CHAPTER 4.

RESULTS
4.1. Construction and characterisation of mammalian expression plasmids carrying hCG subunit cDNAs.

4.1.1. Structure of pHpAPr-1-neo.

A mammalian expression vector pHpAPr-1-neo (Gunning et al., 1987), containing the human β-actin promoter linked to unique restriction sites and a simian virus 40 (SV40) polyadenylation signal, was used for cloning hCG subunit cDNAs. Briefly, this vector consists of 3 kilobases of human β-actin gene 5' flanking sequence, plus 5' untranslated region and intervening sequence 1. This is linked at the 3' splice site to a short DNA polylinker sequence containing unique Sal I, Hind III, and BamH I restriction endonuclease sites followed by simian virus 40 (SV40) polyadenylation signal (Fig. 2). In addition to the ampicillin resistance gene (amp<sup>R</sup>), which enables the maintenance of a selection pressure on the transformed E.coli cells harboring this plasmid, the vector also contains the bacterial neomycin-resistant gene (neo) linked to the SV40 origin of replication plus early promoter, to allow for the selection of cells that integrate this vector into their genomes in a stable manner. Purified vector DNA was digested to completion with restriction endonuclease Hind III, and the digest resolved on a 1% SeaKem agarose gel (Fig. 3 A). Since the vector carries a unique Hind III site, a linearised DNA band of approximately 10 kb was seen on the gel, as expected.

After ascertaining the complete linearisation of the
Figure 2. Structure of the mammalian expression vector pHβAPr-1-neo used in the present study.

The vector contains the 4.3 kb EcoR I - Alu I fragment from the β-actin gene isolate pl4TP-17 (Leavitt et al., 1984), which carries 3 kb of human β-actin 5' flanking sequence including the cap site, plus 78 bp of 5' UTR and 832 bp of IVS1 (hatched region). This is linked at the 3' splice site to a short DNA polylinker sequence containing unique Sal I, Hind III and BamH I recognition sites. The region spanning bp 4320 - 6600 is derived from pcDV1 (Okayama and Berg, 1983) and contains the pBR322 Amp\(^\text{R}\) gene and bacterial origin (ori) plus the SV40 late region polyadenylation signal (Poly A). Nucleotides 6600 - 10,000 is the Pvu II - EcoR I fragment from pSV\(_2\)-neo (Southern and Berg, 1982) containing the bacterial neo gene linked to the SV40 ori plus early promoter (SV\(_2\)-neo). Thin arrows indicate the direction of transcription. Numbers inside the circle indicate the nucleotide positions with respect to the unique EcoR I site taken as 1.
EcoR1

pHBAPr-1-neo

Human B-Actin Promoter

Cap site 5'UTR

SV2-neo

10,000/1

10,000/1

6600

IVS1

AmpR

Ori

NdeI

Poly A

Bam H1

Sal I

Acc I

Hind III

5'UTR
Figure 3. Agarose gel electrophoresis of pHPr-1-neo vector DNA digested with Hind III.

Purified vector pHPr-1-neo DNA was digested to completion with restriction endonuclease Hind III and an aliquot was resolved on 1% SeaKem agarose gel (panel A, lane 2). Undigested pHPr-1-neo DNA was run in parallel as control (panel A, lane 1). A single band of approximate size 10 kb (panel A, lane 2) indicated the complete linearisation of the vector DNA. The linearised vector DNA, after dephosphorylation, was purified by electrophoresis on a preparative 1% SeaPlaque agarose gel (panel B, lane 1). The 10 kb band (marked by an arrow) corresponding to the linearised DNA was cut out and used for elution of DNA. The rest of the gel from which the 10 kb DNA band was cut out (lane 1), is shown in panel C. Molecular weight standards consisted of either a Hind III digest of lambda DNA (panels B and C, lane 2), or a mixture of this and Hae III digest of bacteriophage phiX174 RF DNA (panel A, lane 3). Numbers on the right indicate the size of DNA fragments in base pairs. The gels were stained with ethidium bromide and visualised on a UV transilluminator.
vector DNA, the Hind III digest was treated with bacterial alkaline phosphatase (BAP), to remove the terminal 5' phosphates from the sticky ends created by Hind III. This dephosphorylation by treatment with BAP prevents re-circularisation via the sticky ends, of the vector DNA, which might otherwise lead to a high background of non-recombinants during cloning. The Hind III digested and dephosphorylated vector DNA was electrophoresed on a 1% SeaPlaque agarose gel to resolve any remaining traces of the uncut vector DNA (Fig. 3 B). The 10 kb band corresponding to the linearised vector DNA was cut out (Fig. 3 C) and DNA eluted, as described in the methods section. The re-circularisation efficiency of the vector DNA prepared in this manner was checked by setting up a self-ligation of this DNA using T4 DNA ligase. The self ligated vector DNA was then used to transform competent E.coli cells, the transformants plated on amp plates and incubated O/N at 37°C. No colonies were obtained. This indicated that the vector DNA had been dephosphorylated efficiently and was free of any undigested vector DNA molecules. This DNA could then be used for cloning the gonadotropin cDNAs.

4.1.2. Cloning of cDNAs encoding alpha (α) and beta (β) subunits of hCG into pHBAPr-1-neo.

The strategy used for cloning cDNAs encoding alpha hCG and beta hCG respectively, into pHBAPr-1-neo vector is outlined in Fig. 4.
Figure 4. Schematic outline of the strategy used to clone alpha and beta hCG cDNAs in pHJAPr-1-neo vector.

Plasmids pBR-α hCG and pBR-β hCG were cut at Hind III sites (H) to isolate cDNAs encoding alpha and beta subunits, respectively, of hCG. The cDNAs (hatched) for alpha hCG (621 bp) and beta hCG (539 bp) with Hind III sticky ends (H) were purified by preparative agarose gel electrophoresis. Similarly, the vector pHJAPr-1-neo (pJ3ACT) was linearised at the unique Hind III site (H), followed by treatment with bacterial alkaline phosphatase (BAP). The linearised, dephosphorylated vector DNA was purified and ligated to hCG subunit cDNAs, and used to transform competent E.coli HB101 cells. The transformants were plated on ampicillin plates and ampicillin resistant colonies screened with radiolabelled alpha or beta hCG cDNA probes. Clones hybridising to the respective probes were isolated and grown in 3 ml cultures. Miniprep DNA isolated from the recombinants was analysed by digestion with appropriate restriction endonucleases to determine the presence as well as the orientation of the cloned insert. Finally, recombinant clones pAM1 and pAM2 which carried alpha and beta hCG subunit cDNAs, respectively, cloned in the correct orientation with respect to the β-actin promoter, were selected for further studies. Stippled region indicates the neo gene cloned under the SV40 promoter (SV2-neo). Positions of ampicillin resistance gene (AmpR) and the SV40 polyadenylation sequence (poly A) are also indicated. Arrows inside the circle indicate the direction of transcription.
Hind III
Purify α-hCG cDNA

VECTOR

Purify linearised vector DNA

pBR-α hCG

LIGATE

Transform E. coli HB101

Probe: α-hCG
Screen AmpR colonies with 32P-labelled probe
Pick up +ve (hybridisation signal) clones
Restriction analysis of DNA to determine orientation of insert

Hind III
BAP

pBR-β hCG

LIGATE

pβACT

521 bp

539 bp

539 bp

pAM-1

pAM-2

SV2-neo

β-actin Pr.

AmpR

Poly A

α-hCG

β-hCG

SV2-neo

β-actin Pr.

AmpR

Poly A

β-hCG
4.1.2.1. **Isolation of hCG subunit cDNAs.**

The cDNA encoding the alpha subunit of hCG (alpha hCG) is approximately 621 bp in size (Fiddes and Goodman, 1979). Plasmid pBR - alpha hCG carries the full length coding region from this cDNA, cloned at the Hind III site in pBR322 vector (Bolivar et al., 1977), with the use of Hind III linkers. Thus, the complete cDNA for alpha hCG can be excised out of pBR - alpha hCG as a Hind III fragment. This construct (pBR - alpha hCG) was made available in our laboratory and was used as the source for alpha hCG cDNA, for cloning into the pH~APr-1-neo vector.

*E.coli* HB101 cells transformed with pBR - alpha hCG DNA were grown and plasmid DNA isolated as described in the methods section. Purified pBR - alpha hCG DNA was digested to completion with restriction endonuclease Hind III, and an aliquot of the reaction mixture was resolved on a 1 % agarose gel (Fig. 5 A) to monitor the extent of digestion. As expected, the alpha hCG cDNA was released as an approximately 600 bp fragment, with Hind III sticky ends. After ascertaining the complete digestion of pBR - alpha hCG DNA, the entire Hind III digest was loaded on a 1 % SeaPlaque agarose gel to separate out the fragment of interest from the vector DNA (Fig. 5 B). This fragment was cut out, DNA eluted and used for setting up ligation with the Hind III linearised vector (described in 4.1.1).

The cDNA encoding the beta subunit of hCG (Beta hCG) is approximately 539 bp long (Fiddes and Goodman, 1980).
Purified plasmid pBR-α hCG DNA was digested to completion with restriction endonuclease Hind III and an aliquot was resolved on a 1% agarose gel (panel A, lane 2). Undigested plasmid pBR-α hCG DNA was run in parallel as control (panel A, lane 1). Appearance of an approximately 600 bp fragment corresponding to the α hCG cDNA in the Hind III digest of pBR-α hCG (panel A, lane 2, opposite to the arrow), indicated the complete digestion of this plasmid. The entire Hind III digest was then loaded on a preparative 1% SeaPlaque agarose gel to purify the desired 600 bp fragment of α hCG cDNA (panel B, lane 2). Molecular weight standards consisted of a Hind III digest of lambda DNA (panel A, lane 3; panel B, lane 1). Numbers in the centre indicate the size of DNA fragments in kilobase pairs. The gels were stained with ethidium bromide and visualised on a UV transilluminator.
Figure 6. Agarose gel electrophoresis of plasmid pBR-βhCG DNA digested with Hind III.

