MATERIALS AND METHODS

CHEMICALS AND RADIOISOTOPES

DNA molecular weight markers and ethidium bromide were obtained from Boehringer Mannheim Biochemica, FRG. Trypsin (1:250), coomassie blue, trypan blue, BSA (fraction V), lithium chloride, trizma base, sucrose, urea, Denhardt's solution, salmon sperm DNA, TEMED and protein molecular weight standards were purchased from Sigma Chemical Co. USA. Glycerol, deionized formamide, acrylamide, N,N’-methylene bisacrylamide, β-mercaptoethanol, low melting point agarose and ammonium persulfate were procured from Gibco-BRL, USA. All fine chemicals not mentioned here or elsewhere were of highest purity available locally.

\(^{32}\)P-\(\alpha\)dCTP, carrier free \(^{125}\)I Hyperfilm, cassettes and intensifying screens were purchased from Amersham, UK.

ENZYMES

All restriction enzymes, T4 DNA ligase, DNA polymerase (Klenow fragment), lysozyme, micrococcal nuclease, proteinase K, RNAase A and endoglycosidases were purchased from Boehringer Mannheim Biochemica, FRG, or New England Biolabs, USA.

PLASMIDS AND VECTORS

The plasmid pBlueScript KS- used in intermediate cloning stages was obtained from Stratagene Cloning Systems, USA. Plasmid pBR322-βhCG containing the entire βhCG cDNA in the HindIII site of pBR322 was from laboratory stock (originally a kind gift from Dr VB Reddy, USA). The transmembrane region of rabies glycoprotein gene was taken out from the 3'end of a partial clone of
the glycoprotein gene from the LEP strain of rabies virus and cloned as a 1.5kb fragment at the EcoRI site in the pBMS M13+ vector by Dr S Chandrasekhar at the Institute. Vaccinia virus vector pMJ602, used for cloning of genes into vaccinia virus was a generous gift from Dr B Moss NIH, USA. The fowlpox transfer vector pBHCX402 was provided by Dr Deoki Tripathy, University of Illinois, USA.

MOLECULAR CLONING TECHNIQUES

**Bacterial Growth Media**

Luria Bertani (LB) medium: 10 g Bacto-tryptone, 5 g bacto-yeast extract (DIFCO Laboratories, USA) and 10 g NaCl was dissolved in one liter of deionized water and its pH adjusted to 7.5.

LB-agar: 15g Bacto-agar (DIFCO) per liter of LB medium.

**Transformation of Competent Cells**

Five ml of plain LB medium was inoculated with DH5α or TG1 strain of E.coli (originally obtained from the American Type Culture Collection, USA) and grown overnight with vigorous shaking at 37°C to raise a primary culture. A 50 ml secondary culture was raised with this till it attained an OD of 0.3 at 590nm. The cells were chilled on ice and all subsequent steps performed at 4°C. The cells were spun down at 1100 x g for 10 min, the pellet suspended in 25 ml of 100mM CaCl₂ and incubated for 30 min. Cells were again spun down at 1100 x g at 4°C, resuspended very gently in 2.5 ml sterile 100mM CaCl₂ containing 15% glycerol (final concentration) and stored at -70°C in aliquots of 200 µl each.

Competent cells (100 µl) were added to 10-20 ng of plasmid DNA (10-20 µl) or to the ligation mixture. The cells were then incubated on ice for 30 min, given a heat shock at 37°C for 5 min, and after adding 1 ml of plain LB
medium incubated again at 37°C for 1 hr. The cells were pelleted at full speed for 10 sec in a microfuge before plating out on selective medium (LB Amp plates or additionally in presence of X-Gal and IPTG).

Isolation of Plasmid DNA

Miniprep by alkaline lysis: Rapid minipreps of plasmid DNA were obtained by the alkaline lysis method as described by Birnboim (1983). Briefly, 3 ml of LB medium containing 50 µg/ml of ampicillin (Boehringer Mannheim Biochemica, FRG) was inoculated with a single bacterial colony and an overnight culture was raised at 37°C with vigorous shaking. Cells were spun down in an eppendorf tube at 12,000 x g for 1 min and the pellet suspended in 100 µl of ice-cold TEG solution (25 mM Tris.Cl, pH 8.00; 10mM EDTA [Na salt] pH 8.00, and 50 mM glucose) by gentle pipetting. The mixture was incubated on ice for 15 min, 200µl of freshly prepared NaOH/SDS solution (0.2N NaOH and 1% SDS) was added and mixed by gentle tapping. The mixture was incubated on ice for 10 min and then neutralized by adding 150µl ice cold 3M potassium acetate solution, pH 5.5. The contents were mixed thoroughly by gentle vortexing for 2 sec followed by incubation on ice for 10 min. The bacterial debris and chromosomal DNA were spun down by centrifuging at 12,000 x g for 5 min at 4°C in a microfuge. The clear supernatant was transferred to a fresh tube and an equal volume of phenol/chloroform (1:1) added, mixed by vortexing and centrifuged for 2 min at 12,000 x g. The supernatant was taken and the DNA precipitated with twice the volume of ethanol (EtOH) at -70°C. After 2hr, DNA was pelleted down by centrifugation for 10 min at 12,000 x g at 4°C. The pellet was washed with 70% EtOH, vacuum dried and resuspended in 30µl of TE buffer. The DNA was stored at -20°C until further use.
Large scale preparation: High quality plasmid (CCC) DNA free of most contaminants (including chromosomal DNA and RNA) required for transfection purposes was purified from bulk bacterial cell cultures in liquid medium. The crude lysate enriched in plasmid DNA was prepared as follows:

