CHAPTER 3.

MATERIALS AND METHODS
3.1. MATERIALS

3.1.1. Bacterial strains, cell lines and plasmids.

The bacterial strains used in this study were E. coli K12 strains, HB101 [ F-, hsd S20 ( rB-, mB- ), supE44, ara14, λ-, galK2, lacY1, proA2, rpsL20, xyl-5, mtl-1, recA13 ] (Boyer et al., 1969), and JM105 [ thi, rpsL, endA, sbcB15, hsdR4, ( lac-proAB ), (F', traD36, proAB, lacIqM15 ) ].

The mammalian cell lines used are listed in Table 1. Rat-2 is an established rat fibroblast cell line. FWIL (Larrick et al., unpublished) is a human myeloma cell line derived from the fusion of U266 IgE myeloma cells with WIL-2 lymphoblastoid cells. Rat - 2 and FWIL cell lines were kindly provided by Dr. J.W. Larrick, Cetus Corporation, USA. The other four cell lines were obtained from American Type Culture Collection (ATCC).

The plasmids used in this study are described in Table 2.

3.1.2. Media.

Bacto - tryptone, Bacto - agar and Bacto - yeast extract were from Difco Laboratories, Detroit, USA. Fetal calf serum, Ham's F-12 media, Dulbecco modified Eagle medium (DMEM), Iscove's modified DMEM were from Gibco Laboratories, USA.

3.1.3. Chemicals.

Acrylamide, bisacrylamide, ammonium persulphate, Bio - gel P-4 and TEMED, were from Bio - Rad Laboratories, USA. Agarose (SeaKem) and low gelling agarose (NuSieve
## TABLE 1.

LIST OF MAMMALIAN CELL LINES USED IN THIS STUDY

<table>
<thead>
<tr>
<th>#</th>
<th>CELL LINE</th>
<th>BRIEF DESCRIPTION</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>CHO-K1</td>
<td>Chinese hamster ovary</td>
<td>ATCC # CCL 61</td>
</tr>
<tr>
<td>2.</td>
<td>NIH3T3</td>
<td>NIH Swiss mouse embryo</td>
<td>ATCC # CRL 1658</td>
</tr>
<tr>
<td>3.</td>
<td>HeLa</td>
<td>Human epitheloid carcinoma</td>
<td>ATCC # CCL 2</td>
</tr>
<tr>
<td>4.</td>
<td>Mouse LMtk−</td>
<td>Mouse connective tissue</td>
<td>ATCC # CCL 1.3</td>
</tr>
<tr>
<td>5.</td>
<td>FWIL</td>
<td>Human myeloma cell line</td>
<td>Dr. J.W. Larrick, pers. commun.</td>
</tr>
<tr>
<td>6.</td>
<td>Rat-2</td>
<td>Rat fibroblast</td>
<td>-do-</td>
</tr>
</tbody>
</table>
# PLASMID | GENETIC MARKERS | REFERENCE
---|---|---
1. pBR-αhCG | Amp<sup>r</sup> | Khandekar et al., unpublished
2. pBR-βhCG | Amp<sup>r</sup> | -do-
3. pHβAPr-1-neo | Amp<sup>r</sup>, neo<sup>r</sup> | Gunning et al., 1987
4. pAM-1 | Amp<sup>r</sup>, neo<sup>r</sup> | This study
5. pAM-2 | Amp<sup>r</sup>, neo<sup>r</sup> | This study
6. pSV<sub>2</sub>S | Amp<sup>r</sup> | Michel et al., 1988
7. pSV<sub>2</sub>BS35 | Amp<sup>r</sup> | Munshi et al., communicated
8. pUC18 | Amp<sup>r</sup>, lac<sup>+</sup> | Yanisch-Perron et al., 1985
9. pAM-3 | Amp<sup>r</sup>, lac<sup>−</sup> | Munshi et al., communicated
10. pAM-5 | Amp<sup>r</sup>, neo<sup>r</sup> | Munshi et al., communicated
GTG) were from FMC Bio Products, USA. SDS, ethidium bromide, calf thymus DNA, cesium chloride, tris base, dithiothreitol, IPTG, X-gal, DAB, ficoll, PVP, chloroquine, coomassie brilliant blue, amido black, bovine serum albumin, were from Sigma Chemicals Company (Sigma), USA. DEAE-dextran was from Pharmacia, Sweden. Nick translation kits were from Bethesda Research Laboratories (BRL), USA, and Amersham International plc, UK. Lipofectin was kindly provided by Syntex, Inc., USA. βhCG RIA kit was from ICN Micromedic Systems, Inc., USA. Purified hCG (13,000 I.U./mg) was kindly provided by Dr. Y.Y. Tsong, Population Council, USA. HBsAg detection kit was from Abbott Laboratories, USA. Protein molecular mass standards were from Bio-Rad Laboratories, USA. DNA size markers were from BRL. All other chemicals were from Glaxo Laboratories, India, and E. Merck, India.

3.1.4. Enzymes.

Restriction endonucleases, T₄ DNA ligase, DNA polymerase I large fragment (Klenow), bacterial alkaline phosphatase (BAP) were from BRL, USA and New England Biolabs, USA. Lysozyme and RNase A were from Sigma. Thermus aquaticus thermostable DNA polymerase was kindly provided by Cetus Corporation, California, USA.

3.1.5. Antibiotics.

Ampicillin, tetracycline, chloramphenicol and gentamycin were from Sigma. Geniticin (G418) was from Gibco Laboratories, USA.
3.1.6. **Radioactive chemicals.**

$^{32}\text{p} - \text{dCTP}$ (specific activity 400 or 800 Ci/mmole) was from Amersham, UK or from New England Nuclear division of DuPont, USA. $^{125}\text{I}$ was from Amersham.

3.1.7. **Materials for autoradiography.**

Intensifying screens were from Kiran X-ray Screens, India. X-ray films were from Agfa - Gevaert, Belgium, Kodak, USA, or Hindustan Photo Films, India. Developer and fixer were from Hindustan Photo Films, India.

3.1.8. **Antisera.**

All antisera were obtained from the reagent bank at National Institute of Immunology, New Delhi.

3.1.9. **Other Materials.**

Nitrocellulose membranes (BA85) were obtained from Schleicher and Schuell, Germany. GeneScreen and GeneScreen Plus membranes were from DuPont, USA. Millipore membranes (0.45 um) were from Millipore Corporation, USA. 3 MM and 1 MM chromatography filter papers were from Whatman Ltd, U.K.

3.1.10. **Computer software.**

All computer software facilities were provided by the NII computer centre.

3.2. **METHODS**

3.2.1. **Growth and storage of bacteria.**

Composition of growth media used for culturing *E. coli* is given in Table 3. For routine propagation, *E. coli* cells were grown in Luria - Bertani medium (LB). LB was prepared by dissolving 10 g Bacto-tryptone, 5 g Bacto-
## TABLE 3.