Purified plasmid pBR-βhCG DNA was digested to completion with restriction endonuclease Hind III and an aliquot was resolved on a 1% agarose gel (panel A, lane 2). Undigested plasmid pBR-βhCG DNA was run in parallel as control (panel A, lane 1). Appearance of an approximately 500 bp fragment corresponding to the βhCG cDNA in the Hind III digest of pBR-βhCG (panel A, lane 2, opposite to the arrow), indicated the complete digestion of this plasmid. The entire Hind III digest was then loaded on a preparative 1% SeaPlaque agarose gel to purify the desired 500 bp fragment of βhCG cDNA (panel B, lane 1). Molecular weight standards consisted of a Hind III digest of lambda DNA (panel A, lane 3; panel B, lane 2). Numbers on the right indicate the size of DNA fragments in kilobase pairs. The gels were stained with ethidium bromide and visualised on a UV transilluminator.
Plasmid pBR-βhCG carries the full length coding region from this cDNA, cloned as a Hind III fragment in pBR322, with the use of Hind III linkers. Thus, as in case of alpha hCG, the complete coding region of βhCG cDNA could be excised as a Hind III fragment from pBR-βhCG (Fig. 6 A). The βhCG cDNA was isolated from pBR-βhCG in a manner analogous to that described above for the isolation of the alpha hCG cDNA (Fig. 6 B).

4.1.2.2. Identification of recombinants.

The purified alpha hCG and βhCG cDNAs were ligated to the Hind III cut, dephosphorylated pHBAPr-1-neo vector DNA using T₄ DNA ligase, following the standard procedure described earlier (see Methods section). An aliquot from each ligation reaction was used to transform competent E.coli HB101 cells and the transformants plated on amp plates. For each ligation reaction, the transformation products were plated on multiple agar plates and several hundred transformants were obtained in each case. After 0/N growth of the transformants, colony lifts were performed by overlaying nitrocellulose paper (NC) circles on the plates and gently peeling off the paper after it was wet thoroughly. All the bacterial colonies were thus transferred to the NC as a replica of the plate in which these were grown originally. The above colony lift filters were then processed for colony hybridisation as described in the Methods section.

The transformants obtained with alpha hCG ligation
were screened with a nick translated $^{32}$P-labelled alpha hCG cDNA as probe. The positive control consisted of pBR - alpha hCG DNA spotted in triplicate on a NC paper. The negative control also was spotted in triplicate, on a NC paper and consisted of pHAPr-1-neo DNA. The hybridisation was done at 68°C in 5 X SSPE in aqueous solution for 16 hours. The filters were washed with SSC upto 0.5 X containing 0.1 % SDS. The results of colony hybridisation are shown by a representative panel in Fig. 7. The positive control exhibited intense hybridisation (panel C), while no hybridisation was seen with the negative control, i.e., vector DNA, confirming the specificity of the hybridisation. A large number of transformants exhibited hybridisation signal with the alpha hCG probe (panel A). The hybridisation signals in these colonies indicated the presence of the alpha hCG insert in these recombinants. A few of the colonies exhibiting hybridisation were picked up for further analysis of the plasmid DNA contained in these colonies.

The transformants obtained with pHCG ligation were also screened by performing colony lifts, followed by hybridisation with a radioactive probe. The probe in this case was purified pHCG cDNA, which was nick translated using $^{32}$P-dCTP. The positive control for the hybridisation consisted of pBR - pHCG DNA spotted on a nitrocellulose paper while the negative control was the vector DNA alone spotted in a similar fashion. The hybridisation and washing
Figure 7. Colony hybridisation of recombinant clones carrying alpha hCG cDNA.

Following transformation of *E. coli* with alpha hCG cDNA ligated to pHAPr-1-neo vector, colony lifts of the transformants were performed as described in Methods. The DNA immobilised on the nitrocellulose was hybridised to 32p-labelled alpha hCG cDNA probe. The final wash of the filters was in 0.5 X SSC containing 0.1% SDS. Panel A shows the transformant colonies hybridising to the probe, thereby indicating the presence of cloned alpha hCG cDNA insert in these recombinants. The positive control consisted of pBR-. hCG DNA (panel C) spotted at three places on a nitrocellulose filter (marked with +), and exhibited intense hybridisation. The negative control consisted of pHAPr-1-neo DNA (panel B) spotted at three places on a nitrocellulose filter (marked with -), and exhibited no hybridisation.
Figure 8. Colony hybridisation of recombinant clones carrying beta hCG cDNA.

Following transformation of *E. coli* with \( \beta \text{hCG cDNA} \) ligated to pHBAPr-1-neo vector, colony lifts of the transformants were performed as described in Methods. The DNA immobilised on the nitrocellulose was hybridised to \(^{32}\text{P}\)-labelled \( \beta \text{hCG cDNA} \) probe. The final wash of the filters was in 0.5 X SSC containing 0.1 % SDS. Panel A shows the transformant colonies hybridising to the probe, thereby indicating the presence of cloned \( \beta \text{hCG cDNA} \) insert in these recombinants. The positive control consisted of pBR-\( \beta \text{hCG} \) DNA (panel B, lower half) spotted at three places on a nitrocellulose filter (marked with +), and exhibited intense hybridisation. The negative control consisted of pHBAPr-1-neo DNA (panel B, upper half) spotted at three places on a nitrocellulose filter (marked with -), and exhibited no hybridisation.
conditions were similar to those described above for alpha hCG transformants. Results of the colony hybridisation of βhCG transformants are shown by a representative panel in Fig. 8. As seen in case of the alpha hCG transformants, a large number of transformants were seen to hybridise to the βhCG probe (panel A). The positive control exhibited intense hybridisation (panel B, lower half) while the negative control did not show any hybridisation under similar conditions (panel B, upper half), indicating the authenticity of the above hybridisation signals. The results of the hybridisation experiment thus identified the recombinants carrying the cloned βhCG cDNA. A few of these colonies were picked up and DNA was isolated from them for further analysis, to verify the presence of the complete insert.

4.1.2.3. Analysis of the recombinants.

Based on the results of colony hybridisation, several transformants for alpha hCG as well as βhCG were picked up and grown in 1 ml LB cultures containing amp. Miniprep DNA was isolated from these clones. In addition, a few colonies which did not show any hybridisation, were also grown and their DNA isolated, to serve as the negative control. The miniprep DNA from alpha hCG transformants was electrophoresed on 0.8 % agarose gel in TAE buffer and the DNA from the negative control was also run in parallel. As seen in Fig. 9 A, the unrestricted DNA from the alpha hCG transformants migrated slightly slower (lanes 2-5 and
Figure 9. Agarose gel electrophoresis of DNA from pHpAPr-1-neo recombinants carrying cloned alpha hCG cDNA.

Plasmid DNA was isolated from transformants carrying alpha hCG cDNA cloned in pHpAPr-1-neo, using the miniprep method. In addition, miniprep DNA was also isolated from non-recombinant transformants, to serve as negative control. Unrestricted recombinant plasmid DNAs were electrophoresed on a 0.8% agarose gel (panel A, lanes 2 - 7 and 9 - 14) to compare their relative mobilities with the negative control (lane 8). Panel B shows Hind III digests of DNAs from two of the recombinants. In each case, Hind III digestion released an approximately 600 bp fragment (lanes 2 and 4, indicated by an arrow) which was not present in the corresponding unrestricted DNA (lanes 3 and 5) or in the negative control (lane 6). Molecular weight standards consisted of either a Hind III digest of lambda DNA (panel A, lane 1), or a mixture of this and Hae III digest of bacteriophage phi X 174 RF DNA (panel B, lane 1). Numbers on the left indicate the size of DNA fragments in kilobase pairs. The gels were stained with ethidium bromide and visualised on a UV transilluminator.
10 - 14) compared to that from the negative control (lane 8). Surprisingly, clones in lanes 6, 7 and 9 showed a very fast migrating DNA species. These clones were discarded from further investigation. The slower mobility observed in the rest of the clones was expected corresponding to the larger size of these clones and confirmed the presence of an insert in these clones. This was further verified by digesting the miniprep DNAs with Hind III for 2 hours at 37°C. The reaction was terminated as described in the Methods section and electrophoresed on a 1% agarose gel in TAE buffer. As seen in Fig. 9 B, Hind III digestion of the clones released a band of approximately 600 bp as expected (lanes 2 & 4). In case of the negative control (lane 6), no band corresponding to the insert DNA was seen. This confirmed that the clones picked up on the basis of colony hybridisation did indeed contain the alpha hCG cDNA inserted at the Hind III site of pHAPr-1-neo vector.

Next, these clones were analysed to ascertain the orientation of the alpha hCG insert with respect to the β-actin promoter. The vector pHAPr-1-neo had no site for Xba I and a single site for BamH I, while alpha hCG cDNA had no site for BamH I and a single site for Xba I at position 222 from the 5' end. Digestion of DNA from the recombinant plasmids carrying cloned alpha hCG cDNA, with either BamH I or Xba I was, therefore, expected to linearise the plasmid at 10.6 kb position. Double digestion of the recombinant carrying alpha hCG cDNA in the correct
Figure 10. Agarose gel electrophoresis of DNA digests from recombinant alpha hCG clones, to reveal the orientation of the cloned insert.

