buffer (0.4N NaOH). Air bubbles were removed and the nylon membrane was placed carefully on the gel taking care again to remove air bubbles. 3-4 pieces of Whatman 3MM paper cut to size were placed on the nylon membrane and a stack of filter papers again cut to size were placed on this with a suitable weight. Transfer was carried out for 4 h and the blot neutralized in 6X SSC and air dried before proceeding for prehybridisation.

**Prehybridisation and hybridisation:** Prehybridisation was carried out for 4 h at 55°C in excess of prehybridisation buffer.

**Composition of prehybridisation buffer:**

- **SSC** - 6X
- **Denhardt's** - 5X
- **SDS** - 0.5%
- **Salmon sperm DNA** - 10 µg/ml

Probe DNA (100 ng DNA @ 10^8 cpm specific activity) was denatured by boiling for 10 min and quenching on ice for 10 min. Probe was added after reducing the volume of the prehybridisation buffer to 0.05 ml/cm^2 and hybridisation was carried out for 16 h at 55°C. Probe was then removed and
the blot washed as follows:

- **2X SSC** - 2 X 10 min at room temperature
- **0.2X SSC with 0.5% SDS** - 2 X 20 min at 55°C.
- **0.1X SSC with 0.5% SDS** - 1 X 10 min at 65°C.

The blot was rinsed in 3mM Tris, damp dried and exposed to Amersham MP X-ray film at -70°C.

**SSC (20X)**

- NaCl: 175.3 g/l
- Sodium citrate pH 7.0: 88.2 g/l

**Denhardt’s solution**

(50X) For 500 ml

- Ficoll: 10g
- PVP: 10g
- BSA (Fraction V): 10g

### 2.2.2 Insect cell culture and generation of recombinant virus

**2.2.2.1 Insect cell culture:** Cells were subcultured two to three times a week in TNMFH containing 10% FCS, gentamycin and amphotericin B. Cells were grown either as a monolayer or as a suspension culture. These cells have a doubling time of 18-24 h at 27°C (Summers and Smith, 1989).

**TNMFH:** To make TNMFH, a 1 l pouch of Grace’s medium was dissolved in 700 ml double distilled water. 0.35 g sodium bicarbonate was added and the pH adjusted to 5.9 with potassium hydroxide. 3.33 g each of lactalbumin hydrolysate and yeastolate were added and the volume adjusted to 1l. The medium was sterilized by passing through a 0.22 um filter and sterility checked by incubating at 37°C for three days. Complete medium was
prepared by adding 10% FCS, 50 μg/ml gentamycin and 5 μg/ml amphotericin B to the medium.

Monolayer and suspension cultures: Subculturing in flasks was done by gently pipetting the medium across the surface of a confluent culture to dislodge the cells. The cells were seeded at a density of 2.5X10^6 in a fresh 25cm^2 flask in 5 ml complete medium. Viability of the cells was checked by staining the cells with 10% v/v of trypan blue (0.4% stock solution made up in buffered isotonic salt solution, pH 7.2-7.3). Non-viable cells stain blue. Only those batches of cells with 95-99% viability were used for experimental purposes.

Suspension cultures were maintained in spinner flasks (Bellco, USA). Flasks were sterilized by washing successively in 0.4N NaOH, 0.2N HCl and distilled water thoroughly before autoclaving. Cells were diluted to a final concentration of 0.5X10^6/ml and grown at 27°C. Cells were passaged by pelleting at 2000 rpm for 15 min and diluting with fresh medium again to a density of 0.5X10^6 cells/ml. Sf9 cells are not anchorage dependent and can be transferred between monolayer and suspension cultures repeatedly without noticeable loss of viability.

2.2.2.2 Infection of cells with virus: Monolayer cultures were dislodged and seeded at an appropriate density (see table) in incomplete medium. Cells were allowed to attach for 15-20 min. The medium was removed and appropriate volume of virus inoculum was added. Unless specific multiplicity of infection was desired, the minimum volume of virus inoculum to cover the surface was added and left for 1h at 27°C. The virus inoculum was then removed and fresh medium was added. The cells were incubated for 2-4 days
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and observed for cytopathic effects. Supernatant was separated from the cell pellet by low speed pelleting and ultracentrifuged for recovery of ECVs. The cell pellet was used for DNA or RNA purification, SDS-PAGE electrophoresis of infected cellular proteins etc.