### BACTERIAL GROWTH MEDIA COMPOSITION (PER LITER)

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>LB</th>
<th>TB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto tryptone</td>
<td>10 g</td>
<td>12 g</td>
</tr>
<tr>
<td>Bacto yeast extract</td>
<td>5 g</td>
<td>24 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>10 g</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td></td>
<td>4 ml</td>
</tr>
<tr>
<td>Solution X*</td>
<td></td>
<td>100 ml</td>
</tr>
</tbody>
</table>

*Solution X* : $0.17 \text{ M } \text{KH}_2\text{PO}_4$

$0.72 \text{ M } \text{K}_2\text{HPO}_4$
yeast extract, and 10 g NaCl in distilled water, pH adjusted to 7.5 with NaOH and final volume made up to one liter (Maniatis et al., 1982). Cultures of *E. coli* cells transformed with plasmid DNA were grown in media supplemented with 50 ug/ml of ampicillin. For large scale plasmid DNA isolation, *E. coli* cells were grown in an enriched medium, Terrific Broth (TB). One liter of TB was prepared by adding 100 ml of a sterile solution of 0.17 M KH₂PO₄ and 0.72 M K₂HPO₄ to a sterile solution containing 12 g Bacto-tryptone, 24 g Bacto-yeast extract, 4.0 ml glycerol and water to a final volume of 900 ml (Tartof and Hobbs, 1987). The media were sterilised by autoclaving at 15 psi for 20 minutes. Heat labile compounds and antibiotics were sterilised by filtration through a 0.45 µ nitrocellulose membrane and added to autoclaved media after cooling the same to 55°C. Solid media was prepared by adding 1.5 % bacto-agar prior to autoclaving. Storage of *E. coli* was carried out essentially as described by Maniatis et al., (1982).

3.2.2. **Large scale isolation of DNA.**

3.2.2.A. **Isolation of plasmid DNA.**

Plasmid DNA was isolated using the alkaline lysis method of Birnboim (1979) with slight modifications. One liter of TB supplemented with ampicillin @ 50 ug / ml was inoculated with 10 ml of a freshly grown primary culture and the culture incubated O/N at 37°C, in an incubator-shaker. The cells were pelleted by centrifugation at 4000g for 10 minutes at 4°C. The supernate was discarded and the pellet
resuspended in 20 ml of Tris-Glucose solution (25 mM Tris.HCl, pH 8.0; 50 mM Glucose). The cells were vortexed followed by repeated pipetting to obtain a uniform cell suspension. To this, 6.0 ml of a freshly prepared lysozyme solution (10 mg/ml, prepared freshly in sterile distilled water) was added. The cell suspension was swirled to mix thoroughly and incubated for 5 minutes at room temperature. Next, 0.5 M EDTA was added to a final concentration of 10 mM, the contents swirled to mix and incubated in ice for 20 minutes. Next, 40 ml of a lytic mix containing 0.1% SDS and 0.2 N NaOH was added. This was prepared freshly by mixing 4 ml of 10% SDS solution into 36 ml of 0.22 N NaOH solution. The solution was mixed by vigorous but brief shaking till the cell lysate became clear, followed by incubation on ice for 5 minutes. Finally, 20 ml of 5 M potassium acetate solution, pH 4.8 was added. Again the contents were swirled to mix, followed by incubation in ice for at least 1-2 hours. The lysate was centrifuged at 10,000 rpm for 30 minutes at 4°C. The supernate was filtered through sterilised glass wool kept in a funnel, and collected in a graduated cylinder. The measured volume of the cell lysate was transferred into another centrifuge bottle and two volumes of 95% ethanol added to precipitate the DNA, at -20°C, 0/N. The DNA was pelleted by centrifugation at 10,000 rpm at 4°C for 30 minutes. The supernate was carefully poured off and the pellet resuspended in 25 ml of TE (10 mM Tris.HCl, pH 8.0; 1 mM EDTA). The plasmid DNA was
further purified by centrifugation to equilibrium in a 30 ml cesium chloride - ethidium bromide density gradient, as described by Maniatis et al., (1982). The band corresponding to closed circular plasmid DNA was collected and further purified by a second centrifugation to equilibrium in a 6.5 ml cesium chloride - ethidium bromide density gradient. The final DNA band collected from the gradient was extracted with an equal volume of isopropanol which had been previously saturated with TE and cesium chloride. This extraction was repeated twice to completely remove the ethidium bromide from the DNA sample. The DNA was then dialysed against one liter of TE for at least 8 hours, at 4°C, with several changes of TE. To the dialysed sample, one tenth volume of 3 M sodium acetate, pH 5.2, was added and the DNA precipitated with two volumes of chilled ethanol. The precipitation was carried out 0/N at -20°C. The precipitated DNA was collected by centrifugation at 10,000 rpm, at 4°C, for 10 minutes. The supernate was carefully decanted and the pellet dried briefly under vacuum. The final DNA pellet was resuspended in 500 ul of TE. A 1:50 dilution of the sample was used to measure the absorbance at 260 nm and at 280 nm. The A260 and A280 values were used to estimate the concentration and purity of the sample as described by Maniatis et al., (1982).

3.2.2.B. Isolation of genomic DNA from mammalian cells.

Genomic DNA from cultured mammalian cells was isolated by a rapid procedure, essentially as described by Davis et
al., (1986). Briefly, about $10^8$ cells were pelleted and the pellet washed twice with 10 mM phosphate buffered saline, pH 7.4. The pellet was resuspended in 2 ml of a solution containing 0.1 M NaCl, 0.2 M sucrose, 0.01 M EDTA, and 0.3 M Tris, pH 8.0. To this, 125 ul of 10% SDS was added, mixed by vortexing and the sample incubated at $65^\circ$C for at least 30 minutes. Next, 350 ul of 8 M potassium acetate was added, the contents vortexed to mix and incubated on ice for 60 minutes. The lysate was centrifuged at 5000g for 10 minutes at $4^\circ$C. The supernate was transferred to a new tube and extracted with 2 ml of phenol (saturated previously with TE) and 2 ml of chloroform / isoamyl alcohol (24:1). The extraction was done by gentle rocking or by inverting the tube. The tube was spun at 1500g for 5 minutes to separate the two phases, and the upper aqueous phase was collected. This was re-extracted with 2 ml of chloroform / isoamyl alcohol as described above and the aqueous phase collected. Then 5 ml of ethanol was added to the aqueous phase to precipitate the DNA. The two layers were mixed slowly to prevent shearing of DNA. The DNA was pelleted by centrifugation at 1500g for 10 minutes at $4^\circ$C. The supernate was discarded very carefully, to minimise the loss of the loose DNA pellet. The DNA pellet was washed gently with 5 ml of 80% ethanol. Again, the tube was centrifuged at 1500g to pellet the DNA and the supernate was discarded. The final DNA pellet was dried partially by letting the tube stand at room temperature for 30 minutes. To resuspend the DNA, 300 ul TE
was added followed by gentle shaking for 90 minutes at room temperature. This DNA was stored at 4°C. The DNA prepared by this method was of sufficient purity for restriction endonuclease cleavage and Southern blotting, but because of RNA contamination, this DNA could not be used for accurate absorbance measurements. However, typically a 30 ul aliquot was expected to contain approximately 10 ug DNA.