Miniprep DNA isolated from five recombinant alpha hCG clones was double digested with BamHI and XbaI, and the digests resolved on 0.8% agarose gel (lanes 2, 3, and 5-7). In addition, pHBAFr-1-neo DNA was digested with the same enzymes to serve as negative control (lane 1). All five recombinants upon digestion released approximately 400 bp fragment, as expected from alpha hCG cloned in the correct orientation. No such band was seen in the digest of the vector DNA (lane 1). Molecular weight standards (lane 4) consisted of a Hind III digest of lambda DNA mixed with a Hae III digest of bacteriophage phi X174 RF DNA. Numbers on the right indicate the size of DNA fragments in kilobase pairs. The gel was stained with ethidium bromide and visualised on a UV transilluminator.
orientation, with BamH I and Xba I would release a 398 bp fragment from the alpha hCG cDNA, whereas in the reverse orientation this fragment would be only 222 bp long. Plasmid DNA from five representative recombinants was double digested with BamH I and Xba I and resolved on a 0.8% agarose gel. In addition, pHAPr-1-neo DNA was double digested with the same enzymes to serve as the negative control. As seen in Fig. 10, all the recombinants exhibited a band of approximate size 400 bp (lanes 2, 3, and 5 - 7). No such band was seen in case of the vector DNA (lane 1). This indicated that this band was released from the alpha hCG cDNA and that all these recombinants carried the alpha hCG cDNA cloned in the correct orientation with respect to the promoter.

Of the several clones found to carry alpha hCG insert in the correct orientation, one clone, hereafter designated as pAM1, was randomly selected for further restriction analysis (Fig. 11 A), followed by Southern blot hybridisation (Fig. 11 B), to further confirm the orientation of the inserted cDNA. Nco I had two sites in the neo region on pHAPr-1-neo vector DNA, and Nco I digestion of the vector DNA released two fragments of approximate sizes 9 kb and 1 kb (lane 1). Since the multiple cloning site was located on the 9 kb Nco I fragment, cloning of alpha hCG cDNA at the Hind III site (as evident by release of the 600 bp alpha hCG cDNA from pAM1 upon Hind III digestion, seen in lane 7) was expected to cause an increase in the size of this fragment. This was indeed the case, as shown by
Figure 11. Southern blot hybridisation of restriction endonuclease digests of pAM1 DNA, hybridised with alpha hCG cDNA probe.

Panel A: Restriction endonuclease digests of pAM1 were electrophoresed on a 0.8% agarose gel, stained with ethidium bromide and photographed on a UV transilluminator. Lanes 1 - 9: pHBAPr-1-neo DNA digested with Nco I (lane 1), pAM1 DNA digested with Nco I (lane 2), BamH I (lane 3), Xba I (lane 5), Xba I + BamH I (lane 6), Hind III (lane 7), unrestricted pAM1 DNA (lane 9). Hind III fragments of lambda DNA mixed with Hae III fragments of phi X 174 DNA were used as size markers (lane 4). Lane 8 is blank. Numbers on the right indicate size in kilobase pairs.

Panel B: Autoradiogram of the gel shown in panel A, following hybridisation with radiolabelled alpha hCG cDNA as probe.
Nco I digest of pAM1 DNA (lane 2), exhibiting a 9.6 kb band, besides the other 1 kb fragment. Further, as described above, digestion of pAM1 with BamH I (lane 3) and with Xba I (lane 5) was expected to linearise the plasmid at 10.6 kb position. Finally, double digestion of pAM1 DNA with Xba I and BamH I (lane 6) was expected to release a 398 bp fragment from alpha hCG cDNA when cloned in the correct orientation, the remainder of the alpha hCG cDNA remaining attached to the 10 kb vector DNA.

The gel shown in Fig. 11 A, was capillary blotted on to GeneScreen membrane for Southern blot hybridisation. Purified alpha hCG cDNA nick-translated to a specific activity of 4.9 x 10^7 cpm / ug, was used as probe. Pre-hybridisation and hybridisation was performed as described in the Methods section. The filter was washed upto a maximum stringency of 0.5 X SSC, 1 % SDS at 55°C for 1 hour. Fig. 11 B shows the autoradiogram obtained following O/N exposure of the filter at -70°C. All restriction fragments carrying the alpha hCG cDNA or a portion thereof, were seen to hybridise to the probe at the expected positions (lane 2, 9.6 kb; lane 3, 10.6 kb; lane 5, 10.6 kb; lane 6, 10.2 kb and 0.4 kb; lane 7, 0.6 kb). No hybridisation signal was observed in case of the negative control (lane 1), DNA size markers (lane 4), or with other fragments from pAM1 DNA devoid of alpha hCG cDNA, thereby establishing the specificity of the hybridisation. Results of Southern blot hybridisation thus, were in concordance with the restriction pattern observed in
Fig. 11 A, and confirmed the correct design of this construct. On the basis of all the above evidence, it was concluded that pAM1 construct carried alpha hCG cDNA cloned in the correct orientation with respect to the β-actin promoter and could be used for expression studies.

As in the case of alpha hCG recombinants described above, plasmid DNAs (Fig. 12) were isolated from βhCG transformants selected on the basis of colony hybridisation. When electrophoresed on a 0.8% agarose gel (panel A), these exhibited slower mobility (lanes 5 - 8) compared to that seen in the negative control (lane 2). These clones were selected for further analysis. Plasmid DNAs from two other recombinants (lanes 3 & 4) exhibited the same mobility as seen in the negative control while in lane 1, an unusually fast migrating DNA species was observed. These clones were discarded. Further, upon Hind III digestion (panel B), the selected clones released an insert of approximately 500 bp as expected (lanes 2, 4 & 11). The control DNA (lanes 7 & 9) did not release any such insert upon Hind III digestion (lanes 6 & 8) nor was it seen in the unrestricted DNA from the selected clones (lanes 1, 3 & 10). That this 500 bp fragment corresponded to the βhCG cDNA was confirmed by running a Hind III digest of pBR-βhCG in parallel (lane 12), which released the 500 bp βhCG cDNA insert. This confirmed that βhCG cDNA had been cloned at the Hind III site in the pHBAPl-neo vector.

Several of these clones were analysed by restriction
Figure 12. Agarose gel electrophoresis of DNA from pHAPr-1-neo recombinants carrying cloned pHCG cDNA.

Plasmid DNA was isolated from transformants carrying pHCG cDNA cloned in pHAPr-1-neo, using the miniprep method. In addition, miniprep DNA was also isolated from non-recombinant transformants, to serve as negative control. Unrestricted recombinant plasmid DNAs were electrophoresed on a 0.8% agarose gel (panel A, lanes 1 and 3-7) to compare their relative mobilities with the negative control (lane 2). Panel B shows Hind III digests of DNAs from three of the recombinants along with those from negative controls. In each of the recombinants, Hind III digestion released an approximately 500 bp fragment (lanes 2, 4 and 11, indicated by an arrow) which was not present in the corresponding unrestrained DNA (lanes 1, 3 and 10) or in the negative control, before (lanes 7 and 9) or after digestion with Hind III (lanes 6 and 8). A Hind III digest of pBR-pHCG DNA was also run as positive control (lane 12), and confirmed the position of pHCG cDNA released as a 500 bp fragment. Molecular weight standards consisted of either a Hind III digest of lambda DNA (panel A, lane 8), or a mixture of this and Hae III digest of bacteriophage phi X 174 RF DNA (panel B, lane 5). Numbers on the right indicate the size of DNA fragments in kilobase pairs. The gels were stained with ethidium bromide and visualised on a UV transilluminator.
Figure 13. Agarose gel electrophoresis of DNA digests from recombinant βhCG clones, to reveal the orientation of the cloned insert.

Miniprep DNA isolated from three recombinant βhCG clones was digested with Sma I, and the digests resolved on 1% agarose gel (lanes 2, 4 and 5). In addition, pHAPr-1-neo DNA was digested with the same enzyme to serve as negative control (lane 1). Two of the recombinants upon Sma I digestion released an approximately 800 bp fragment carrying part of βhCG cDNA (lanes 2 and 4), as expected from βhCG cloned in the correct orientation. No such band was seen in the digest of the vector DNA (lane 1), or in the third recombinant (lane 5) which appeared to have βhCG cDNA cloned in the reverse orientation. Molecular weight standards (lane 3) consisted of a Hind III digest of lambda DNA mixed with a Hae III digest of bacteriophage φX174 RF DNA. Numbers on the right indicate the size of DNA fragments in kilobase pairs. The gel was stained with ethidium bromide and visualised on a UV transilluminator.
digestions to check the orientation of cloned βhCG cDNA with respect to the β-actin promoter. βhCG cDNA had a single Sma I site located asymmetrically towards the 3' end at position 487. The vector DNA also had six sites for Sma I, and therefore, at first Sma I did not seem to be the ideal choice for doing the orientation analysis. However, lack of better alternatives determined this choice. Moreover, the multiple cloning site of the vector was located within the largest Sma I fragment of pHBAPr-1-neo. This, to some extent, eased the potentially difficult situation of multiple Sma I sites being present on the vector DNA. DNA from recombinant clones as well as the vector DNA, was digested with Sma I and the digestion products resolved on a 1 % agarose gel (Fig. 13). In case of the vector DNA (lane 1), Sma I digestion released four major fragments of approximate sizes 4.3 kb, 3.2 kb, 1.8 kb, and 0.64 kb. As mentioned above, insertion of βhCG cDNA as a Hind III fragment occurred in the largest Sma I fragment (4.3 kb), and therefore, the recombinants carrying βhCG cDNA exhibited an altered mobility of this fragment. With βhCG cDNA cloned in the correct orientation, the size of 4.3 kb fragment was expected to be reduced to 4.05 kb approximately, along with the appearance of an additional 813 bp fragment carrying most of the βhCG cDNA. In the reverse orientation, an additional fragment of 378 bp carrying 52 bp portion from the 3' end of βhCG cDNA was expected to be released. In addition, an increase in the size of the 4.2 kb fragment to about 4.5 kb was expected.
owing to the presence of an additional 487 bp from the \( \beta \)hCG cDNA. Sma I digests of DNA from representative clones 1 and 2 (lanes 2 and 4, respectively) revealed an additional fragment of approximately 813 bp, which was not seen in the negative control (vector DNA, lane 1) and presumably corresponded to the fragment carrying most of the \( \beta \)hCG cDNA. This indicated that clones 1 and 2 carried \( \beta \)hCG cDNA cloned in the correct orientation. Clone 3, however, exhibited a different restriction pattern following Sma I digestion. In this case, there was an increase in the size of the largest fragment from 4.2 kb to approximately 4.5 kb (lane 5), while there was no difference in the size of the other three fragments, as expected. In addition, no fragment corresponding to 813 bp was seen. The additional 378 bp fragment expected to arise in this case is not seen in this gel. This indicated that \( \beta \)hCG cDNA was cloned in the reverse orientation in clone 3. Thus, recombinant clones were identified carrying \( \beta \)hCG cDNA inserted in correct as well as reverse orientation. Of the several clones found to carry \( \beta \)hCG cDNA in the correct orientation, one clone designated pAM2, was randomly selected for further restriction analysis (Fig. 14A) followed by Southern hybridisation (Fig. 14B) to further confirm the orientation of the inserted cDNA. pAM2 DNA was digested (panel A) with BamH I (lane 2), Sma I (lane 4), and Sal I (lane 5) and run on a 1% agarose gel. \( p \)HPAPr-1-neo DNA digested with Sma I was used as the negative control (lane 1).
Figure 14. Southern blot hybridisation of restriction endonuclease digests of pAM2 DNA, hybridised with βhCG cDNA probe.