1. The bacterial cells from a 500ml culture were harvested by centrifugation at 6000 x g for 15 min at 4°C. The cell-pellet was completely dispersed in 5ml of Tris/EDTA/Glucose (TEG) solution containing hen egg white lysozyme to a final concentration of 25 mg/ml and allowed to stand for 10 min at room temperature (RT).

2. 10ml freshly prepared SDS/NaOH solution (1% and 0.2N respectively) was added to above and mixed by stirring gently. Following incubation on ice for 10 min, 7.5ml of potassium acetate solution was added, mixed gently for neutralization and incubated on ice for 10 min.

3. The cellular debris, chromosomal DNA and SDS-protein complexes were pelleted out by centrifuging at 20,000 x g for 10 min at 4°C. The supernatant was decanted into another tube and an equal volume of isopropanol added. The contents were mixed by inversion and allowed to stand at RT for 10 min.

4. The nucleic acids were recovered by centrifugation at 15,000 x g for 10 min at RT. The pellet of nucleic acid was washed with 70% EtOH, dried and dissolved in 4 ml TE buffer.

Purification of Plasmid DNA: The crude lysate was purified by the Cesium chloride/ethidium bromide gradient centrifugation. Binding of ethidium bromide (EtBr) to DNA lowers its density. Since binding capacity of covalently closed circular (CCC) plasmid DNA is lesser than chromosomal DNA, it forms a band in a region of greater density (lower in the tube) than does chromosomal DNA.
4.4g of CsCl and 4 mg EtBr (0.4 ml) were added to the crude lysate of nucleic acids (4 ml) and final density adjusted to 1.55 g/ml either by the addition CsCl or TE. The solution was transferred to a 5ml ultracentrifuge tube, filled upto the brim with paraffin oil and sealed. The plasmid was banded by centrifuging for ≈ 14 hr at 20°C at 350,000 x g (65,000 rpm in VTi 80 rotor). After the run a 20G needle was inserted gently near the top and the plasmid band was recovered by suction with a 3ml syringe fitted with another 20G needle. EtBr was removed from the DNA-CsCl solution by repeated extractions with isoamyl alcohol. The plasmid DNA was reprecipitated with twice the volume of 100% EtOH at RT and centrifuged for 10 min at 4°C at 10,000 x g to get rid of CsCl. The pellet was washed with 70% EtOH, dried under vacuum, resuspended in TE buffer and stored at 4°C.

Quantitation of DNA

The amount of DNA in the sample was estimated by taking the OD_{260}. An OD_{260} of 1 ≈ 50μg/ml of ds DNA, ≈ 40 μg/ml of ss DNA and RNA, and ≈ 20 μg/ml of ss oligonucleotide. The purity of the nucleic acid was ascertained by taking the ratio of OD_{260} and OD_{280}. An OD_{260}/OD_{280} = 1.8 for DNA and 2.0 for RNA. Contaminants like protein or phenol lower the ratio.

Small quantities (<250ng/ml) of DNA were estimated visually by running the DNA in an agarose gel along with known amount of a standard DNA (lambda DNA HindIII digest). As the amount of fluorescence is proportional to the total mass of DNA, the quantity of the DNA in the sample was estimated by comparing the fluorescent intensity of the sample DNA with the intensity and concentration of lambda DNA bands.
Digestion and Ligation Reactions

All the digestion and ligation reactions were carried out as per the manufacturer's guidelines. For cloning or restriction analysis the DNA was incubated with restriction enzymes (1 unit/μg DNA) in such a way that the glycerol content in the digestion mixture was never more than 5%. The buffer supplied along with the restriction enzyme by the manufacturer was used to a concentration of 1x in the final reaction volume. Digestion volume was made up with water and the reaction mixture incubated for 2 hr at a temperature appropriate for the respective restriction enzymes. Ligation reactions were carried out in the presence of T₄ DNA ligase under appropriate reaction conditions. Typically, ligation of cohesive ends was carried out at 15°C and blunt end ligation at 20°C for 16 hr in a total reaction volume of 10 μl.