3.2.3. Polymerase chain reaction (PCR).

Polymerase chain reaction was carried out as described by Scharf et al., (1986), with some modifications. 23 mer oligonucleotide primers synthesised by the solid phase triester method were designed to flank the target DNA desired to be amplified. A primer was designed to be complementary to the 5' end of the βhCG cDNA (+) strand and was termed 5' primer (also see Fig. 20). Another primer was designed to be complementary to the sequence flanking the translation termination codon (TAA) of HBsAg (-) strand and this was termed 3' primer. The 5' primer also included the recognition sequence for restriction endonuclease Sal I as an overhang, while the 3' primer included the recognition sequence for Hind III as an overhang. In addition, two extra bases (G or C) flanking the restriction site were included in the sequence of the primers, to improve the enzyme digestion. Thus, the nucleotide sequence of the 5' primer read as: 5'-GGCGTCGACATGGAGATGTTCCA-3', while that for the 3' primer read as: 5'-CCAAGCTTTTAAATGTATACCCA-3'.

A standard PCR reaction contained 500 ng to 1 ug
plasmid DNA in a 100 ul mixture having 10 ul of 10 X PCR buffer, 10 ul of 10 mM dNTPs, 3 ul of each primer to give a final primer concentration of 1 uM, and 2.5 units of the Thermus aquaticus thermostable DNA polymerase. PCR reaction buffer (10 X) contains: 500 mM KCl, 100 mM Tris.Cl, pH 8.3, 15 mM MgCl₂ and 0.1% gelatin. The mixture was subjected to PCR amplification in a programmed thermal cycler block set for 30 cycles. The procedure is diagramatically outlined in Fig. 1 and involved four steps: a) The reaction was heated to 95°C for 30 seconds to separate the two strands of the target DNA; b) the reaction was then cooled to 37°C for one minute to allow annealing of the two primers to the template DNA to occur; c) next, the temperature was raised to 72°C and the reaction maintained at this temperature for 10 minutes, for primer extension to occur; d) at the end of the cycle, the temperature was again raised to 95°C as in step (a) to start a new cycle. In between the steps (a) to (d), one minute ramp times were used to allow the efficient realisation of the set temperature. In the last cycle, the duration of step (c) was extended to ensure the conversion of all single strands into double stranded DNA. At the end of 30 cycles of PCR, a 10 ul aliquot from the PCR reaction was electrophoresed on a 1% SeaKem / 3% NuSieve agarose gel in TBE buffer, to resolve the PCR products. The amplified DNA was purified by electrophoresing the entire PCR mixture on a 1% preparative agarose gel in TAE buffer.
A typical PCR consists of repetitive cycles of multiple temporal segments (designated here as A - G) with distinct target temperatures. After making the desired cocktail of template DNA, primers, Taq polymerase and the enzyme buffer, the reaction tube is incubated in a programmable thermal cycler, to incubate the reaction contents at pre-set temperatures for designated periods of time. Segments A - B, template denaturation; C - D, primer annealing; E - F, strand synthesis; G, ramp to the completion of the first cycle prior to the start of the next cycle (dotted line). The duration and target temperature for each segment in an amplification cycle can be varied to suit the desired objectives.
All fine chemicals and the thermal cycler used for PCR, were kindly provided by Cetus Corporation, California, USA.

3.2.4. Digestion of DNA with restriction enzymes.

DNA samples were digested with restriction endonucleases in the appropriate digestion buffers as recommended by BRL. The digestion buffers were in most cases, supplied by BRL. Composition of the 1 X buffers is given in Table 4.

3.2.4.A. Routinely, 0.2 - 1 ug DNA was made up to 10 ul with sterile double distilled water in an autoclaved eppendorf tube. 2 ul of 10 X buffer and 2 - 5 units of restriction endonuclease were added. The reaction components were mixed well and incubated in a 37°C water bath for 1 - 2 hours. The digestion reaction was terminated by the addition of 2 ul of 10 X tracking dye ( 0.25 % xylene cyanol, 0.25 % bromophenol blue, 0.1 M EDTA, pH 8.0, and 50 % glycerol ) followed by brief vortexing to mix, after which the sample was loaded on to the gel.

3.2.4.B. Digestions involving more than one restriction endonuclease were carried out with 2 - 4 ug DNA in a final reaction volume of up to 50 or 100 ul. In these cases, if the two enzymes had radically different optimal assay conditions, the DNA was digested first with the enzyme requiring a lower salt concentration. After incubating for one hour, a 5 ul aliquot from the digestion reaction was electrophoresed on a mini gel to monitor the extent of digestion. Once the digestion was complete, appropriate amount of salt and the
TABLE 4.

BUFFERS FOR RESTRICTION ENDONUCLEASES (1 X)

<table>
<thead>
<tr>
<th>CODE</th>
<th>Tris.HCl (mM)</th>
<th>pH</th>
<th>MgCl2 (mM)</th>
<th>NaCl (mM)</th>
<th>KCl (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>REact 1.</td>
<td>50</td>
<td>8.0</td>
<td>10</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>REact 2.</td>
<td>50</td>
<td>8.0</td>
<td>10</td>
<td>50</td>
<td>--</td>
</tr>
<tr>
<td>REact 3.</td>
<td>50</td>
<td>8.0</td>
<td>10</td>
<td>100</td>
<td>--</td>
</tr>
<tr>
<td>REact 4.</td>
<td>20</td>
<td>7.4</td>
<td>5</td>
<td>--</td>
<td>50</td>
</tr>
<tr>
<td>REact 5.</td>
<td>10</td>
<td>8.2</td>
<td>8</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>REact 6.</td>
<td>50</td>
<td>7.4</td>
<td>6</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>REact 7.</td>
<td>50</td>
<td>8.0</td>
<td>10</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>REact 8.</td>
<td>20</td>
<td>7.4</td>
<td>10</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>REact 9.</td>
<td>50</td>
<td>8.5</td>
<td>5</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>REact 10.</td>
<td>100</td>
<td>7.6</td>
<td>10</td>
<td>150</td>
<td>--</td>
</tr>
<tr>
<td>REact 11.</td>
<td>10</td>
<td>9.0</td>
<td>12</td>
<td>--</td>
<td>100</td>
</tr>
</tbody>
</table>

(The above buffers and their composition were provided by BRL Inc., USA.)
second enzyme were added and the incubation continued in an increased final reaction volume, to offset any increase in the glycerol concentration in the new reaction. Alternatively, the DNA was extracted once with phenol/chloroform, once with chloroform, and then precipitated with one half volume of 7.5 M ammonium acetate and two volumes of ethanol. The precipitation was done for 30 minutes at room temperature, and the DNA spun down for 30 minutes at room temperature. The supernate was discarded, pellet washed with 70% ethanol, recentrifuged, dried briefly under vacuum and finally resuspended in 18 ul distilled water. The DNA purified in this manner could then be used for setting up digestion with a second enzyme or for setting up a ligation.