Panel A: Restriction endonuclease digests of pAM2 were electrophoresed on a 0.8% agarose gel, stained with ethidium bromide and photographed on a UV transilluminator. Lanes 1-7: pHBAPr-1-neo DNA digested with Sma I (lane 1), pAM2 DNA digested with BamH I (lane 2), Sma I (lane 4), Sal I (lane 5), unrestricted pAM2 DNA (lane 7). Lanes 2 and 5, and to some extent lane 4, showed incomplete digestion. Hind III fragments of lambda DNA mixed with Hae III fragments of phiX 174 DNA were used as size markers (lane 3). Lane 6 is blank. Numbers on the right indicate size in kilobase pairs.

Panel B: Autoradiogram of the gel shown in panel A, following hybridisation with radiolabelled βhCG cDNA as probe.
The Sma I digestion patterns of vector DNA and pAM2 DNA, respectively, were as described above. Digestion of pAM2 DNA with BamH I (lane 2) and Sal I (lane 5) was expected to linearise the plasmid since there were only single sites for these enzymes on the vector DNA and no sites on βhCG cDNA. Although these digestions appeared to be partial, a band corresponding to the linearised DNA was visible in each case. The gel shown in Fig. 14A was capillary blotted on to GeneScreen membrane for Southern blot hybridisation. Purified βhCG cDNA nick translated to a specific activity of 8.0 x 10^7 cpm/μg was used as probe. Pre-hybridisation, hybridisation and washing conditions were similar to those described for Southern blot with pAM1 DNA. Fig. 14B shows the autoradiogram obtained following 6 hours' exposure at -70°C. As expected, only two Sma I fragments (lane 4, 813 bp and 4.05 kb) hybridised to the probe, confirming that these carried portions of the cloned βhCG cDNA, as expected and also that the βhCG cDNA had been cloned in the correct orientation in this plasmid. None of the other fragments from pAM2 DNA, nor those from the vector DNA (lane 1) exhibited any hybridisation to the probe, proving that these did not carry βhCG cDNA. On the basis of above characterisation by restriction analysis followed by Southern blot hybridisation, it was confirmed that clone pAM2 carried βhCG cDNA cloned in the correct orientation and could be used for expression studies.
4.2. Construction and characterisation of a βhCG-HBsAg gene fusion.

Plasmid pSV₂S has been described previously (Michel et al., 1988) and carries the S gene of hepatitis B virus (HBV) cloned downstream from SV40 early promoter, in a mammalian expression vector (Fig. 15). The pre-S1 region is absent in this vector while the HBV sequences downstream of the S gene allow polyadenylation of HBsAg messenger RNA. The S gene cloned in pSV₂S was used to construct a chimaera with βhCG cDNA. The strategy used for making this fusion is outlined in Fig. 16.

4.2.1. Preparation of vector DNA.

Plasmid pSV₂S was digested to completion with restriction endonuclease Xba I which had a single site at position 245 in the HBV DNA and thus linearised the plasmid DNA. This DNA was then treated with DNA polymerase I large fragment (Klenow polymerase) and dNTPs, to fill-in the recessed 3'-ends, generating blunt ended DNA. The fill-in reaction was carried out as described (Maniatis et al., 1982) and monitored by including ³²P-dCTP in the mixture of dNTPs such that the radioactive label got incorporated into the formation of blunt ends. An aliquot from the fill-in reaction was electrophoresed on a very thin 1% agarose gel in TAE buffer along with Hind III digest of lambda DNA as size marker. After the run was over, the gel was stained with ethidium bromide and seen on a UV transilluminator (Fig. 17 panel A). A single band of

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Figure 15. Structure of HBsAg expression vector pSV₂S.

Plasmid pSV₂S (Michel et al., 1988) carries a portion of pBR322 DNA (pBR) including the beta lactamase gene (\textit{Amp}^\textit{R}), and a gene cassette (open box) for the expression of HBsAg. This cassette consists of a fragment of HBV DNA which includes the \textit{preS}₂ (pS₂) and \textit{S} regions of HBsAg, cloned downstream of the SV40 early promoter (SV40). The boundaries of the SV40 promoter and the \textit{preS}₂ regions are indicated inside the open box. Initiator (ATG) and termination (TAA) codons are indicated outside the open box. Arrows indicate the direction of transcription. Positions of Hind III and Xba I sites are also indicated outside the open box. Numbers in the centre indicate the size of the plasmid in base pairs.
Figure 16. Schematic outline of the strategy used to construct a βhCG-HBsAg gene fusion.

Plasmid pBR-βhCG (pβhCG) was digested with EcoR I and BamH I to release a fragment carrying the entire βhCG cDNA along with some flanking regions. This fragment, after purification, was further cut with Sau3A I followed by filling in of the recessed 3' ends with Klenow polymerase. Finally, following Hind III digestion, the larger fragment carrying the coding region of βhCG from a.a. 1 - 142, was purified. This had a Hind III sticky end at the 5' end of βhCG gene and a blunt end at the 3' end of the βhCG gene. Plasmid pSV2S was linearised with Xba I, filled in with Klenow, and recut with Hind III, to create a sticky end proximal to the 3' end of the SV40 promoter. The fragment carrying βhCG cDNA as described above, was ligated to this vector DNA, to obtain pSV2BS35 carrying the desired βhCG-HBsAg gene fusion. Nucleotide sequence of the fusion junction is indicated at the bottom, and was deduced from published nucleotide sequences for βhCG cDNA and HBV DNA (ayw subtype), respectively. Numbers under the triplets indicate the codon position in the native βhCG and HBsAg proteins, respectively. Translational start (ATG) and stop (TAA) codons are indicated inside the circle. Key restriction sites are also indicated; B, BamH I; H III, Hind III; R I, EcoR I, S3A, Sau3A I.
Purified plasmid pSV$_2$S DNA was digested to completion with Xba I to linearise the plasmid. The recessed 3' ends were filled in with Klenow polymerase and dNTPs which included $^{32}$P-dCTP, to label the blunt ends. An aliquot of the fill-in reaction was electrophoresed on a thin 1% agarose gel. Panel A shows the ethidium bromide staining pattern of this gel. The linearised pSV$_2$S DNA migrated as a 4.8 kb band (lane 2). The unrestricted plasmid DNA did not show any such band (lane 1). Panel B shows an autoradiogram of lane 2. The linearised band of 4.8 kb gave a signal (marked with an arrow), confirming the incorporation of radioactive dCTP in the blunt ends created in the vector DNA. Lane 3 shows the DNA size markers and consisted of Hind III fragments of lambda DNA along with Hae III fragments of phi X RF DNA. Numbers in the centre indicate the size of DNA fragments in kilobase pairs.
approximate size 4.8 kb, corresponding to the linearised pSV₂S DNA was seen (lane 2). Positions of various bands in the DNA size marker lane were marked by making small holes in the gel. The gel was then sealed in a plastic bag and exposed to an X-ray film at room temperature. The film was developed after 1 hour. Again a single band of about 4.8 kb size was seen in the autoradiogram and corresponded to the expected size of the linearised vector (panel B). This indicated that the recessed 3'-ends on the linearised vector DNA had been filled up and the radioactive dCTP had been incorporated. Thus, only after ensuring the filling in of the vector DNA, could the next step in the cloning scheme be attempted. The blunt ended vector DNA was precipitated with ammonium acetate and ethanol, and resuspended in TE. This DNA was then digested with Hind III to release a 197 bp fragment of HBV DNA which encoded the first 31 a.a. of HBsAg and also included the pre-S₂ region. The vector DNA thus had a Hind III sticky end created just downstream of the SV40 promoter while the distal end was blunt. This vector DNA was then purified by electrophoresing on an agarose gel to separate out the smaller fragment as well as other undesired contaminants. The desired vector DNA band was cut out from the gel and DNA eluted as described in the methods section.

4.2.2. Construction of plasmid pSV₂BH₃₅.

Plasmid pBR-βhCG DNA was digested with restriction endonucleases EcoR I and BamH I to release an approximately 914 bp fragment containing the βhCG cDNA. This fragment was
purified by electrophoresis on an agarose gel, to separate out the rest of the vector DNA. The EcoRI - BamHI fragment was further digested with Sau3A I which cut at a single site towards the 3'-end of the βhCG cDNA, releasing the cDNA fragment encoding a.a. 143 - 145 of βhCG along with the 3' flanking sequences, from the rest of βhCG cDNA. The Sau3A I digest was treated with Klenow polymerase to fill in the recessive 3'-ends to generate a blunt ended βhCG cDNA fragment, encoding a.a. 1 - 142. This was further digested with HindIII to release the βhCG cDNA as a 507 bp fragment with a HindIII sticky end at the 5'-end and a blunt end at the 3'-end. The purified βhCG cDNA was then ligated to the vector DNA prepared as described above. An aliquot of the ligation reaction was used to transform competent E.coli HB101 cells and the transformants plated on LB plates containing amp. The transformants were grown O/N in 1 ml LB/amp cultures and DNA isolated by the miniprep method. Several clones were found to carry the desired insert. Since this was a directional cloning, it was expected that most of the clones would have the insert in the correct orientation with respect to the promoter. Detailed characterisation of one of the clones, pSV2BS35, was undertaken by digestion with several diagnostic enzymes to verify the cleavage pattern. pSV2BS35 DNA had two sites for PstI, such that two fragments of sizes 2.8 kb and 2.3 kb, could be expected from the PstI digest. There were two sites for BamHI and digestion with BamHI was expected to release two fragments of sizes 4.19 kb
**Figure 18.** Agarose gel electrophoresis of restriction endonuclease digests of pSV₂BS35 DNA.