Purification of DNA Fragments

Fragments of DNA needed for cloning or for preparation of probes were purified from regular agarose gels (Maniatis et al 1982). For most runs 1% gels were made in TAE buffer (1x working solution contains 40mM Tris-acetate and 1mM EDTA, pH 8.0). After the run, the gel was stained by immersing in EtBr solution (0.5 μg/ml) and the target band was cut out with a scalpel. The cut band was placed on top of siliconized glass wool packed in a 1.5 ml eppendorf tube pierced with a 21G needle at its bottom. This tube was placed in a second 1.5ml tube and the assembled tubes were spun at full speed for 7 min to elute DNA from the cut fragment. The eluate collected in the lower tube was dried under vacuum, washed with 70% EtOH and dissolved in 20 μl of TE. This DNA was suitable for cloning reactions without any need for extraction with organic solvents or further purification.
Preparation of Probe

DNA fragments were labelled using the commercially available kit for random priming by following the manufacturer's instructions (Boehringer Mannheim Biochemica). For each labelling reaction 50-100ng of DNA fragment was denatured in a boiling water bath for 5 min followed by quenching on ice for 10 min. The primers, dNTPs-(dCTP*) were added and the volume made upto 20μl with autoclaved water. The reaction was carried out at 37°C for 30 min after the addition of Klenow fragment of DNA polymerase.

Purification of probe: The labelled DNA was separated from unincorporated dNTP precursors by gel filtration. A 1 ml tuberculin syringe plugged at its bottom with autoclaved glass wool was packed with TE equilibrated Sephadex G-50 (Pharmacia, Sweden) inserted in a polypropylene tube and centrifuged at 800 x g for 2 min. The labelling reaction volume was made upto 100μl with TE, loaded onto the column and spun under same conditions. The unincorporated nucleotides were retained in the column while the labelled probe eluted out.

Purified probe (1 μl) was loaded onto a small piece of Whatman filter, air dried and radioactivity on the filter measured in a LKB-liquid scintillation counter using a toluene-based scintillation fluid (4% PPO and 0.1% dimethyl POPOP). The probe was stored at -20°C until further use.

Transfer of DNA onto Nitrocellulose Membrane

Colony lift: Bacterial colonies (≈ 100/82mm petriplate) were grown till they attained a diameter of 0.1-0.2 mm and lifted by a procedure adopted by Grunstein & Hogness (1975) and Benton & Davis (1977). The plates were chilled at 4°C prior to lifting. The hybridization transfer membrane (Colony/Plaque Screen™, DuPont, USA) was placed on the surface of agar in
contact with the bacterial colonies without entrapping any air bubble and its position marked by stabbing with a needle through it and the agar. After 2-3 min the disc was peeled off without smearing and treated first with denaturing solution (0.5N NaOH) for 5 min followed by 1M Tris (pH 7.5) for 2 min and finally with neutralizing solution (0.5M Tris, pH 7.5 and 1.5M NaCl) for 15 min. During each treatment step the disc with the colony side facing up was placed on 3MM Whatman sheet saturated with the respective solution and blot dried on another filter before proceeding to the next solution. Finally, the filter was air dried at RT for 30 min followed by baking at 80°C for 2 hr in order to fix the DNA onto the filter.

**Southern blot (Rapid alkali blot):** The agarose gel was soaked in 0.25N HCl for 15-30 min after electrophoretic separation of DNA fragments, the change in colour to yellow of tracking dye indicating the completion of depurination process. The acid solution was decanted, the gel rinsed with distilled water and treated with 10 gel volumes of 0.4M NaOH for 20 min at RT for denaturing the DNA. Nylon membrane (Gene Screen Plus, DuPont USA) cut to the same dimensions as that of the gel was soaked in 0.4N NaOH for 10 min. The transfer of the DNA to the nylon membrane was carried out by traditional capillary blotting in 0.4N NaOH for 5 hr. On completion the blot was air dried and neutralized in 6x SSC before proceeding for hybridization.