For those double digestions where one of the enzymes was known to be active over a broad range of ionic strength conditions, including those required for the optimal activity of the second enzyme, both the enzymes were added simultaneously in the digestion reaction, which was carried out using the optimal conditions of the second enzyme having more stringent assay requirements.

3.2.4.c. For digestions aimed at purification of restriction fragments, 10 - 20 ug of DNA was digested in a reaction mixture of about 100 - 200 ul volume. Aliquots from the digestion reaction were checked on a minigel after one hour to monitor the extent of digestion. After the digestion was complete, one tenth volume of the 10 X tracking dye was
mixed with the sample by vortexing, and the DNA loaded on a preparative agarose gel.

When digesting vector DNA in preparation for ligation, the DNA was first purified from the digestion reaction as described in 3.2.4.B. This DNA was then treated with bacterial alkaline phosphatase as described by Maniatis et al., (1982). The dephosphorylated DNA was run on a preparative agarose gel to purify the linearised, dephosphorylated vector DNA. The efficiency of dephosphorylation was monitored by self ligation, followed by transformation of competent E.coli cells. Only after achieving efficient dephosphorylation of the vector DNA, was it used for ligation with the insert DNA.

3.2.5. **Agarose gel electrophoresis.**

3.2.5.A. **Electrophoresis of DNA.**

3.2.5.A.1. **Minigel electrophoresis:** For rapid electrophoretic analysis of plasmid DNA prepared by miniprep protocol, or to monitor the progress of digestion during various cloning procedures, the DNA was resolved on short agarose gels, taking less than one hour for the run. The electrophoresis was carried out in TAE buffer using 8 cm long gels with a comb of teeth size 0.4 x 0.2 cm. The width of the gel was variable, depending on the number of samples to be analysed. Gels were run at 50 - 100 volts, till the bromophenol blue dye migrated to within 0.5 cm of the edge of the gel.

3.2.5.A.2. DNA digested with restriction enzymes was
electrophoresed on 0.7 % - 1.2 % agarose gels in TAE or TBE buffer. Choice of the percentage of agarose and the electrophoresis buffer system was made following the guidelines of Maniatis et al., (1982). In general, upto 1 kb fragments were resolved on 1.2 % agarose gels using TBE buffer. For most other purposes, TAE buffer was used. Agarose gel electrophoresis was carried out as described by Maniatis et al., (1982). The run was stopped when the bromophenol blue dye migrated to within 1 cm - 1.5 cm from the edge of the gel, except when the sample had fragments smaller than 500 bp, in which case the electrophoresis was terminated at an earlier stage. The gel was immersed in water containing 0.5 ug / ml ethidium bromide, for 30 minutes, to stain the DNA. When detecting very low amounts of DNA, the staining was done for 60 minutes followed by destaining in 1 mM MgSO₄ for one hour at room temperature. The DNA bands were visualised on a short wavelength UV transilluminator (Fotodyne, Inc., USA) and photographed with a Polaroid MP-4 camera using Polaroid type 667 film.

3.2.5.B. **Electrophoresis of RNA.**

Total RNA was resolved in formaldehyde - agarose gels as described by Maniatis et al., (1982). In general, the electrophoresis was performed using 1.2 % agarose gels containing 2.2 M formaldehyde and 1 X running buffer (0.04 M morpholinopropanesulfonic acid - MOPS, pH 7.0; 0.01 M sodium acetate; 0.001 M EDTA). RNA samples (upto 20 ug in 5 ul) were incubated at 55°C for 15 minutes in 5 X gel buffer
containing 2.2 M formaldehyde and 50 % V/V formamide. The samples were chilled on ice for 5 mins. and loading buffer added. A Taq I digest of phi X 174 DNA, filled-in with Klenow polymerase using $^{32}$P-dCTP, was used as size marker for electrophoresis. The gels were run at <5 V/cm.

3.2.6. **Isolation of restriction fragments of DNA.**

After digestion of the plasmid DNA with appropriate restriction enzymes, the DNA fragments were resolved by electrophoresis on preparative agarose gels of a suitable percentage, and stained with ethidium bromide as described above. Depending upon the amount of DNA to be resolved on the gel, the size of the sample well varied from 1.5 cm - 5 cm x 0.3 cm, such that the desired fragment could be cut out with a minimum of agarose accompanying it. The DNA bands were visualised under long wave UV (366 nm), using a hand held monitor (model UVGL-58 Mineralight Lamp, UVP, Inc., California, USA), and the desired fragment cut out as a thin agarose slice (keeping the size of the slice as small as possible). DNA was eluted from the agarose slice by the method of Zhu et al., (1985). Briefly, a GeneScreen (NEN) or Durapore (Millipore, GVWP 04700) membrane was wetted with 200 ul of elution buffer (0.1 % SDS + 50 mM Tris. HCl, pH 7.5), and folded over to form a cone. Meanwhile, the conical lower half of an eppendorf tube was cut off and a hole pierced in the bottom with a hot wire or needle. The membrane was placed into this cone, pushing it as far as possible. This assembly was then transferred to

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a 1.5 ml eppendorf tube and the gel slice put into the paper cone. The tube was centrifuged for 10 minutes at room temperature, to elute the DNA into the filtrate. The filtrate was extracted with one volume of phenol / chloroform (1:1 v/v), and the DNA precipitated from the aqueous phase by the addition of 5 M NaCl to a final concentration of 1 M, and 2 - 3 volumes of ethanol at -20°C, for a few hours. The centrifuged DNA pellet was dissolved in an appropriate volume of TE.

3.2.7. Ligation of DNA fragments.

Wherever possible, the cloning of DNA fragments was achieved by ligation of compatible sticky ends generated on the vector as well as the insert by digestion with the same enzyme. Self ligation of the linearised vector with compatible sticky ends was minimised by dephosphorylation of the vector DNA using bacterial alkaline phosphatase.

The ligation conditions for each batch of T4 DNA ligase were standardised using Hind III generated fragments of lambda DNA as a test sample for sticky end ligation.

Routinely, 200 ng of vector DNA was mixed with 2 - 5 fold molar excess of the insert fragment DNA, 2 ul each of the 10 X ligase buffer (500 mM Tris.HCl, pH 7.5, 100 mM MgCl2), 10 mM ATP, and 200 mM DTT. The final reaction volume was adjusted to 15 - 20 ul with sterile double distilled water, and 0.5 - 1 ul of T4 DNA ligase (103 units / ml) was added. The contents were mixed well and incubated at 13°C for 12 - 16 hours. An aliquot of 2 ul was electrophoresed on a
minigel along with unligated vector to test the ligation. The ligated DNA was used to transform competent E. coli cells.