Plasmid pSV₂BS35 DNA was digested to completion with different restriction endonucleases, and the digestion products were resolved on 1% agarose gel, stained with ethidium bromide and visualised on a UV transilluminator (panel A). Some of the digestion products which are poorly reproduced in panel A are shown again in panel B. The digests of pSV₂BS35 DNA are: 1, Pst I; 3, Hind III (also in lane 3, panel B); 4, Hind III + BamH I (also in lane 2, panel B); 5, BamH I (also in lane 4, panel B). Hind III fragments of lambda DNA were used as size markers (panel A, lane 2; panel B, lane 1). Numbers in the centre indicate the size of DNA fragments in kilobase pairs.
and 912 bp, respectively. pSV₂BS35 DNA had only a single site for Hind III, so the plasmid would be linearised at 5.1 kb. Double digestion of the plasmid with Hind III and BamH I was, however, expected to release three fragments of approximate sizes 3.4 kb, 912 bp, and 748 bp, respectively. pSV₂BS35 DNA was digested with Pst I, BamH I + Hind III, Hind III, BamH I restriction endonucleases, and the digests resolved on 1 % agarose gel. As seen in Fig. 18 A, the cleavage pattern of pSV₂BS35 DNA with Pst I (lane 1), Hind III (lane 3), BamH I (lane 5) and BamH I + Hind III (lane 4), was in agreement with the expected cleavage pattern for this construct, as described above. Fig. 18 B reiterates some of the above digests to clearly show the smaller fragments, which are not so distinctly visible in panel A. The above restriction analysis confirmed that βhCG cDNA had been cloned downstream of the SV40 promoter and was contiguous with the region of HBV DNA following the Xba I site.
4.3. **Cloning of βhCG-HBsAg cassette under β-actin promoter.**

In order to use an alternate system to express the βhCG-HBsAg cassette, this gene fusion was cloned downstream of human β-actin promoter (using the vector described in section 4.1.1.) followed by transfection of the resulting construct into appropriate mammalian cell lines.

pHβAPr-1-neo vector had unique sites for Sal I, Hind III and BamH I which could be used for cloning purposes. However, it was not possible to excise the βhCG-HBsAg cassette from pSV₂βS₃₅ using these enzymes. This problem was circumvented by recourse to the polymerase chain reaction (PCR).

**4.3.1. Strategy for PCR cloning.**

The strategy used for accomplishing this PCR derived cloning is outlined in Fig. 19. Using appropriate primers, the desired target DNA (βhCG-HBsAg ORF) could be amplified selectively from the pSV₂βS₃₅ DNA template, thus effecting the excision of the desired fragment with convenient sticky ends. This fragment could then be easily cloned into a high copy number vector like pUC18. The resulting clone would serve as the source for preparing the insert DNA in bulk, which could then be easily cloned in pHβAPr-1-neo vector as a Sal I – Hind III fragment.

**4.3.2. PCR primer design.**

Two primers were designed to flank the βhCG-HBsAg ORF and included recognition sequences for Sal I (primer for the 5'-end of the ORF) and Hind III (primer for the 3'-end of
Figure 19. Schematic outline of the strategy used to clone βhCG-HBsAg gene fusion in pHAPr-1-neo vector.

A 1093 bp fragment of βhCG-HBsAg gene fusion, flanked by the two primers (shown outside the circle) carrying Sal I (5' end) and Hind III (3' end) recognition sequences, was amplified from pSV₂JS35 by PCR. The sequence of the fusion junction is indicated, with numbers under the triplets indicating the codon positions in the native βhCG and HBsAg proteins, respectively. The PCR amplified fragment was cut with Sal I and Hind III and cloned into pUC18, to generate pAM3. The βhCG-HBsAg gene fusion was isolated from pAM3 as a Sal I - Hind III fragment and cloned into pHAPr-1-neo, to generate pAM5. β-actin promoter (β-actin), polyadenylation sequence (AA), and the neo cassette (SV₂neo) are indicated. Arrows indicate the direction of transcription. SV40 promoter (stippled), lac Z region (shaded arrow), βhCG cDNA (hatched) and HBsAg gene (striped), are also indicated. ATG and TAA inside pSV₂JS35 indicate the initiator and terminator codons, respectively, of the βhCG-HBsAg ORF.
1. PCR with Soli + Hind III

2. Cut with Soli + Hind III

T4 DNA Ligase

1. Cut with Soli + Hind III

pUC18

lacZ

1. Cut with Soli + Hind III

T4 DNA Ligase

1. Cut with Soli + Hind III

pAM3

Hind III

Hind III

Hind III

Hind III

Hind III
the ORF) as overhangs. Nucleotide sequences of the two primers and their alignment with the target DNA, are shown in Fig. 20. The choice of restriction enzyme sites to be included in the two primers as overhangs was based on the following considerations. First, these sites did not appear in the target DNA to be amplified, while there was a single BamH I site present in the target DNA. Second, these sites occurred as cloning sites in the pH3BAPr-1-neo vector. Third, use of different sites at the two ends of target DNA permitted directional cloning of the amplified DNA, thereby facilitating the isolation of the desired recombinants. The portion of HBV sequence 3' to the translation termination codon (TAA) was not included in the target DNA to be amplified, in order to keep the size of target DNA as small as possible, for efficient amplification.

4.3.3. PCR amplification and fragment purification.

About 500 ng - 1 µg pSV2BS35 DNA was used for each PCR reaction as described in the Methods section. Results from two initial exploratory experiments to standardise PCR methodology, are shown in Fig. 21. Two types of the starting plasmid DNA, supercoiled or linearised, were tried for PCR (panel A) to evaluate their relative suitability for PCR amplification. Linearised plasmid DNA (lane 2) gave a slightly better amplification compared to the supercoiled form of the same plasmid DNA (lane 3). Therefore, all subsequent PCRs were performed with linearised plasmid DNA. Another parameter that was standardised was the temperature
Figure 20. Primer design for PCR amplification of PhCG-HBsAg ORF.

23-mer oligomers were designed for PCR amplification of PhCG-HBsAg ORF from pSV2BS35. The top panel shows the double stranded sequence of part of the 5' end region of PhCG cDNA. The 5' primer was designed to be partly complementary (dotted vertical lines) to this region, starting from the initiator codon (ATG) of PhCG, which constituted the 5' end of the PhCG-HBsAg ORF. This primer also included at its 5' end, the recognition sequence for Sal I (underlined), as an overhang. The lower panel shows the double stranded sequence of part of the 3' end region of HBsAg coding region. The 3' primer was designed to be partly complementary (dotted vertical lines) to this region, ending at the termination codon (TAA) of HBsAg, which constituted the 3' end of the PhCG-HBsAg ORF. This primer also included at its 5' end, the recognition sequence for Hind III (underlined), as an overhang. The cleavage site for each restriction enzyme is indicated by an arrow. The 5' and 3' ends of the two primers are also indicated. Flanking regions of the target DNA are shown as dotted lines. In the upper panel, these consisted of SV40 DNA at the 5' end and HBsAg DNA at the 3' end. In the lower panel, these consisted of PhCG cDNA at the 5' end and pBR DNA at the 3' end.
**5'-PRIMER WITH SalI SITE**

\[
\text{SV40} \quad \text{SalI site}
\]

**3'-PRIMER WITH Hind III SITE**

\[
\begin{align*}
\text{Hind III site} \\
3'-\text{ACCCATATGTAATT TCGAACC - 5'} \\
\text{HBsAg}
\end{align*}
\]
Figure 21. PCR amplification using pSV2B35 DNA template and oligonucleotide primers with varying parameters.

Aliquots from a 100 microliter PCR mixture were applied to a 1% SeaKem agarose / 3% NuSieve agarose minigel for electrophoresis in TBE buffer, and the gel stained in ethidium bromide and visualised on a UV transilluminator. Panel A shows the efficiency of PCR with linearised (lane 2) versus supercoiled (lane 3) template DNA. Panels B and C show the specificity of PCR with annealing temperature 37°C (panel B, lane 1) compared with the annealing temperature of 42°C (panel C, lane 2). In each case, the major product of amplification is indicated by an arrow (the varying intensities of this band in panels B and C reflect different amounts of amplified DNA loaded in the two gels). Hae III fragments of phi X 174 DNA were used as size markers (panel A, lane 1; panel B, lane 2 and panel C, lane 1). Numbers on the left and between panels B and C, indicate the size of DNA fragments in base pairs.
used for primer annealing. Two sets of PCRs were done in parallel, with the primer annealing temperature as 37°C in one, while in the other, this was 42°C. Rest of the parameters were identical for these two reactions. At the annealing temperature of 37°C, the amplified DNA consisted of many bands other than the desired product of approximately 1 kb size (panel B, lane 1), whereas at the annealing temperature of 42°C, only the desired target DNA was specifically amplified (panel C, lane 2). The figure shown in panel C should not be taken to be indicative of lesser amplification occurring at the higher primer annealing temperature, but only reflects a lower amount of the amplified product loaded in this lane. Thus, at lower temperature, the primer annealing and the consequent target amplification may not be very specific and may include undesired products of amplification. These problems may be circumvented at a higher annealing temperature, without compromising the amplification yield. After having established the optimal conditions for efficient amplification of the desired target DNA, PCR was carried out using these conditions for 30 cycles, following which an aliquot of the PCR mix was electrophoresed on a 1% SeaKem / 3% NuSieve agarose gel (Fig. 22, panel A) to analyse the products of PCR. As seen in lanes 2 & 3, a very efficient and specific amplification of the desired 1095 bp target DNA was obtained. After ascertaining the amplification of the desired target DNA, the amplified DNA was digested
Figure 22. PCR amplification using pSV₂PS35 DNA template and oligonucleotide primers under standardised conditions.