**Dot blot:** Nylon membrane and Whatman filters were fitted in commercially available dot blot manifold after soaking in 0.4M Tris.Cl pH 7.5 for 30 min and clamped tightly. The DNA was denatured with 0.25N NaOH for 10 min, chilled on ice, diluted to the desired concentration in 0.125x SSC (20x SSC stock: 17.5% M NaCl, 8.8% sodium citrate, pH 7.0) and loaded onto the membrane via the wells of the manifold. The solution was allowed to remain on the membrane without any suction for 30 min after which a slight suction was applied for ≈ 30 sec. The membrane was recovered and air-dried at RT.
DNA Hybridizations

Prehybridization: Once the denatured DNA was immobilized on the nylon membrane via colony lift, southern blot or dot blot, it was prehybridized for 2 hr at 42°C with constant agitation in a sealed plastic bag in a buffer containing 5x Denhardt’s solution (0.1% each of Ficoll, PVP and BSA), 5x SSC, 1% SDS, 50% formamide and 100 µg/ml of denatured salmon sperm DNA.

Hybridization: The double stranded labeled DNA probe was denatured in a boiling water for 10 min, added to prehybridization bag (≈ 500,000 cpm/ml hybridization solution) from the corner without removing the prehybridization solution and incubated with constant agitation for 12-18 hr at 42°C.

Post hybridization: The hybridization solution was poured out and stored at -20°C for reuse. The blot was washed for 10 min at RT in a small volume of 1x SSC containing 0.1% SDS to remove any excess probe. This was followed by three high stringency washes (15 min each) in 0.1x SSC, 0.1% SDS at 60°C with constant agitation. The membrane was blotted briefly onto filter paper, sealed in a damp condition in a plastic bag, then exposed to an X-ray film at -70°C in a cassette lined with intensifying screens and finally the film developed after appropriate exposure time.

In-gel hybridization: After the DNA samples were electrophoresed in an agarose gel, stained and photographed, the gel was dried under vacuum on a 3MM Whatman paper at RT for 30-45 min followed by drying at 70-75°C for 1 hr. The dried gel was stored at RT till further use. The gel was subjected to hybridization directly after denaturation and neutralization. The DNA in the gel was denatured at RT for 30 min with 0.5N NaOH and 1.5 M NaCl, rinsed in water, neutralised with 0.5M Tris pH 8.0 and 0.15M NaCl again for 30 min and finally rinsed with distilled water before transferring it to the hybridization buffer (6x SSC, 0.1% SDS, 5x Denhardt’s and 1µg/ml salmon sperm DNA).
The denatured probe (= 50,000 cpm/cm²) was added to the hybridization buffer. After incubation for 18-20 hr at 55°C, the gel was washed at RT first with 2x SSC for 15-30 min and then twice with 2x SSC containing 0.1% SDS at 65°C, each wash being of 15 min duration. The gel was exposed to the X-ray film as mentioned earlier.

**PREPARATION OF CELL CULTURES AND VIRUS STOCKS**

**Media and Media Supplements**

The media (Dulbecco's modified Eagle's medium, DMEM, and medium 199, Gibco-BRL, USA) were prepared in deionized water and sterilized by filtering through 0.22μm filter. Initial start up medium, maintenance medium, and medium used for infection purposes contained fetal bovine serum (FBS, Hyclone labs inc. USA) to a final concentration of 20%, 10% and 2.5% respectively. Penicillin and streptomycin were used at a concentration of 100 U/ml and 100 μg/ml, respectively (Antibiotic-antimycotic, Gibco-BRL, USA) and BrDU (Boehringer Mannheim Biochemica, FRG) at 25 μg/ml.

**Cell Lines**

CV1, BSC1 and HuTK-143B cell lines, made available by Dr B Moss, were grown in DMEM supplemented with 10% FBS. For HuTK-143B cell line, BrDU was used at a final concentration of 25μg/ml.

**Monolayer Cell Subculture:** Cells were revived from frozen stocks in DMEM containing 20% FBS. When the cells became confluent, the medium was aspirated out and the cells treated for 2 min at 37°C with 0.025% trypsin (1:250) containing 0.01% EDTA. After removing trypsin, cells were harvested, diluted with growth medium supplemented with 10% FBS, made into a uniform suspension by repeated pipetting and dispensed into the required number of fresh culture flasks.
Primary chick embryo fibroblast cultures: Chick embryo fibroblast (CEF) cell cultures were prepared from pathogen free embryonated eggs (Government Poultry Farm, Satbari, New Delhi). Ten to 12 days old embryos were trypsinized after the removal of their hair and other cornified tissue (claws, beak, eyes) and viscera (Solomon, 1975) and disaggregated cells grown in Medium 199 supplemented with 10% FBS and antibiotics.