Suspension cultures were pelleted at 2000rpm and the cell density and viability checked by trypan blue staining. The cell pellet was resuspended in the desired volume of the virus inoculum and incubated for 1h at 27°C. Complete medium was then added to achieve a final density of 0.5X10^6 cells/ml, transferred back to the spinner flask and incubated for 3-5 days at 27°C. Infection was monitored by examining a small aliquot of the suspension for CPE. ECVs and cell pellet were harvested as described before.

<table>
<thead>
<tr>
<th>Container</th>
<th>Cell density</th>
<th>Virus vol</th>
<th>Final vol</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 well plate</td>
<td>2X10^4 /well</td>
<td>10 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>24 well plate</td>
<td>3X10^5 /well</td>
<td>50 μl</td>
<td>500 μl</td>
</tr>
<tr>
<td>6 well plate</td>
<td>2X10^6 /well</td>
<td>200μl</td>
<td>2 ml</td>
</tr>
<tr>
<td>T25 flask</td>
<td>3X10^6 /flask</td>
<td>1 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td>T75 flask</td>
<td>9X10^6 /flask</td>
<td>2 ml</td>
<td>12 ml</td>
</tr>
<tr>
<td>Spinner</td>
<td>0.5X10^6 /flask</td>
<td>4 ml</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Coinfections: Viruses carrying different foreign genes were titrated by plaque assay. Cells were seeded in a 24-well plate at a density of 0.3X10^3 cells per well. The cells were infected with different viruses of identical titre and the cell pellet and culture supernatant harvested 72 h pi. The supernatant was
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assayed by RIA for the presence of the different subunits of hCG and then checked for bioactivity by the mouse Leydig cell assay.

Microscopic observation for cytopathic effects in infected cells: Infected cells were observed periodically under light microscope. Typical cytopathic effects include enlarged cells with enlarged nuclei, tendency to detach from the monolayer, irregular shapes and in the final stages of infection, lysis.

2.2.2.3 Co-transfection: 1.5X10^6 cells from a confluent culture of at least 99% viability were seeded in a 35mm petridish. A transfection mix was made as follows:

50mM Hepes buffer pH 7.1 - 475 µl
100mM glucose - 95 µl
AcNPV DNA - x µl (1 µg)
Recombinant plasmid DNA - y µl (25 µg)
Sterile distilled water - z µl
Total volume - 950 µl

AcNPV DNA was added very carefully to avoid shearing. 50 µl of CaCl₂ (2.5M) was added in a dropwise fashion and the mix incubated for 30 min.

The mix was added to the seeded cells and further incubated for 60-90 min after which it was replaced by 2 ml of complete medium. The cells were incubated at 27°C for four days and regularly checked for the presence of CPE. Appearance of polyhedra indicates that transfection has indeed occurred. The transfection supernatant was then screened for presence of recombinant virus either by dot blot hybridisation or by plaque assay.

A modified approach (Goswami and Glazer, 1991) was also used for cotransfection. Sf9 cells were seeded at a density of 1.8 X 10^6 cells in a 35mm
petridish. The cells were infected with AcNPV at an moi of 0.01 for 2 h at RT. The transfection mix was made as above (omitting the viral DNA) and incubated for 30 min. The virus inoculum was removed and the transfection mix added on the infected monolayer. Transfection was carried out for 1 h using a transfection mix without viral DNA after which it was removed and replaced with 2 ml of complete medium. On the fifth day pi the culture supernatant was harvested. This was termed as transfection supernatant and used for dot blot hybridisation or plaque assay as usual.