3.2.8. Transformation of E. coli.

All glassware/plasticware used for transformation procedure was sterile and prechilled.

3.2.8.A. Preparation of competent E. coli cells.

A single E. coli colony taken from an agar plate was used to inoculate 10 ml of LB and incubated O/N at 37°C in an incubator-shaker. Next day, 0.5 ml of this freshly grown culture was used to inoculate 100 ml of LB in a 500 ml flask. The culture was incubated at 37°C in an incubator-shaker and absorbance of the growing culture was monitored at 620 nm. When the A_{620} reached 0.4 - 0.5 (in about 120 - 150 minutes), the flask was rapidly chilled by shaking in ice. The cells were harvested in sterile, chilled centrifuge bottles at 4,000g for 10 minutes at 4°C. The pellet was gently resuspended in 50 ml sterile, ice cold 100 mM CaCl₂ and the cells incubated in ice for 30 minutes. The cells were again centrifuged as above and the pellet resuspended in 6.5 ml of sterile, chilled, 100 mM CaCl₂ containing 15% glycerol. The cells were resuspended very gently, and a 200 ul aliquot was transformed with a standard plasmid DNA to check the competence of the cells. Meanwhile, the rest of the competent cells were incubated in ice for 16 - 18 hours, to increase the competence of the cells a further few fold. After ascertaining high transformation efficiency of the competent cells, the cells were dispensed as 200 ul aliquots into prechilled, sterile 1.5 ml eppendorf tubes. These cells
were stored at -70°C for at least six months without any significant loss in the competence.

3.2.8.B. Transformation procedure.

Transformation was performed in chilled 1.5 ml eppendorf tubes, using 200 ul of competent cells and about 50 ng of ligated plasmid DNA. Frozen competent cells were thawed in ice and the DNA was added immediately after thawing. The DNA volume was always kept under 30 ul. The DNA was mixed well with the cells by gentle tapping, and the tube incubated in ice for 30 minutes with occasional gentle shaking. The tube was then immersed in a 42°C water bath for 2 minutes, to give a heat shock to the cells. The cells were then incubated in ice for 10 minutes. Next, 1 ml LB was to the cells, and the cells incubated in a 37°C water bath without shaking, for one hour. 50 ul aliquots were plated in triplicate from the transformed cell mixture on suitable antibiotic containing agar plates and incubated 0/N at 37°C to select the transformants. In case of JM105 cells, the transformed cells were plated on antibiotic containing agar plates on which 50 ul of 2 % X-gal (made in dimethyl formamide), and 10 ul of 100 mM IPTG had been spread in advance, to select for the lac⁻ phenotype. The lac⁻ colonies appeared colourless while the lac⁺ colonies were blue.

For each batch of transformations, a negative control was included in which no DNA was added to the cells while keeping the rest of the procedure the same as for the test transformations.
3.2.9. **Plasmid DNA minipreps.**

From an O/N grown culture, 1 ml cells were pelleted in a 1.5 ml eppendorf tube. The cells were washed once with 100 ul of solution I (50 mM glucose in 25 mM Tris.HCl, pH 8.0). The cells were pelleted again and resuspended in 70 ul of solution I. To this, 20 ul of a freshly prepared solution of lysozyme (10 mg/ml in distilled water) was added. The tube was vortexed to mix the contents and incubated in ice for 5 minutes. Next, 10 ul of 0.1 M EDTA, pH 8.0, was added, vortexed and the tube incubated in ice for 5 minutes. Next, 200 ul of solution IV (0.2 N NaOH + 1 % SDS) was added, the contents vortexed quickly but briefly to mix and incubated in ice for 5 minutes. Finally, 150 ul of 5 M potassium acetate, pH 4.8 was added and the tube incubated in ice. After 60 minutes, the tube was centrifuged for 10 minutes at 10,000 rpm, at 4°C. 450 ul of the supernate was removed to another tube and DNA precipitated with two volumes of ethanol at -70°C for 15 minutes. The DNA pellet was collected by centrifugation and after draining off the supernate, the pellet was washed with 80 % ethanol. The pellet was dried briefly under vacuum and finally resuspended in 150 ul TE. From this, a 10 ul aliquot was used for checking on gel or for setting up digestions with restriction endonucleases.

3.2.10. **Isolation of RNA.**

Total RNA was isolated from cultured mammalian cells by the method of Chomczynski and Sacchi (1987), with slight modifications. Briefly, cells from a 3.5 cm petri-dish were
lysed directly in 1.5 ml of solution D (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5 % sarcosyl and 0.1 M 2-mercaptoethanol). For every 2 ml of the lysate, 0.2 ml of chloroform was added, followed by vigorous mixing for 15 seconds, and incubation on ice for 15 minutes. The lysate was spun at 12,000g, at 4°C for 15 mins., and the aqueous phase transferred to another tube. RNA was precipitated with an equal volume of isopropanol and incubation at -20°C for 45 mins. The samples were then spun at 12,000g for 15 mins. at 4°C, and the supernate discarded. The RNA pellet was washed twice with 75 % ethanol. Finally, the pellet was dried briefly under vacuum for 10 - 15 mins. and dissolved in 0.5 % SDS. All chemicals and glassware used for handling RNA were treated with diethylpyrocarbonate (DEPC).

3.2.11. Immobilisation of DNA / RNA on a solid support.

3.2.11.A. Transfer of DNA.

3.2.11.A.1. Colony lifts.

Colony lifts were performed essentially as described by Maniatis et al., (1982). Recombinant colonies were grown O/N at 37°C to have well separated colonies. The colonies were overlaid with 80 mm diameter nitrocellulose filter circles (BA 85, S & S) and after the filter became wet throughout, it was peeled off in a single, smooth motion, avoiding the smearing of the bacterial colonies. The plate was reincubated at 37°C for a few hours to regenerate the colonies. The colonies transferred to the filter were lysed to bind the liberated DNA to the nitrocellulose.
3.2.11.A.2. **Southern blot.**

Restriction fragments of DNA resolved on agarose gel were transferred to nylon membrane (GeneScreen or GeneScreen Plus) by the capillary blotting procedure of Southern (1975) as described by Maniatis et al., (1982). After the completion of electrophoresis, the gel was stained and photographed as described earlier. Position of the various bands obtained in the DNA size marker lane were marked by piercing small holes at the two ends of each band in the gel with a yellow tip. The gel was then denatured, neutralised and blotted essentially as described by Maniatis et al., (1982). Locally available coarse absorbent paper was used to make the paper towels of the appropriate size. In case of genomic DNA from mammalian cells, the agarose gel was first treated with 0.25 M HCl for 10 minutes, followed by the rest of the procedure as mentioned above. The transfer buffer was 20 X SSPE in all cases. To prevent the absorption of fluid from the 3 MM paper under the gel directly to the blotting paper atop the nylon membrane, the gel was surrounded with polythene sheets to minimise the direct contact between the blotting paper and the 3 MM paper placed under the gel. The blotting was performed for 18 - 24 hours. After the transfer was over, the paper towels and the 3 MM papers on top of the nylon filter were peeled off. The gel along with the attached membrane, was turned over and kept on a clean sheet of 3 MM paper with the gel side up. The position of the gel slots was marked with a ball - point pen. Also, the positions of the
bands seen in the DNA size marker, were marked with a ball-point pen at the places where small holes had been pierced in the gel earlier (see above). Thus it was easy to monitor the size of the fragments showing hybridisation to the probe. The gel was then peeled off and the membrane washed in 6 X SSC with gentle rocking for 10 minutes to wash away any residual agarose sticking to the membrane. After air drying at room temperature, the membrane was baked at 80°C for two hours. The baked filter was stored at room temperature in a dessicator, if not used immediately.