Preparation of Virus Stocks

CV1 cells were used for growing Wyeth strain of vaccinia virus (a generous gift of Dr B Moss), while primary CEFs were employed for growing CEVA strain of fowlpox virus (obtained from Dr Deoki Tripathy). Infected cells were harvested 48 hr post-infection (PI) in 1mM Tris (pH 9.0) and the virus released from them by three cycles of freeze-thawing followed by sonication in a cup sonicator. Large debris was removed by low speed centrifugation for 10 min and the supernatant layered over a 36% sucrose cushion followed by centrifugation in an SW-27 rotor at 26,000 x g for 2 hr at 4°C. Virus in the pellet was collected in sterile 1mM Tris, pH 9.0, and disaggregated by indirect sonication before yield was assessed by plaque titration on CV1 or CEF monolayers. The stocks were stored in aliquots of 200μl at -70°C.

Virus titration: Virus quantitation was done by plaque assay (Mackett et al 1985). For vaccinia virus 6-well tissue culture plates were seeded with 5x10⁵ CV1 cells/well/2ml of medium and the cells allowed to attain confluency by growing at 37°C in a humidified atmosphere containing 5% CO₂. Ten-fold serial dilutions of the virus were made in DMEM containing 2.5% FBS. The medium from the cells was aspirated out and the cells were infected in duplicate with serial dilutions of the virus in a volume of 0.5 ml/well. The plates were incubated for 2 hr with rocking at 15-30 min intervals in order to
keep the cells moist. The cells were overlaid with 2ml of DMEM containing 2.5% FBS and incubated for 48 hr at 37°C in a humidified atmosphere supplemented with 5% CO₂. They were stained with 0.1% crystal violet (in methanol) after removing the culture supernatant. Virus plaques in each well were counted and the titer determined by the following formula:

\[ \text{pfu/ml} = 2 \times \text{dilution factor} \times \text{No. of plaques/well} \]

For fowlpox virus, plaque assays were performed on CEF monolayers overlayed with medium 199 with Earle's salts containing 1% low melting point agarose and 2% FBS. Plaques were stained with MTT tetrazolium (Klebe & Harriss, 1984) on fifth day after inoculation. Virus stocks were disaggregated by digestion for 30 min at 37°C with trypsin (1mg/ml) prior to dilution for infection of cell cultures.

**Transient Expression**

Expression of ßhCG genes using vaccinia virus promoters was ascertained employing protocol as described by Cochran et al (1985). Briefly, confluent monolayers of CV1 cells were infected at an MOI of 5 pfu/cell of the wild type (WT) virus. After 2 hr, the viral inoculum was aspirated out, cells washed with serum free medium and transfected with the desired DNA using lipofectin reagent (Gibco-BRL). Lipofectin gave a better transfection efficiency as compared to the calcium phosphate method (Graham & Van der Eb, 1973) and was therefore used for all transfection experiments.

Lipofectin reagent, a 1:1 (w/w) liposome formulation of the cationic lipid N[1-(2,3-dioleyloxy)propyl]-n,n,n trimethylammonium chloride (DOTMA) and dioleoyl phosphatidylethanolamine in water, interacts spontaneously with DNA to form a lipid-DNA complex (Felgner et al 1987), fusion of which with cells results in efficient uptake of DNA and its expression.
In all transfection experiments, CsCl purified DNA was used. To prepare the lipofectin-DNA complexes two polystyrene tubes were taken, in one tube 10 μg of DNA was diluted to 100 μl with serum-free medium and in the other 25 μl of the lipofectin reagent was diluted to 100 μl. The contents of the tubes were mixed gently and incubated at RT for 15 min. The volume of the mixture was made up to 1 ml with serum-free medium, added over the cells infected with the WT virus and incubation carried out at 37°C in the presence of 5% CO₂. In case of vaccinia virus the cells were harvested 24 hr PI and the expression of βhCG assayed in the culture supernatant. βhCG anchored on membrane surface (fowlpox virus constructs) was estimated in the cell-pellet.

**Generation of Recombinant Virus**

For generation of recombinant virus standard protocol (Mackett et al 1984) was employed. The gene for βhCG subcloned into plasmid transfer vectors (pMJ 602 for vaccinia and pBHCX 402 for fowlpox virus) was inserted into the viral genome by homologous recombination. The process essentially consisted of three steps: 1. infection of cells with WT virus, 2. transfection of cells with purified recombinant plasmid, and 3. plaque purification of the recombinant virus in appropriate selective media.

A T-25 flask was seeded with 1x10⁶ CV1 and CEF cells (for vaccinia and fowlpox recombinants respectively) and incubated overnight at 37°C for the cells to attain confluency. 3x10⁶ cells were infected with respective WT virus at MOI of 0.05 pfu/cell (CV1) or 0.01 and 0.05 pfu/cell (CEF) in 1 ml of the medium containing 2.5% FBS. After 2 hr of incubation with intermittent rocking the inoculum was aspirated, the cells washed with serum-free medium and overlaid with 10μg of DNA complexed with lipofectin (as described above). Four different concentrations (5, 10, 20 and 40μg) of plasmid DNA
Construction of vaccinia virus recombinants.
were used for each titre of WT virus employed for generating fowlpox virus recombinants. Following incubation for 5 hr at 37°C in a humidified atmosphere containing 5% CO₂, the DNA containing medium was replaced with 2 ml of normal growth medium supplemented with serum. After incubation for 2 days (vaccinia recombinant) or 5 days (avipox recombinants), the cells were scraped, centrifuged at 1800 x g at 4°C for 5 min, suspended in 0.5 ml DMEM with 2.5% FBS and lysed by three cycles of freeze-thawing. The lysate was stored at -70°C until used for plaque purification.