Panel A: Five microliters from a 100 microliter PCR mixture was applied in duplicate (lanes 2 and 3) to a 1% SeaKem agarose/3% NuSieve agarose minigel for electrophoresis in TBE buffer, and the gel stained in ethidium bromide and visualised on a UV transilluminator. In each case, the major product of amplification was a 1.1 kb band (indicated by an arrow). Panels B and C: The PCR amplified DNA, after digestion with appropriate restriction enzymes, was purified on a preparative 1% agarose gel. Panel B (lane 1) and panel C (lane 2) show the products of separate PCRs for the same target DNA (arrow). Hae III fragments of phi X 174 DNA were used as size markers (panel A, lane 1; panel B, lane 2 and panel C, lane 1). Numbers on the left and between panels B and C, indicate the size of DNA fragments in base pairs.
with Hind III, purified as described in the methods section, and then digested with Sal I, to generate the corresponding sticky ends on the target DNA. Following this, the entire PCR mix digested with the two restriction enzymes, was loaded on a preparative agarose gel to purify the amplified DNA (panels B and C). The DNA eluted from the agarose gel was purified by phenol / chloroform extraction, followed by precipitation with ammonium acetate and ethanol. This DNA was then used for cloning into pUC18 vector.

4.3.4. Cloning of BhCG-HBsAg cassette in pUC18.

Plasmid pUC18 was selected as an intermediate vector for cloning the BhCG-HBsAg cassette for several reasons. First, pUC18 is a small, high copy number E.coli plasmid with multiple cloning sites, facilitating the directional cloning of foreign DNA. Second, pUC18 has a selectable marker (lac Z) which gets inactivated when foreign DNA is cloned into the polylinker region of this vector. Thus, scoring of recombinants following cloning into pUC18 is very simply accomplished by a visual colour screening, the recombinants being colourless while the wild type colonies being blue. This also precludes the need to perform more tedious screening procedures like colony hybridisation to identify the recombinants.

pUC18 DNA was digested with Hind III and Sal I enzymes, followed by purification of the linearised vector DNA by preparative agarose gel electrophoresis (Fig. 23). The vector DNA was not treated with any alkaline phosphatase.
Purified pUC18 DNA was double digested with restriction endonucleases Hind III and Sal I, to prepare the vector DNA for a directional cloning experiment. After ensuring the complete linearisation of the vector DNA, the entire DNA was loaded on a 1 % SeaPlaque agarose (low-melting) gel and electrophoresed O/N. The sample well was created by sealing several adjacent teeth of the comb with a tape. The gel was stained with ethidium bromide and photographed on a UV transilluminator (panel A, track 2). Panel B shows the same gel after the DNA of interest was cut out from the gel as a thin slice of agarose (track 2). Lane 1 in each panel shows the DNA size markers which consisted of Hae III fragments of phi X 174 DNA mixed with Hind III fragments of lambda DNA. Numbers on the left indicate the size of DNA fragments in kilobase pairs.

Figure 23. A representative preparative agarose gel electrophoresis of linearised DNA.
since the two sticky ends (Sal I and Hind III) created on the vector were incompatible and no recircularisation of the vector DNA was likely to occur. However, the vector DNA preparation was checked by self ligation, followed by transformation into E.coli, to assess the background if any. Following extended digestion with each of the above two enzymes, the purified vector DNA did not show any background in the transformation. This vector DNA was then used for ligation with the amplified \( \beta \)hCG-HBsAg cassette.

Because of the ease in cloning foreign DNA in pUC18, a few deviations from the usual cloning procedures were adopted to test simple but rapid cloning procedures. Accordingly, the linearised vector DNA band was cut out from the preparative agarose gel but the DNA was not eluted as usual. Instead, "in gel" ligations were set as described by Struhl (1987). The preparative gel was made using SeaPlaque agarose (FMC BioProducts, Rockland, USA) which is a low gelling/melting temperature agarose of high purity. The desired DNA band was cut from the agarose gel in a minimal volume, not exceeding 50 ul. The gel slice was melted at 70°C for 5 - 15 minutes. From this, an appropriate aliquot was transferred to another tube and mixed with the appropriate volume of \( \beta \)hCG-HBsAg DNA fragment. Care was taken to keep the total volume of the two fragments upto 10 ul. The mixture was allowed to cool to 37°C and 10 ul of ice cold, 2 X concentrated ligase buffer, containing T₄ DNA ligase enzyme, was added. The mixture was incubated at 15°C for 4 - 24 hours. During this time, the gel
slice resolidified but this did not inhibit the ligation. Finally, the gel slice was remelted at 70°C and diluted 25 fold with ice cold TCM buffer (10 mM Tris.HCl, pH 7.5, 10 mM MgCl₂, 10 mM CaCl₂). After this step, the gel slice did not resolidify. The molten, diluted gel slice was then used for transformation as usual. Using this method, the PCR amplified βhCG-HBsAg ORF was ligated to pUC18 vector and the ligated DNA used to transform E.coli cells, following which a large number of recombinants were obtained as white colonies.

4.3.5. Analysis of pUC18-βhCG-HBsAg recombinants.

Recombinant colonies scored on the basis of white colour were inoculated into 2 ml LB/amp medium and grown O/N. From this, 1 ml culture was used to isolate plasmid DNA by the miniprep procedure. Two blue colonies were also grown as negative control, and their DNA isolated. Fig. 24 A shows the unrestricted plasmid DNAs from pUC18 recombinants, electrophoresed on 1% agarose gel in TAE buffer. As expected, the plasmid DNA from the recombinants (lanes 2 & 3) migrated slowly compared to the negative control (lane 1). This indicated the presence of the βhCG-HBsAg insert in these clones. Upon digestion with Sal I and Hind III (panel B), these clones released the insert fragment of approximately 1 kb (lanes 4 & 6). No such band was observed in case of the negative control (lane 2) pUC18 DNA double digest (lane 8) or in any of the unrestricted plasmid DNAs (lanes 1, 5, 7 & 9). This confirmed that the recombinant clones selected on the basis of colour selection
Figure 24. Agarose gel electrophoresis of DNA from pUC18 recombinants carrying cloned βhCG - HBsAg gene fusion.

Plasmid DNA was isolated from recombinant transformants (white colonies) carrying βhCG-HBsAg gene fusion cloned in pUC18, using the miniprep method. In addition, miniprep DNA was also isolated from non-recombinant transformants (blue colonies), to serve as negative control. Unrestricted recombinant plasmid DNAs were electrophoresed on a 1% agarose gel (panel A, lanes 2 and 3) to compare their relative mobilities with the negative control (lane 1). Panel B shows Hind III + Sal I digests of DNAs from two of the pUC18-βhCG-HBsAg recombinants. In each case, double digestion released an approximately 1.1 kb fragment (lanes 4 and 6, indicated by an arrow) which was not present in the corresponding unrestricted DNA (lanes 5 and 7). The negative control (lane 1) did not show this 1.1 kb band after the double digestion (lane 2). An additional negative control, pUC18 DNA alone (lane 9) also did not show this band after double digestion with the above enzymes (lane 8). Molecular weight standards consisted of a Hind III digest of lambda DNA mixed with a Hae III digest of bacteriophage φX174 RF DNA (panel A, lane 4; panel B, lane 3). Numbers in the center indicate the size of DNA fragments in kilobase pairs. The gels were stained with ethidium bromide and visualised on a UV transilluminator.
Figure 25. Preparative agarose gel electrophoresis of Hind III + Sal I digested pHBAFr-1-neo and pAM3 plasmid DNAs.

Purified plasmid DNA was double digested with restriction endonucleases Hind III and Sal I, to prepare the pHBAFr-1-neo vector DNA (panel A, lane 1) for directional cloning of βhCG-HBsAg gene fusion released from the double digest of pAM3 DNA as a 1.1 kb fragment (panel A, lane 2). After ensuring the complete digestion of the two plasmid DNAs, the entire DNA in each case was loaded on a 1% SeaPlaque agarose (low-melting) gel and electrophoresed O/N. The gel was stained with ethidium bromide and photographed on a UV transilluminator. The DNA bands of interest are marked with arrows. Panel B shows the same gel after the DNA bands of interest were cut out from the gel. Lane 4 in each panel shows the DNA size markers which consisted of Hae III fragments of phi X 174 DNA mixed with Hind III fragments of lambda DNA. Numbers on the right indicate the size of DNA fragments in base pairs.
did indeed carry the desired insert. Since this was a forced orientation cloning, there was only one way for the insert to get cloned with respect to the promoter, that is, the correct orientation. Nevertheless, determination of orientation of the insert was of little importance in this case since the objective of the cloning was to obtain a clone from which the desired insert could subsequently be purified.

One of the pUC18-βhCG-HBsAg recombinants, pAM3, was selected for preparing plasmid DNA on a large scale. The plasmid DNA was purified by two density gradient centrifugations in CsCl - ethidium bromide solution. Finally, the purified DNA was digested to completion with Hind III followed by Sal I, and the digest electrophoresed on a preparative 1 % agarose gel in TAE buffer (Fig. 25). The βhCG-HBsAg cassette migrated as a 1 kb band (panel A, lane 2) and was cut out from the gel (panel B, lane 2) and DNA eluted as described in the Methods section.