The dehydrated gel was restained in water containing 0.5 ug/ml ethidium bromide for 30 minutes and examined on a short wave UV transilluminator to check for the presence of any DNA fragments that escaped blotting. The absence of any residual bands indicated that the transfer was complete.

3.2.11.B. **Northern blot.**

Following electrophoretic resolution of total RNA, the gels were blotted on to GeneScreen membrane as described by Maniatis et al., (1982). The RNA gel to be used for blotting was not stained with ethidium bromide. The blotting was performed in 20 X SSC or 20 X SSPE, O/N.

3.2.12. **$^{32}P$ - labelling of DNA.**

3.2.12.A. **Nick translation.**

DNA was labelled using the nick translation kits supplied by BRL or NEN, USA, or Amersham, UK. The $^{32}P$-dCTP was from either NEN or Amersham, UK, at a concentration of 10 mCi/ml. The specific activity of the label ranged from
400 Ci / mmole to 3000 Ci / mmole. The nick translation reaction was set up as recommended by the manufacturer of the kit, using about 0.5 ug DNA. The reaction was incubated at 12 - 14°C for 90 minutes, except in the case of small fragments ( 500 bp ) when the reaction was incubated for 45 minutes only. The reaction was terminated by the addition of stop buffer supplied with the kit.

3.2.12.B. Purification of the probe.

The nick translated probe was purified by a spun column procedure to remove the unincorporated nucleotides. A sterile 1 ml syringe was plugged at the lower end with siliconised glass wool. The syringe was then filled with Bio-gel P-4 ( Bio Rad Laboratories, USA ) equilibrated in advance with TE. For doing this, 30 grammes of Bio-gel P-4 was slowly added into 250 ml of TE ensuring a good dispersion of the powder. This was then autoclaved at 15 psi for 20 minutes. After cooling, the supernate was decanted and replaced with an equal volume of sterile TE. The slurry was stored at 4°C. The slurry was poured upto the 1 ml mark in the syringe. The syringe was placed into a centrifuge tube and spun at 2000 rpm for 3 minutes. The column was packed by repeating this process till the packed column volume reached 1 ml mark. Next, 50 ul of 2 mg / ml denatured salmon sperm DNA was loaded as carrier and the column spun as before. 100 ul of TE was then added to the column and it was respun as before. Finally, the nick translation reaction was diluted to 100 ul with TE and loaded on to the column. A sterile 1.5 ml
Eppendorf tube was put at the bottom of the column to collect the eluate. The column was respun as before and the purified probe collected in the eppendorf tube, the unincorporated nucleotides remaining within the column. One ul aliquot from the purified probe was diluted 100 fold, mixed well and 1 ul aliquots were put in triplicate into 3 ml scintillation fluid containing vials which were counted in a Beckman Liquid Scintillation Counter. The total radioactivity of the probe was calculated by multiplying the mean radioactivity of the three diluted samples with a factor of $10^4$ (dilution factor, $10^2$, total reaction volume, $10^2$). The specific activity of the probes ranged from $1 \times 10^7$ to $5 \times 10^7$ cpm / ug DNA. The probe purified by the above method did not require any further purification.

3.2.13. Hybridisation of DNA / RNA bound to nylon membranes.

3.2.13.A. Colony hybridisation.

Colonies bound to nitrocellulose filter (NC) were lysed to liberate the DNA which was hybridised as described by Maniatis et al., (1982). To obtain sharper autoradiography signals, the nitrocellulose filter bearing colonies was first overlaid on a 3 MM Whatman paper impregnated with 10 % SDS till the NC wetted evenly. The NC was peeled off and overlaid on another 3 MM paper impregnated with the denaturing solution. In this manner, the NC was successively treated with denaturing and neutralising solutions. Finally, the NC filter was air dried, sandwiched between two sheets of 3 MM paper and baked at 80°C for two
hours.

The NC filters having bound DNA liberated from bacterial colonies, were set up for hybridisation with radioactive probes as described by Maniatis et al., (1982). The filters were washed thoroughly with a solution containing 50 mM Tris.Cl, pH 8.0, 1 M NaCl, 1 mM EDTA, 1 % SDS, at 42°C, for 1 hour, to wash off any residual bacterial debris and agar etc. Prehybridisation and hybridisation was performed in aqueous solution without formamide in 5 X SSPE. The filters were washed up to a stringency of 0.2 X SSC at 65°C.

3.2.13.B. **Hybridisation of Southern filters.**

The prehybridisation and hybridisation of the Southern filters was carried out as described by Maniatis et al., (1982), with some modifications. In all stages, the SDS concentration was maintained at 1 % to minimise the background likely to occur on the nylon membrane. Prehybridisation was done at 68°C, for 4 - 6 hours, with 0.1 ml of prehybridisation buffer for each square centimeter of the membrane. The probe was denatured by immersing the eppendorf tube in a boiling water bath for 10 minutes and added directly to the bag containing prehybridisation mix. Hybridisation was done in aqueous system, at 68°C, without the use of formamide, for 18 - 24 hours, in a plastic bag kept submerged in a water bath, without any shaking. At the end of hybridisation, the filter was taken out of the bag and quickly immersed in a plastic box containing 5 X SSC, 1 % SDS at room temperature. After 15 minutes, the filter was
transferred to another plastic box containing 2 X SSC, 1 % SDS and washed at room temperature by gentle rocking for 15 minutes. The buffer was then changed and the washing continued at 60 - 68°C in a shaking water bath for 30 minutes. Depending on the homology between the probe and the immobilised DNA, the washing conditions were varied. The stringency ranged from 1 X SSC, 1 % SDS, at 65°C to 0.2 X SSC, 1 % SDS, at 65°C. After the washing, the filters were immediately sealed into plastic bags and put for autoradiography. Special care was taken to not to allow the filters to dry during any stage which might otherwise cause permanent binding of the probe to the filter preventing the reprobing of the same filter with a different probe at a later time.