**Plaque Purification of Recombinant Virus**

*Vaccinia virus recombinants:* 5x10⁵ HuTK-143B cells seeded in a 6-well plate grown to confluency in DMEM supplemented with 10% FBS and BrDU (25μg/ml) were infected with the cell lysate obtained after transfection and incubated at 37°C with rocking at 30 min intervals. After 2 hr the virus inoculum was aspirated out and 1.5 ml of selective agarose (LMP agarose in DMEM with 2% FBS and 25μg/ml BrDU) was added to each well. The agarose was allowed to solidify at 4°C followed by incubation for 48 hr at 37°C. For visual selection of the recombinant virus a second selective agarose overlay containing X-gal (300 μg/ml) was added after 48 hr of incubation. After an overnight incubation 5-6 blue plaques were picked by suction with a pasteur pipet, transferred to microfuge tubes containing 500 μl of DMEM supplemented with 2.5% FBS, vortexed and subjected to three cycles of freeze-thaw followed by indirect sonication for 30 sec in an ice-water bath. 3 to 4 rounds of plaque purification were carried out to ensure a clonally pure recombinant virus after which the recombinant plaque isolate was amplified by infection of successively large number of cells. The selective drugs were added during the initial amplification steps after which no selection was required.
Fowlpox virus recombinants: The purification of recombinant FPV plaques was beset with difficulties because of some tricky problems described below:

1. There are no good avian TK- cell lines, hence only a single selection system based on the blue color afforded by the lacZ gene system in the presence of chromogenic substrate X-gal had to be employed.

2. CEFs have an inherent β-galactosidase activity and turn blue uniformly in the presence of X-gal irrespective of being infected with the recombinant virus or not. Therefore, Bluo-gal (Gibco-BRL) an analog of X-gal was added to the selective agarose to a final concentration of 400 μg/ml (from a 2% stock solution in dimethylformamide). Only the recombinant virus plaques turned blue and were picked up in the same way as vaccinia virus recombinants. Unlike vaccinia virus, however, the plaques started appearing only by third day of infection and the selective agarose was added only on day 5 PI.

3. The plaques were diffuse and appeared as a loose mass of rounded cells so that every time a recombinant plaque was picked it invariably comprised of a mixture of recombinant and wild type viruses. Hence, the recombinant viruses were purified by immunoselection.

Preparation of antibody coated dishes: To each 60mm dish, 3 ml of 10μg/ml (in 50mM Tris.Cl, pH 9.5) solution of affinity purified anti-βhCG specific antibody was added and incubated at RT with swirling at regular intervals. After 90 min the antibody solution was aspirated out and the plates washed thrice with 0.15M NaCl. The unbound sites were blocked by incubating with 1% BSA solution (3ml/60mm dish) overnight at RT and dishes stored at -20°C till further use.

Immunoselection of cells infected by the recombinant virus: The culture medium from CEFs infected by an impure population of recombinant viruses
was aspirated 48 hr PI and the cells incubated for 10-20 min at RT with 0.5 mM EDTA in PBS to dislodge the cells. The cells were transferred to a centrifuge tube and spun for 4 min at 200 x g and the pellet resuspended in 0.5ml PBS/EDTA solution. After passing through a nylon mesh the single cell suspension was layered over antibody coated dishes and incubated at RT for 1-3 hr. The infected cells expressing βhCG on membrane surface adhered to the dish while the non-adherent cells were removed by washing 3 times with DMEM + 5% FBS by gentle swirling. Fresh medium was added to the panned dish and incubated overnight at 37°C in a CO₂ incubator. The cells were scraped and subjected to three cycles of freeze thaw followed by sonication. The lysate obtained was used for infecting fresh cells for second round of immunoscreening. The procedure was repeated until a clonally pure recombinant virus plaque was obtained.