4.3.6. Cloning of βhCG-HBsAg cassette in pHBAPr-1-neo vector.

pHBAPr-1-neo vector DNA was digested to completion successively with Hind III followed by Sal I. The linearised, double digested vector DNA was purified by preparative agarose gel electrophoresis (Fig. 25, panels A & B, lane 1). The purity of this DNA preparation (that is, the presence of single cut vector molecules) was checked by self ligation followed by transformation of competent E.coli cells. No transformants were obtained. This confirmed that
Figure 26. Colony hybridisation of recombinant \textit{pHpAPr-1-neo} clones carrying \textit{pHCG-HBsAg} gene fusion.

Following transformation of \textit{E.coli} with \textit{pHCG-HBsAg} gene fusion ligated to \textit{pHpAPr-1-neo} vector, colony lifts of the transformants were performed as described in Methods. The DNA immobilised on the nitrocellulose was hybridised to \textit{\textsuperscript{32}P}-labelled \textit{pHCG} cDNA probe. The final wash of the filters was in 0.5 X SSC containing 0.1 % SDS. Panel A shows representative transformant colonies hybridising to the probe, thereby indicating the presence of cloned \textit{pHCG-HBsAg} cassette in these recombinants. The positive control consisted of \textit{pBR-HbCG} DNA (panel B) spotted at three places on a nitrocellulose filter (marked with +), and exhibited intense hybridisation. The negative control consisted of \textit{pHpAPr-1-neo} DNA (panel B) spotted at three places on the same nitrocellulose filter (marked with -), and exhibited no hybridisation.
the vector DNA had been completely digested with both the enzymes and there was no recircularisation of the vector molecule. This vector DNA preparation was then used for setting up the ligation with purified \( \beta \text{hCG-HBsAg} \) insert DNA. The ligated DNA was used to transform competent \( E. \text{coli HB101} \) cells and the transformants plated on LB/amp plates. Screening of the transformants was done by colony hybridisation as described earlier. The probe used in this case consisted of \( ^{32} \text{P} \) labelled, nick translated \( \beta \text{hCG cDNA} \). The pre-hybridisation, hybridisation and washing conditions were the same as described in section 4.1. The positive and negative controls also were the same as described earlier. Results of the colony hybridisation are presented as a representative panel in Fig. 26. The positive control exhibited intense hybridisation, while the negative control did not show any hybridisation to the probe (panel B), confirming the specificity of hybridisation. A number of colonies were seen to hybridise to the probe (panel A). A few of these colonies were picked up and grown up in 2 ml LB/amp media for DNA isolation by the miniprep method. Also, a few colonies not hybridising with the probe, were included as negative control. Unrestricted plasmid DNA isolated from these clones was electrophoresed on 0.8 % agarose gel (Fig. 27 A). The DNA from the clones exhibiting hybridisation to the probe (lanes 2 - 6, and 9 - 14) migrated slowly compared to the negative control (lane 7) or the vector DNA (lane 8), thereby suggesting its bigger
Figure 27. Agarose gel electrophoresis of DNA from pHpAPr-1-neo recombinants carrying cloned βhCG-HBsAg gene fusion.

Plasmid DNA was isolated from recombinant transformants (colonies giving +ve hybridisation signal) carrying βhCG-HBsAg gene fusion cloned in pHpAPr-1-neo, using the miniprep method. In addition, miniprep DNA was also isolated from non-recombinant transformants (non-hybridising colonies), to serve as negative control. Unrestricted recombinant plasmid DNAs were electrophoresed on a 0.8% agarose gel (panel A, lanes 2-6 and 9-14) to compare their relative mobilities with the negative control (lane 7) or the vector DNA alone (lane 8). Panel B shows Hind III + Sal I digests of DNAs from two of the pHpAPr-1-neo/βhCG-HBsAg recombinants. In each case, double digestion released an approximately 1.1 kb fragment (lanes 2 and 4, indicated by an arrow) which was not present in the corresponding unrestricted DNA (lanes 1 and 3). The negative control (lane 6) did not show this 1.1 kb band after the double digestion. Molecular weight standards consisted of either a Hind III digest of lambda DNA (panel A, lane 1) or a mixture of this with a Hae III digest of bacteriophage phi X 174 RF DNA (panel B, lane 5). Numbers at the sides indicate the size of DNA fragments in kilobase pairs. The gels were stained with ethidium bromide and visualised on a UV transilluminator.
size due to the presence of the insert. Upon double
digestion with Hind III and Sal I (panel B), these
clones released the 1 kb insert (lanes 2 and 4), while
no such band was seen in the negative control (lane
6). This confirmed that the βhCG-HBsAg cassette had been
cloned as a Sal I - Hind III fragment in pHBAPr-1-neo vector.
As mentioned earlier, since this was a forced orientation
cloning, the insert was expected to be cloned in the correct
orientation with respect to the β-actin promoter.

One of the clones, pAM5, was selected for further
analysis.

4.3.7. Characterisation of pAM5.

Plasmid pAM5 DNA was characterised by detailed
restriction analysis and Southern blot hybridisation, to
further confirm the orientation of insert with respect to
the promoter (Fig. 28). pAM5 had single sites for EcoR
I, Sal I and Hind III. There was a BamH I site in the HBV
region of the βhCG-HBsAg ORF, such that in all, pAM5 had two
BamH I sites. Digestion of pAM5 DNA with the above single
cutters was expected to linearise the plasmid at
approximately 11.1 kb position, while with BamH I, a 345 bp
fragment was expected to be released, with the rest of the
DNA migrating at 10.75 kb position. Double digestion of pAM5
DNA with EcoR I and Sal I, was expected to release two
fragments of 6793 bp and 4300 bp, respectively, with the
larger fragment carrying the cloned insert DNA. Further,
double digestion of pAM5 DNA with BamH I and EcoR I, was
Figure 28. Southern blot hybridisation of restriction endonuclease digests of pAM5 DNA, hybridised with βhCG cDNA probe.

Panel A: Restriction endonuclease digests of pAM5 were electrophoresed on a 0.8% agarose gel. Lanes 1–9, pAM5 DNA digested with BamH I (lane 1), EcoRI + BamH I (lane 2), EcoRI (lane 3), EcoRI + Sal I (lane 4), Hind III + Sal I (lane 6), Sal I (lane 7), Hind III (lane 8), unrestricted pAM5 DNA (lane 9). Hind III fragments of lambda DNA were used as size markers (lane 5). Numbers on the right indicate size in kilobase pairs.

Panel B: Autoradiogram of the gel shown in panel A, following hybridisation with radiolabelled βhCG cDNA as probe.
expected to give three fragments of 5700 bp, 5048 bp and 345 bp, respectively. Of these, the 5048 bp fragment would carry the cloned insert, while the 345 bp fragment would only carry the HBV portion of the cloned insert, as described above. As seen in panel B, digestion of pAM5 DNA with either Hind III (lane 8), or Sal I (lane 7) linearised the vector, while double digestion with Hind III and Sal I released the 1 kb βhCG-HBsAg insert (lane 6), as expected. Further, digestion with EcoR I also linearised the vector (lane 3) while double digestion with EcoR I and Sal I gave two fragments of the expected size (lane 4). Single digestion with BamH I released a 345 bp fragment from pAM5 (lane 1), while double digestion with EcoR I and BamH I gave three fragments of the expected size (lane 2). Thus, restriction digestion pattern of pAM5 DNA conformed to the expected pattern with the βhCG-HBsAg insert cloned in the correct orientation in pHJAPr-1-neo.

The gel shown in Fig. 28 A, was Southern blotted and probed with nick translated,\textsuperscript{32}P labelled βhCG cDNA probe. The conditions for pre-hybridisation, hybridisation and washing were the same as described for Fig. 14. Fig. 28 B shows the results of this hybridisation. No hybridisation was observed with Hind III fragments of lambda DNA (lane 5), which served as the negative control, while intense hybridisation was obtained in case of unrestricted pAM5 DNA (lane 9), which served as the positive control. Specific hybridisation signal was obtained in all fragments carrying the
Figure 29. Southern blot hybridisation of restriction endonuclease digests of pAM5 DNA, hybridised with HBV DNA as probe.

Panel A: Restriction endonuclease digests of pAM5 were electrophoresed on a 0.8% agarose gel. Lanes 1-6, pAM5 DNA digested with Sal I (lane 1), EcoR I + BamH I (lane 2), EcoR I (lane 3), EcoR I + Sal I (lane 4), Hind III + Sal I (lane 6). Hind III fragments of lambda DNA were used as size markers (lane 5). Numbers on the right indicate size in kilobase pairs. Panel B: Autoradiogram of the gel shown in panel A, following hybridisation with radiolabelled HBV DNA as probe. Arrow at the bottom indicates the position of 345 bp fragment released from the HBV DNA cloned in pAM5, which was not seen by ethidium bromide staining but exhibited weak hybridisation to the probe, probably because of less DNA.
whole (lane 3, 7 & 8, 11.09 kb; lane 6, 1.09 kb), or subparts of the βhCG-HBsAg insert containing the βhCG sequence (lane 1, 10.75 kb; lane 2, 5.05 kb; lane 4, 6.79 kb). The Southern hybridisation pattern with the βhCG probe thus confirmed the restriction pattern seen after ethidium bromide staining, thereby further confirming the identity of this clone.

In a similar experiment (Fig. 29, panel A), pAM5 DNA was digested with Sal I (lane 1), BamHI and EcoRI (lane 2), EcoRI (lane 3), EcoRI and Sal I (lane 4), and Hind III and Sal I (lane 6) restriction endonucleases. Digestion pattern obtained in this case was the same as described above for Fig. 28 A. This gel was used for Southern blot hybridisation, using nick translated, $^{32}$P-labelled HBV DNA as probe. As seen in panel B, hybridisation was obtained with Hind III fragments of lambda DNA, which served as negative control. Intense hybridisation was seen in case of the 1.1 kb insert released from the vector DNA following digestion with Hind III and Sal I (lane 6). In other digests of pAM5 DNA, all those fragments which were earlier seen to hybridise to the βhCG probe (Fig. 28 B), also exhibited hybridisation to the HBV probe, thereby confirming the fact that these fragments carried sequences from HBV as well as βhCG DNAs. In addition, faint hybridisation was also seen corresponding to the 345 bp fragment released from pAM5 DNA following digestion with EcoRI and BamHI (lane 2, near the edge of the gel). This fragment was expected to be released from the
HBV DNA, and, therefore, did not hybridise to the βhCG probe (Fig. 28 B, lanes 2 & 3), but selectively hybridised to the HBV probe. The results of Southern blot hybridisations with the βhCG and HBV DNA probes, were thus complementary, and taken together, confirmed the orientation and restriction map of insert DNA in the construct pAM5. Having thus verified the structure of this construct, this clone could then be used to express the desired gene product in mammalian cells.
4.4. Generation of cell lines transfected with various plasmid constructs.