For autoradiography, the plastic bag containing the washed filter was fixed on a 3 MM Whatman sheet and placed securely inside a X-ray cassette with one or two intensifying screens, and a X-ray film was placed over the filter in a dark room. The cassette was kept at -70°C for the desired length of exposure. The film was taken out in the dark room, developed for approximately 3 minutes, washed in water for one minute to wash off all the developer adhering to the film, and fixed for 5 minutes. Finally, the film was washed in cold water for 10 minutes and air dried.

3.2.14. Growth and maintenance of cell lines.

CHO-K1 cells were cultured in Ham's F-12 media. NIH3T3, mouse LMtk− and HeLa cells were cultured in DMEM supplemented
with glutamine. Rat-2 and FWIL cells were cultured in IMDM supplemented with glutamine. All media were supplemented with 10% fetal calf serum. The cells were maintained in a 5% CO₂ atmosphere and were split after 72 hours in culture, at a ratio of 1:15 approximately. For splitting the adherent cells, the cells were washed once with HBSS and 0.1% trypsin in PBS added to the cells. The flask was shaken briefly to ensure a uniform distribution of trypsin over the cells. The cells were incubated in trypsin for 1-2 minutes after which 2 ml of FCS was added to the cells to inactivate the trypsin. The trypsin was carefully aspirated from the flask and fresh culture medium was added into the flask. The cells were dislodged from the bottom of the flask by gently tapping the flask against the working bench. Alternately, the cells were resuspended by vigorous pipetting up and down of the medium. The cells were centrifuged at 1500g for 5 minutes at room temperature and the supernate was discarded aseptically. The cells were resuspended in a known volume of fresh culture medium, an aliquot counted on a haemocytometer, and then accordingly seeded at the desired density in a fresh flask.

For long term storage, the cells were frozen in a mixture of 95% culture medium and 5% DMSO in liquid nitrogen.

3.2.15. Transfection.
3.2.15.A. Transient expression.

Transient expression of the cloned gene product was studied by transfection performed with
1 - 5 ug of plasmid DNA using the DEAE - dextran procedure. DEAE - dextran ( M.Wt. 500,000 ) was used to perform transient transfection by the method of Luthman and Magnusson ( 1983 ), with modifications as described by Gorman ( 1986 ). Six cell lines ( described above ) with two petri dishes ( 60 mm ) for each cell line were used.

In case of FWIL, 5.4 ug plasmid DNA was used to transflect approximately 5 x 10^6 cells. No exposure to chloroquine was given. The cells were treated with the DNA / DEAE - dextran mixture for 20 minutes at 37°C in a tightly capped tube, mixed gently and reincubated at 37°C for 10 minutes. The sample was then diluted with 3 ml of IMDM supplemented with 10 % FCS, centrifuged and the pellet washed once with normal growth medium. Finally, the pellet was resuspended in 4 ml of growth medium and transferred to a T-25 flask. After incubating for 24 hours at 37°C, 3 ml of fresh medium was added to the cells. The cells were harvested after 72 hours post transfection and the culture supernate was assayed for BhCG activity by RIA.

In case of the other five monolayer forming cell lines, a slightly different protocol was used. Only 1.8 ug plasmid DNA was used for each transfection using 0.5 x 10^6 cells, and 70 uM chloroquine was included in the DNA / DEAE-dextran mixture. Cells were fed 3 hours prior to transfection and washed twice with serum free medium just before exposure to DNA. Cells were exposed to DNA / DEAE-dextran mix for approximately 3 hours at 37°C. Following this, the cells were
rinsed twice with serum free medium and replenished with 4 ml of DMEM containing 10 % FCS and 100 µM chloroquine. The incubation was continued for another 3 hours at 37°C, the cells were washed and fed with the normal growth medium containing 10 % FCS. As in the case of FWIL cells, the supernate was collected after 72 hours of transfection and assayed for BhCG activity by RIA.

3.2.15.B. Stable transfection.

Stable transfection was performed into CHO-K1 cells by the following procedures:

3.2.15.B.1. Using calcium phosphate.

Calcium phosphate mediated stable transfections were performed by the method of Graham and Van der Eb (1973) with modifications as described by Gorman (1986). For each plasmid, two petri dishes each containing $0.5 \times 10^6$ CHO-K1 cells were used, with 10 µg of cesium purified DNA for each transfection. A mock transfection which did not contain any DNA, was performed simultaneously as negative control. Precipitation of the DNA was done with great care to ensure the obtention of a fine, translucent precipitate rather than a dense and opaque precipitate. The calcium phosphate / DNA precipitate was added in 4 ml medium to the cells and the cells incubated for 3 hours at 37°C. At this stage, the cells were examined under the microscope and a fine precipitate appeared as small grains all over the cells. The cells were washed once with serum free medium and a glycerol shock given for 3 minutes at 37°C. The cells were washed twice again with
PBS and then replenished with the complete medium. Two days following transfection, the cells were subcultured into the appropriate selective medium for selection of stable clones as described below.

3.2.15.C. Using lipofectin.

Lipofectin was kindly provided by Syntex, Inc., USA as an aqueous solution containing 1 mg/ml of lipid (DOTMA : DOPE; 50 : 50). The procedure used was as described by Feigner et al., (1987) with appropriate modifications as suggested in the user's notes. Lipofection was done with 0.5 x 10^6 cells seeded on a 60 mm plate. For each plasmid, the lipofection was performed in duplicate. The amount and quality of the plasmid DNA used ranged from 400 ng of crude DNA prepared by the miniprep method, to 5 ug of highly purified, cesium banded DNA. The appropriate amount of DNA was suspended in 1.5 ml of serum free DMEM. In another tube, 30 ug of lipofectin was suspended in 1.5 ml of serum free DMEM. The two solutions were mixed. The cells were washed twice with HBSS to totally wash off all traces of serum. The DNA / lipofectin mix was then applied to the cells and the cells incubated for 4 hours at 37°C. Next, 3 ml of media containing 10 % FCS was added and the incubation continued at 37°C for 16 hours. The culture supernate was then aspirated off and fresh medium added to the cells. The selection for stable clones was started after 48 hours by the procedure described below.
3.2.16. **Isolation of stable clones.**

Following transfection with calcium phosphate or lipofectin, the cells were selected for neomycin resistance by using the analogue G418 (Geniticin) in the culture medium. After 48 hours post-transfection, the cells were harvested and replated at a lower density (0.5 x 10^4 cells/60 mm dish). Culture medium containing G418 was then added to the cells. G418 was used at two concentrations - 400 ug/ml and 500 ug/ml. The cells were cultured in G418 containing medium for 2-3 weeks. During this period, the mock transfected cells and the cells transfected with plasmid lacking neo gene, died and the transformed cells formed colonies. Individual G418 resistant colonies were picked up and propagated as independent clones. The culture supernates from these clones were analysed by RIA for BhCG. The stability of the BhCG secreting clones was assessed by culturing with several passages over a few weeks in media with or without G418.