Isolation of Vaccinia Virus DNA

Confluent monolayer of CV1 cells in a 60mm plate were infected with vaccinia virus recombinants at an MOI of 5pfu/cell. After overnight incubation at 37°C, the cells were scraped, centrifuged, washed once with phosphate buffer saline (PBS) and resuspended in 450 μl hypotonic buffer (10mM Tris.Cl, pH 7.8 and 12mM KCl) for 20 min at 0°C. The cell suspension was homogenized with a glass Dounce homogenizer and transferred to a 1.5 ml eppendorf tube. 50μl of 10x reaction buffer (final concentration 60mM KCl, 15mM NaCl, 20 mM Tris.Cl, pH 7.8) were added followed by the addition of Ca²⁺ to a final concentration of 2mM. In order to digest the cellular DNA in situ, 150 units of micrococcal nuclease (Boehringer Mannheim), reconstituted from lyophilized powder with 50 mM Tris.Cl pH 7.8 containing 10 mM NaCl, 1mM EDTA, 50 μM DTT, 0.1% Nonidet P-40 and 50% glycerol, were added and the mixture was incubated for 1 hr at 25°C. The reaction was stopped by adding EGTA to
25mM final concentration (micrococcal nuclease is selectively activated by 
Ca\(^{2+}\) and inactivated by EGTA). Viral DNA was extracted by adding 50\(\mu\)l of 
5\% Sarkosyl\textsuperscript{TM} (Sigma) and 10\(\mu\)l of Proteinase K (1mg/ml) and incubated for 
1hr at 25°C followed by phenol extraction and ethanol precipitation.

PURIFICATION OF THE RECOMBINANT PRODUCT

\(\beta hCG\) secreted into culture supernatant of cells infected with vaccinia virus 
recombinants was purified on an affinity column. The culture supernatants 
were harvested, pooled and ultracentrifuged at 32,900 x g for 2 hr at 4°C 
when the virus particles pelleted down. The supernatant containing secreted 
\(\beta hCG\) was dialyzed against 20mM Tris.Cl, pH 7.0, and 0.2M NaCl and 
circulated through the affinity column anti-\(\beta hCG\)-Sepharose column overnight 
at 4°C. The column was then washed with 10mM Tris.Cl, pH 8.0, containing 
0.14M NaCl and twice with 50mM Tris.Cl, pH 8.0, containing 0.5M NaCl 
and 0.1\% Triton X-100. The recombinant protein was eluted out with 0.5M 
glycine buffer (pH 2.3) containing 0.15M NaCl. Peak fractions were pooled 
and dialysed.

Carbohydrate Analysis

The total carbohydrate content of the expressed protein was determined by 
L-Cysteine sulphuric acid reagent. 200\(\mu\)l each of the standard neutral 
carbohydrate (D-glucose) at concentrations ranging from 1-100\(\mu\)g/ml and two 
different concentrations of the expressed recombinant glycoprotein were added 
in clean assay tubes. 1ml of freshly prepared L-cysteine 
hydrochloride-sulphuric acid solution (L-cysteine HCl added to ice cold 
sulphuric acid, 86\% v/v to give a final concentration of 700mg/l) was added to 
each tube followed by immediate and thorough mixing on ice. The stoppered 
assay tubes were heated at 100°C in a water bath for 3 min and rapidly cooled
to RT. A_{415nm} was measured and the carbohydrate content of the expressed protein determined from the standard curve.

Western Blot

The culture supernatant of CV1 cells infected with the recombinant virus was harvested 72 hr PI, fractionated on a 12.5% denaturing SDS-PAGE (Laemli, 1970) and the resolved bands transferred (Towbin et al 1979) onto nitrocellulose membrane (Amersham, UK) in a transfer buffer containing 20mM Tris.Cl, 150mM glycine pH 8.0 and 20% methanol. After the transfer was complete the membrane was removed and the non-specific sites on it blocked by incubating with PBST (50mM phosphate buffer saline, pH 7.4 and 0.05% tween 20) containing 0.2% BSA for 1 hr at RT with gentle rocking. The filter was washed with PBST and incubated with the primary antibody
antibody specific for βhCG reacted with labelled hCG in the presence of known (standard hCG) or unknown amounts of βhCG (recombinant βhCG in the culture supernatant). All assays were carried out in duplicate in 9x75 mm disposable polystyrene tubes. All dilutions were made in assay buffer (50 mM phosphate containing 0.1% sodium azide and 0.1% BSA; pH 7.4).

**Iodination:** hCG (≈10,000 IU/mg) was radioiodinated by Iodogen method (Fracker & Speck 1978) and labelled hCG was separated from free radiiodine on a Sephadex G-75 column (bed volume ≈10 ml). Peak fractions representing the iodinated hCG were diluted appropriately. The specific activity of iodinated hCG ranged between 40-60 μCi/μg (100 μCi = 37 MBq).