2.2.2.4 Dot blot hybridisation: Sf9 cells (99% viability) were seeded at an appropriate density in a 96 well plate. Transfection supernatant was used to make serial dilutions up to $10^{-6}$ in complete medium. Dilutions from $10^{-3}$ to $10^{-6}$ of the transfection supernatant were used for identification of the recombinant virus. 50 µl of each dilution was used to carry out the infection for 1 h at $27^0$C. 50 µl of complete medium was added to each well and incubation was carried out for four days at $27^0$C. Medium from each well was saved in a duplicate 96 well plate to serve as a future source of ECVs. The cells were then lysed by the addition of 200 µl of 0.5M NaOH followed by vigorous pipetting. The alkali was neutralized by the addition of 20 µl of 10M ammonium acetate. Nitrocellulose paper was wetted in warm water and then in a wash solution of 1M ammonium acetate and 0.02N NaOH. The cell lysate was blotted on the nitrocellulose using a manifold. The blot was air dried and then baked for 2 h at $80^0$C before proceeding for prehybridisation and hybridisation as described before.
2.2.2.5 Plaque assay: Sf9 cells from a confluent culture of 99% viability were seeded in 35mm petridishes and allowed to attach for 15 min. Transfection supernatant was serially diluted in complete medium and 100 μl of 10⁻⁵, 10⁻⁶ and 10⁻⁷ dilutions were used to infect the monolayer for 1 h at 27°C. The viral inoculum was then removed and a 3% solution of low gelling temperature agarose (diluted in an equal volume of 2X complete medium) was added over the monolayer. When the overlay had polymerized, 1 ml of complete medium was added and the plates incubated at 27°C and regularly checked for appearance of CPE. On the fourth day pi, the medium was decanted and 2 ml of neutral red in plaque assay buffer (0.6 ml in 10 ml of plaque assay buffer) was added on the agarose overlay. Staining was carried out for 1 h after which the stain was decanted. The plates were observed on the light box for presence of occlusion negative plaques which appear as bright, refractile clearing zones. The plaques were further confirmed under the light microscope, picked up with a 100 μl yellow tip attached to an Eppendorf pipetman and suspended in 200 μl of complete medium.

Low gelling temperature agarose overlay: A 3% solution of LGT made in double distilled water was autoclaved and diluted with complete medium just prior to use.

Plaque assay buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</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 water, pH adjusted to 7.3 and volume made up to 1 l. It was filter sterilized and stored at 4°C.
Determination of virus titre by plaque assay: Serial dilutions of virus were made from $10^{-2}$ to $10^8$. Plaque assay was carried out as described earlier and the number of plaques on each plate was counted—the optimum number of plaques being 50-100/plate. Virus titre was calculated using the formula:

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

2.2.2.6 Virus amplification: Sf9 cells were grown in spinner flasks and infected with virus as described. Cultures were harvested when 90-95% of the cells exhibited CPE and pelleted at low speed to remove cell debris. The supernatant was ultracentrifuged at 24K rpm in a Beckman SW41 rotor. The supernatant was discarded and the pellet dissolved in minimum volume of TE overnight. The viral suspension was layered on a discontinuous sucrose gradient (10%-50%) and ultracentrifuged at 24K rpm at 4°C. The virus banded at the interphase and was collected by pipetting. The band was diluted with TE and pelleted at 24K rpm and the purified virus pellet dissolved in TE. This was filter sterilized prior to use.

2.2.2.7 Isolation of total cellular DNA: 2X10⁶ cells were infected with recombinant virus at moi greater than 10. The cells were harvested 72 h pi and treated with lysis buffer along with vigorous vortexing for 5 min. Nuclei were pelleted at 2000 rpm for 3 min and the nuclei were washed with ice cold PBS and repelleted at low speed. 450 µl of extraction buffer along with 2 µg of proteinase K was added and incubated at 50°C for 1 h. 50 µl of 10% sarkosyl was added and the incubation continued for 2 h to overnight. Phenol extraction was carried out and the genomic DNA was precipitated by adding two vol of EtOH at RT. Precipitation was completed at -70°C for 10 min. The
DNA was dissolved in TE overnight, digested with the appropriate restriction enzyme and analysed on a 0.7% agarose gel.

2.2.3 Larval culture and infection

2.2.3.1 Maintenance of larval cultures: Colonies of Spodoptera sp were maintained at a temperature of 27°C on a diet of castor leaves in a moisture free and virus free environment in our insectary.

2.2.3.2 Infection of larvae with recombinant virus: Third or fourth instar caterpillars were starved for 2 hours prior to infection. They were immobilized by placing them on ice flakes for a few minutes. The larvae were injected in the second last segment with 10-20 μl of 10^8 pfu/ml of recombinant virus, or mock injected, using a Hamilton syringe or a 1 ml syringe with a 30G needle. The larvae were then allowed to feed and observed for symptoms of infection every day. There was no mortality during the injection process. Typical symptoms included a pink tinge on the underside of the caterpillar and extraordinary growth.