3.2.17. **Immunocytochemistry.**

Cells were grown to subconfluence on polylysine coated glass coverslips contained in plastic dishes. After washing with NKH buffer (145 mM NaCl, 5 mM KCl, 15 mM Hepes, pH 7.4), the cells were fixed for 10 mins. in 70 % ethanol at room temperature. The cells were again rinsed extensively in NKH buffer and the appropriate dilution of primary antibody added. Following O/N incubation at 4°C, the cells were washed 3 X with NKH buffer and the appropriate second antibody,
conjugated to fluorescein isothiocyanate (FITC) or horse radish peroxidase (HRP), added. In case of the FITC staining, the cells were finally mounted in a medium containing 0.1% para-phenylenediamine (PPD) and 90% glycerol in NKH buffer, to retard fading of the fluorescent label. In case of HRP staining, the colour was developed with 0.05% DAB (3',3-diaminobenzidine tetrahydrochloride) in NKH buffer with 0.002% hydrogen peroxide. The colour was developed for 15 mins. and the reaction stopped by rinsing in phosphate buffer. The cells were examined under a standard light microscope (for HRP staining) or a fluorescent microscope (for FITC staining).

3.2.18. Radioimmunoassays.

Quantitative determination of alpha hCG or βhCG was performed by RIA using subunit specific antisera.

3.2.18.A. RIA for βhCG.

βhCG was estimated using either a commercial RIA kit (Micromedic βhCG RIA kit, ICN Biomedicals, Inc., USA) or by the procedure developed at NII, the basic principle of estimation being the same in both assays, i.e., competitive inhibition. The Micromedic kit was used as detailed by the manufacturer. Briefly, 200 ul of the sample was incubated with 100 ul of the given antiserum solution for 30 minutes at room temperature. 100 ul of the tracer (125I-hCG) solution was then added and incubation continued for another 30 minutes. Subsequently, 1.0 ml of the precipitating solution (containing anti-rabbit serum with PEG) was
added and the sample vortexed thoroughly. The sample was then centrifuged for 10 minutes at 1000 x g. The supernate was carefully decanted, the rims of the tube wiped to absorb all residual supernate, and the precipitate counted on a gamma counter set for the detection of $^{125}$I. A standard curve was plotted with each assay by using different concentrations of purified hCG, starting from 0 mIU / ml. The percent binding of the sample was estimated as a fraction of the zero standard and the hCG activity of the sample calculated from the standard curve of the known concentrations.

The other RIA procedure used has been described previously by Salahuddin et al., (1976). This procedure employed a monoclonal antibody shown to be specific to $\beta$hCG (Gupta et al., 1982). The use of this antibody made this assay much more sensitive compared to the commercial assay described above.

3.2.18.B. RIA for alpha hCG.

The RIA for alpha hCG was similar to that used to estimate $\beta$hCG. A monoclonal antibody specific to alpha hCG (Gupta et al., 1985) was used for the assay. The standard used was total hCG.

3.2.19. Radioreceptor assay.

Culture supernate from stable clones secreting alpha hCG was mixed with the culture supernate from stable clones showing $\beta$hCG activity. The mixing was done with shaking at 37°C for 16 hours, to allow the two subunits to associate. The presence of the heterodimer in the culture supernate was
then estimated by monitoring its competitive binding to the hCG receptor in the presence of radiolabelled hCG. The testes homogenates from 10 - 14 week old Wistar outbred rats were prepared by the method of Dighe and Moudgal (1983), and the receptor assay carried out as described by Catt et al., (1975), with slight modifications as described by Pal (1989).

3.2.20. **Leydig cell bioassay.**

The culture supernates containing the associated hCG heterodimer as described above, were tested for their ability to inhibit hCG induced testosterone production by Leydig cells. Leydig cell suspensions were prepared from NMRI inbred adult male mice essentially by the procedure of Van Damme et al., (1974) with modifications. The procedure has been described previously (Pal, 1989) and involved the measurement of the testosterone produced by the Leydig cells following stimulation with culture supernate containing the associated alpha and beta subunits of hCG.

3.2.21. **Western blot.**

Electrophoretic separation of protein samples was carried out on 12.5% SDS-PAGE in a discontinuous system as described by Laemmli (1970). The samples were reduced by boiling for 3 minutes in sample buffer containing β-mercaptoethanol. The samples were then centrifuged for 5 minutes at 10,000 rpm to pellet down all particulate matter, prior to loading on the gel. Electrophoresis was carried out at 100 volts in a LKB vertical slab gel electrophoresis.
apparatus. At the end of the run, the gel was equilibrated for 15 minutes in the transfer buffer (25 mM Tris base, 192 mM glycine, 20% V/V methanol). Immunoblotting was performed essentially as described by Towbin et al., (1979). The proteins were blotted on to nitrocellulose (Schleicher and Schuell) for 16 hours in LKB Transphor apparatus. After the completion of electric transfer of proteins, the nitrocellulose paper was washed with PBS (10 mM sodium phosphate buffer, pH 7.4, 0.9% saline) for 10 minutes at room temperature, with gentle rocking to wash off any adhering traces of acrylamide gel. The filter was then stained with a dilute solution of amido black. This was made by diluting 5 fold with water, a solution of 0.2% amido black containing 45% V/V methanol and 10% V/V acetic acid. The staining was done for 5-10 seconds and the filter washed immediately with distilled water. The proteins transferred to the gel could be seen at this stage. The lane containing protein molecular weight standards was cut out and preserved by drying and storage in dark. The filter could be cut into appropriate lanes at this stage. The filter was destained by repeated washing with PBS containing 0.2% Tween-20 (PBST). After the filter destained completely, it was incubated with 3% BSA made in PBST for 1 hour at room temperature. During this and in all the subsequent steps, incubation of the filter was performed on a rocker platform to ensure a uniform treatment. The filters were then incubated in the appropriate dilution of the primary antibody
for 3 hours at room temperature or O/N at 4°C. The excess antibody was washed by washing the filters with PBST for 15 minutes with at least three changes. The filters were subsequently incubated in the appropriate dilution of the second antibody for one hour at room temperature. Dilutions of the primary as well as secondary antibody were made in 3% BSA in PBST. Following incubation with the second antibody, the filters were washed vigorously with PBST for 5 minutes. The washing was repeated 5 - 6 times. Finally, the filters were washed twice in PBS and colour developed with DAB (0.5 mg/ml in PBS) containing 6 ul/10 ul of 30% H2O2. The colour reaction was stopped after 5 - 10 minutes by washing the filters with distilled water.

3.2.22. Computer analysis.

βhCG and HBsAg sequences were accessed from GenBank or NBRF database on a Microvax II computer and sequence analysis performed using the HPLLOT and AMPHI programmes. HPLLOT is based on the algorithm of Kyte and Doolittle (1982) and plots the hydrophobic and hydrophilic segments of the protein by scanning the whole length of the sequence in blocks of a few residues. The window size used was 6 amino acids. AMPHI is based on the algorithm of Margalit et al., (1985) and predicts the amphipathic segments of the protein which correspond to alpha helices and are therefore, likely candidates for being T cell epitopes.