**Assay protocol:** 100μl each of standard hCG (1.25, 2.5, 5, 10, 20, 40 ng/ml) or doubling dilutions of the culture supernatant containing recombinant βhCG and 125I-hCG (≈ 10,000 cpm) were incubated with 100 μl of an appropriate dilution of βhCG specific antibody (binding 30-40% labelled hCG in the absence of the competing cold hormone) and 100 μl 20% horse serum (carrier serum) in a total volume of 500 μl made up with assay buffer. The tubes were vortexed gently and incubated for 20 hr at 4°C. Antibody bound fraction was precipitated by adding 500 μl PEG 8000 (25%). The tubes were vortexed and centrifuged at 4°C for 20 min at 1500 x g. Supernatant was decanted and the radioactivity in the pellet measured in a γ-counter (LKB multigamma). The counts were corrected for non-specific binding and concentration of recombinant βhCG was determined from the standard curve.

**Bioassay**

The biological activity of recombinant βhCG associated with native αhCG (rβhCG/αhCG) was determined by two methods: in a receptor assay by inhibiting the binding of labelled hCG to rat testicular receptor, and in Leydig cell bioassay by stimulation of steroidogenic response of mouse Leydig cells.
αβ heterodimer was generated by incubating the medium containing expressed βhCG with a 10-fold excess of αhCG in an end-to-end shaker for 24 hr at RT.

**Testicular receptor assay:** Rat testicular receptor were prepared essentially as described (Pal et al 1990). Testes of 2- to 3 month-old Wistar outbred rats were decapsulated and homogenized in a polytron homogenizer in 50mM Tris.Cl buffer pH 7.4 containing 5mM MgCl₂, 0.1% BSA and 0.1% sodium azide. The homogenate was filtered through a nylon mesh and centrifuged at 500 x g for 10 min at 4°C. The resulting pellet was resuspended in 8 ml of Tris buffer per pair of testes.

50μl of testicular homogenate was incubated with 100μl of various concentrations of standard hCG or medium containing rhCG/αhCG dimer and 50μl of ¹²⁵I-hCG (≈ 20,000 cpm, 1 ng hCG) at 37°C for 2hr. The assay was terminated by the addition of 1ml cold Tris buffer. After centrifugation at 2000 x g the pellet was washed in Tris buffer and the receptor bound counts taken in a γ-counter. αβ-dimer concentrations were read off hCG standard curve.

**Leydig cell bioassay:** The protocol for bioassy was that of Van Damme et al (1974) as modified by Rae et al (1988). Testes of 8-week old NMRI mice were decapsulated, minced into DMEM supplemented with 2% FBS and 0.025% NaHCO₃, and gently stirred magnetically for 10 min. The suspension was filtered through a nylon mesh and supernatant containing Leydig cells preincubated in a metabolic shaker at 34°C for 1 hr to enable cells to secrete endogenous testosterone. Cells were pelleted down at 500 x g for 10 min at 4°C, resuspended in 10ml fresh medium per pair of testes and stirred for 10 min under cold conditions.

200μl of the cell suspension (≈ 8x10⁴ viable cells) in each siliconized tube was incubated with 200 μl (0 to 200 pg) of standard hCG (diluted in 50mM
PBS, pH 7.4, containing 0.1% BSA, 50µg/ml streptomycin and 50U/ml penicillin) or with doubling dilutions of medium containing αβ-dimer for 3 hr at 34°C in a 5% CO₂. The assay was terminated by heating the tubes at 90°C for 30 min in a water bath, the tubes cooled on ice and 500µl of 50mM PBS containing 0.1% gelatin added to each tube and vortexed. 300µl aliquots were withdrawn to estimate the amount of testosterone released by RIA using WHO protocol and reagents.

**Immunofluorescence**

The expression of anchored βhCG on the membrane surface of mammalian cells by recombinant fowlpox viruses was demonstrated by immunofluorescence. CV1 cells were grown to confluency in sterile tissue culture chambers on glass slides (NUNC). The cells were infected with the recombinant virus at an MOI of 10pfu/cell. Twenty four hr PI the cells were washed with PBS and the first antibody (anti-βhCG) diluted 1:200 was layered over the slide in such a way that the cells were just covered. Incubation was carried out for 1 hr at RT followed by 2 hr at 4°C for in a humid chamber. The cells were washed with PBS thoroughly 4-5 times with gentle twirling. The fluorescein-isothiocyanate conjugated second antibody (goat anti-mouse IgG-FITC conjugate, USB) was microfuged for 2 min at 13,500 x g at 4°C and layered over the cells at a dilution of 1:100. After incubation in dark for 30 min at RT, cells were washed thoroughly with PBS to remove the unbound second antibody and viewed under the fluorescence microscope. 90% glycerol-PBS containing 1 mg/ml of p-phenyldiamine was added as an anti-quenching agent.