2.2.3.3 Harvesting of recombinant proteins from infected larvae: Larvae were bled by cutting the prolegs and hemolymph was collected while the body tissue was homogenized in 300 μl of PBS (50mM, pH 7.3) containing 2mM PMSF. Hemolymph and body tissue were assayed for βhCG by RIA and the immunoreactivity and biological activity of the synthesized βhCG in both body tissue and hemolymph were determined by western blotting and bioassays as described.
2.2.4 Analysis and characterisation of recombinant protein

2.2.4.1 SDS-PAGE: Sf9 cells were seeded at an appropriate density in a 24 well plate and infected with the recombinant virus as described. Culture supernatant and cells were harvested at 72 h pi. A small aliquot of each was boiled with an equal volume of sample buffer and fractionated on a 12.5% acrylamide gel. The gel was stained in a 0.25% bromophenol blue solution for 1 h and then destained for 18-20 h in destaining solution (40% methanol, 10% Tris in water).

<table>
<thead>
<tr>
<th>Gel composition:</th>
<th>Tris (1.5M) pH 8.8</th>
<th>-</th>
<th>3.75ml</th>
<th>Resolving gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5% Acrylamide (30%)</td>
<td>-</td>
<td>-</td>
<td>6.25ml</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>-</td>
<td>-</td>
<td>4.8ml</td>
<td></td>
</tr>
<tr>
<td>SDS (10%)</td>
<td>-</td>
<td>-</td>
<td>150μl</td>
<td></td>
</tr>
<tr>
<td>APS (10%)</td>
<td>-</td>
<td>-</td>
<td>75μl</td>
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</tr>
<tr>
<td>TEMED</td>
<td>-</td>
<td>-</td>
<td>10μl</td>
<td></td>
</tr>
</tbody>
</table>

| Stacking gel, 4.5%     | Tris (0.5M) pH 6.8 | - | 1.25ml  |              |
| Acrylamide (30%)       | -                  | - | 0.66ml  |              |
| Water                  | -                  | - | 3ml     |              |
| SDS (10%)              | -                  | - | 50μl    |              |
| APS (10%)              | -                  | - | 37.5μl  |              |
| TEMED                  | -                  | - | 5μl     |              |

| Running buffer: (250 ml)| Tris                | - | 0.75g   |              |
|                        | Glycine             | - | 3.6g    |              |
|                        | SDS (10%)           | - | 2.5ml   |              |

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

Coomassie blue staining solution was made by dissolving 0.25g coomassie blue
in 100 ml of destaining solution.

**Silver staining:** The gel was treated with destaining solution overnight for fixing. It was then transferred to a solution containing 34mM potassium dichromate and 32mM nitric acid for 15 min at RT. It was washed thoroughly in deionized water and then transferred to a solution of silver nitrate (0.102g in 50 ml) made freshly. It was left in silver nitrate solution for 30 min at RT and washed thoroughly in deionized water. Colour was developed using a solution of sodium carbonate (1.37g sodium carbonate in 50 ml water + 25μl formaldehyde). Colour development was stopped with a 10% solution of acetic acid in water.

**Western blotting:** Sf9 cells were infected with recombinant virus and harvested at 72 h pi. Total cellular proteins were fractionated on a 12.5% SDS-PAGE. The proteins were then transferred overnight to nitrocellulose membrane (Towbin et al., 1979) at 30mA in transfer buffer. The membrane was first blocked with a 1% solution of non-fat milk for 1 h at RT. It was washed thoroughly in 50mM PBS containing 0.05% Tween 20 and then incubated in an appropriate dilution of the monoclonal antibody in PBS for 1 h at RT. Excess unbound antibody was washed off with PBS Tween. Second antibody conjugated to peroxidase was diluted in PBS Tween and the blot incubated for 1 h at RT. Unbound conjugate was washed off with PBS Tween and the colour developed with diaminobenzidine (4 mg in 20 ml of PBS and 20 μl H₂O₂ was added). The reaction was stopped by washing the membrane in distilled water.

**Transfer buffer:**

| (per l) | Tris | Glycine | 3.025 g | 14.4 g |
2.2.4.2 Radioimmunoassay: Culture supernatant was analysed for the presence of α or 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 $^{125}$I-hCG (40-60 $\mu$ci/μg, 10000 cpm) in the absence of the competing hormone was incubated with 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 ng/ml of standard αhCG or 1.25, 2.5, 5, 10, 20 and 40 ng/ml of standard βhCG (and culture supernatant dilutions), 40% normal horse serum and tracer. Incubation was carried out for 16 h at 4°C and immune complexes were precipitated using 66.6% 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 assay.

2.2.4.3 Rat testicular receptor binding assay: Medium containing specific concentrations 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 of 10000 IU/mg) for 16 h at 37°C. Formation of the αβ dimer was analysed by a rat testicular homogenate radioreceptor assay (Dighe and Moudgal, 1983). Rat testicular homogenate was prepared as follows. Testes from adult Wistar rats were decapsulated and homogenized in 50mM Tris HCl buffer pH 7.4 containing 5mM MgCl$_2$, 0.1% BSA and 0.1% sodium azide. The homogenate was centrifuged at 500 g at 4°C and the pellet was resuspended in 8 ml Tris buffer per pair of testes. 50 μl of the homogenate was incubated with 100 μl of different concentrations of standard hCG or medium containing the dimer and 50 μl of $^{125}$I hCG (100,000 cpm, 40-60 μCi/μg) at 37°C for 2 h.
The assay was terminated by adding 1 ml of cold Tris buffer. After centrifugation at 2000 g, the pellet was washed in Tris buffer and counted in a gamma counter (LKB, Sweden). Dimer concentration was calculated by comparison with the inhibitory response of standard hCG. The capacity of the dimer to stimulate testosterone production was analysed by a mouse Leydig cell assay.

2.2.4.4 Mouse Leydig cell bioassay: Adult NMRI mice were sacrificed by cervical dislocation, testes were removed and minced in cold DMEM (Dulbecco's Minimal Essential Medium) containing 0.38% NaHCO₃ and 2% FCS (Van Damme et al., 1974). 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 g for 10 min at 4°C. The supernatant was discarded and 10 ml fresh medium was added per pair of testes. 200 μl of this cell suspension was added into tubes containing 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 World Health Organization (WHO) matched reagents as described in WHO Methods Manual.

2.2.4.5 Affinity purification of recombinant protein: 100 ml of a suspension culture of infected cells were harvested for the purpose of purification of the expressed protein (Chen et al., 1991a). Cells were first pelleted at low speed and the culture supernatant was then centrifuged at 100,000g for 1 h at 4°C to
pellet virus particles. The supernatant was then passed through an affinity column (CNBr activated Sepharose) carrying immobilized monoclonal antibody specific to βhCG pre-equilibrated with 0.01M Tris-HCl pH 8.0, containing 0.14M NaCl which was recirculated overnight. The column was then washed once with 10mM Tris-HCl, pH 8.0 containing 0.14M NaCl and twice with 50mM Tris-HCl, pH 8.0 containing 0.5M NaCl and 0.1% Triton X-100. The recombinant protein was eluted with 0.5M glycine buffer pH 2.3 containing 0.15M NaCl. Peak fractions were pooled, dialysed and the concentration of the eluted protein determined by RIA.

2.2.4.6 Total carbohydrate analysis of purified protein: The percentage of total carbohydrates was analysed in identical amounts of affinity purified recombinant protein (Dubois et al, 1956). The colour developed was read at 550 nm.

2.2.4.7 Endoglycosidase analysis of purified protein: 300 ng of affinity purified recombinant or native βhCG were digested with Endo H or neuraminidase in 10 μl of digestion buffer as per manufacturer’s specifications. The protein was denatured by boiling for 3 min in sodium acetate buffer containing 0.5% SDS and 1% β-mercaptoethanol. Detergent NP40 was added to a final concentration of 1.25% and enzyme digestion was carried out for 20 h at 37°C. The reaction mixture was fractionated on a 12.5% SDS-PAGE and stained with silver nitrate. Commercially available sialic acid detection kit was used, as per manufacturer’s recommendations, for quantitative estimation of sialic acid residues.
2.2.4.8 Luc assay of infected cells and larvae: Individual larvae were weighed and homogenized on ice in 300 μl of PBS containing protease inhibitor. Aliquots of this homogenate were stored at -70°C and used for luc assay. The assay was carried out in a 96 well plate on a small aliquot of the homogenate in an assay mixture containing 14mM MgCl₂, 14mM glycine buffer, 6mM ATP and 40μM luciferin. In the presence of the substrate (luciferin) and ATP, luciferase forms a complex. This enzyme bound complex, on its return to the ground state, emits a photon which can be visualized as a flash of light. The emitted light was first visualized and then captured on X-ray film (Kodak OG1 film, USA). The larval homogenate was also centrifuged at high speed to pellet debris and then the proteins fractionated on SDS-PAGE as described previously.

Luc assay in cells was carried out under identical conditions except that cells do not require the addition of ATP due to the presence of endogenous supplies. Hence, 40 μM luciferin was added to infected cells and the cells exposed to X-ray film.