After the cloning of BhCG cDNA fused to HBsAg, downstream of the SV40 early promoter in the construct pSV₂BS35, or alternately, downstream of human ß-actin promoter in the construct pAM5, the next step was to check the expression of this gene fusion in mammalian cells. However, in order to establish optimal conditions for the expression of this gene product, it was imperative to first determine the relative merits of SV40 promoter versus the ß-actin promoter for the expression of BhCG-HBsAg gene fusion, prior to undertaking transfection experiments with mammalian cell lines. This could easily be accomplished by comparing the transient expression of BhCG driven by the two vectors in mammalian fibroblast cell lines, which are commonly used for these purposes.

4.4.1. Comparative transient expression studies on pSV₂BS35 and pAM5 constructs.

DEAE-dextran mediated transient transfections were performed into mouse L Mtk− cells, with pSV₂BS35 and pAM5 plasmid DNAs, under identical conditions. In each case, the transfection was performed in duplicate, using 0.5 x 10⁶ cells per 60 mm petri-dish, with 5 ug plasmid DNA used for each transfection. In addition, two dishes were used for mock transfection where no DNA was used. In all cases, the culture supernate was harvested 72 hours following transfection and assayed for BhCG activity by a competitive RIA. Results of
the RIA are shown in Fig. 30. No βhCG activity was detectable in
the mock transfected control, as expected. In case of
transfection with pSV₂βS35 DNA, only a low level of βhCG
activity was detectable in the culture supernate. In contrast
to this, transient transfection with pAM5 DNA was seen to
yield approximately two fold higher level of βhCG expression
in the culture supernate. Thus, β-actin promoter appeared to
have a better potential for driving optimal expression of the
cloned gene product, and could be used for further
experiments. The culture supernates from the above transient
transfections, were also tested for the presence of HBsAg by
RIA for HBsAg (Abbott Laboratories, USA). However, using
this assay, no HBsAg was detectable in the culture
supernates. The precise reason for the failure to detect
HBsAg in the culture supernates, even by using a highly
sensitive RIA, was not very clear at this stage. However,
this situation was resolved as discussed later.

Although βhCG expression from pAM5 was detectable in
mouse L Mtk^- cells as described above, this however, might
not be reflective of the ideal conditions required for the
high expression of this promoter / gene combination. In order
to further optimise the conditions for efficient expression
of the cloned gene product in a eukaryotic system, more
probing experiments were conducted to test the comparative
expression levels of pAM5 DNA in various mammalian cell
lines, in order to identify the cell line where maximal
expression of the desired gene product was obtained. This too

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Figure 39. Radioimmunoassay for βhCG expressed transiently in mouse LMtk<sup>-</sup> cells following transfection with either pSV<sub>2</sub>BS35 or pAM5 DNA.

Culture supernates were collected from mouse LMtk<sup>-</sup> cells after 72 hrs. following DEAE-dextran transfection with either of pSV<sub>2</sub>BS35 or pAM5 DNAs. In each case, the transfection was performed in duplicate. In addition, a mock transfection was performed in parallel, where no plasmid DNA was used, to serve as negative control. Aliquots of the culture supernates were used for the βhCG RIA in a competitive assay, as described in the methods section. Standard hCG (40 ng / ml) was used as positive control (+ve control) for RIA while plain culture media was used as the negative control (-ve control). The results plotted represent mean of percent inhibition values from replicate experiments.
was accomplished by performing transient expression studies of pAM5 plasmid in different cell lines, as described below.

4.4.2. **Transient expression of pAM5 in mammalian cell lines.**

Since in pAM5 construct, the desired gene-fusion was placed under the transcriptional control of a human gene promoter (that of β-actin gene), it was desirable to test this construct in different cell lines, including those of human origin, besides those from other species. For this purpose, the representative cell lines selected for transfection were: HeLa, FWIL, CHO-K1, mouse L Mtk−, NIH 3T3, and Rat-2. The major characteristics of these cell lines have been listed in **Table 1**. Plasmid pAM5 DNA was introduced into the above cell lines by DEAE-dextran mediated transfection as described in the Methods section. In addition, a mock transfection was performed for each cell line. This included all the ingredients used for other transfections, except the plasmid DNA. The culture supernates were harvested 72 hours following transfection and assayed for βhCG activity by RIA. The results are shown in **Fig. 31**. Very low βhCG activity was detected in 4 cell lines, namely, NIH 3T3, Rat-2, HeLa, and FWIL, while none of the mock transfected controls exhibited any βhCG activity. A higher level of βhCG activity was seen in mouse L Mtk− and CHO-K1 cells, with the highest activity obtained in case of CHO-K1 cells. Therefore, for the purposes of obtaining stable cell lines capable of expressing the cloned gene product on a long
Figure 31. Radioimmunoassay for βhCG expressed transiently in mammalian cells following transfection with pAM5 DNA.

Culture supernates were collected from various cell lines after 72 hrs. following DEAE-dextran transfection with pAM5 DNA. For each cell line, a mock transfection was performed in parallel, where no pAM5 DNA was used, to serve as negative control. Aliquots of the culture supernates were used for the βhCG RIA, as described in the methods section. Standard hCG was used in varying concentrations, as positive control (+ve control) for RIA while plain culture media was used as the negative control (-ve control). Levels of βhCG present in the samples were calculated based on the standard curve plotted for the different concentrations of the standard hCG control. The results plotted represent mean of replicate experiments.
\( \beta hCG \ (\text{ng} \times 10^2 / \text{ml}) \)

- + VE CONTROL
- CHO-K1
- MOCK TRANSFECTED CHO-K1
- NIH 3T3
- MOBILE TRANSFECTED NIH 3T3
- MOUSE LMtk⁻
- MOCK TRANSFECTED MOUSE LMtk⁻
- RAT-2
- MOCK TRANSFECTED RAT-2
- HeLa
- MOCK TRANSFECTED HeLa
- FWIL
- MOCK TRANSFECTED FWIL
- - VE CONTROL
<table>
<thead>
<tr>
<th>#</th>
<th>PARAMETER</th>
<th>CALCIUM PHOSPHATE COPRECIPITATION</th>
<th>LIPOFECTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Amount of plasmid DNA</td>
<td>10 ug</td>
<td>1 - 10 ug</td>
</tr>
<tr>
<td>2.</td>
<td>Quality of DNA</td>
<td>Highly pure DNA (twice run on cesium chloride density gradient)</td>
<td>Mini prep DNA -- Highly pure DNA</td>
</tr>
<tr>
<td>3.</td>
<td>Carrier</td>
<td>Sheared salmon sperm DNA</td>
<td>None</td>
</tr>
<tr>
<td>4.</td>
<td>G418 concentration</td>
<td>i) 400 ug / ml</td>
<td>i) 400 ug / ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ii) 500 ug / ml</td>
<td>ii) 500 ug / ml</td>
</tr>
</tbody>
</table>
term basis, it was decided to use CHO-K1 cells, for performing stable transfections with various constructs carrying the desired genes placed under the transcriptional control of human β-actin promoter.

4.4.3. Stable transfection of CHO-K1 cells.

The plasmids used for stable transfections of CHO-K1 cells were pAM1, pAM2 and pAM5. Mainly, two methods were used for performing stable transfections - using calcium phosphate or using lipofectin (DOTMA). The calcium phosphate procedure has been used conventionally and has generally low efficiency. This method was used initially to transfect pAM1 into CHO-K1 cells. Transfections with pAM2 DNA were done by the lipofection procedure. For comparison between the two transfection procedures, pAM5 DNA was transfected using both the procedures. In addition, several variations were introduced in different parameters in both the transfection procedures. The range of variation in the parameters employed in the transfections is shown in Table 5. Calcium phosphate coprecipitation was essentially performed with 10 μg of highly purified (purified twice on cesium chloride - ethidium bromide density gradient) plasmid DNA with the use of sheared salmon sperm DNA as carrier, to aid precipitation. On the other hand, lipofectin was used with DNA ranging in amount from 1 μg to 5.4 μg. The quality of DNA used ranged from highly purified (the same quality as that used for calcium phosphate coprecipitation) to crude DNA prepared by the miniprep.
TABLE 6.

NUMBER OF STABLE (neo^R) CLONES AND TRANSFECTION FREQUENCY (PERCENT) OBTAINED WITH VARIOUS CONSTRUCTS

<table>
<thead>
<tr>
<th>#</th>
<th>CLONED DNA</th>
<th>TRANSFECTION PROCEDURE</th>
<th>CALCIUM PHOSPHATE</th>
<th>LIPOFECTION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>G418 CONCENTRATION</td>
<td>G418 CONCENTRATION</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>400 ug/ml</td>
<td>500 ug/ml</td>
</tr>
<tr>
<td>1</td>
<td>pAM-1</td>
<td>3</td>
<td>(0.004)</td>
<td>(0.005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>2</td>
<td>pAM-2</td>
<td>N.D.</td>
<td>N.D.</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.238)</td>
</tr>
<tr>
<td>3</td>
<td>pAM-5</td>
<td>4</td>
<td>2</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.005)</td>
<td>(0.002)</td>
<td>(0.05)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.05)</td>
</tr>
<tr>
<td>4</td>
<td>pSV2BS35</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>No DNA</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

N.D. : Not done

Numbers in parentheses represent transfection frequency expressed as percent per microgram DNA used for transfection.
method. Also, no carrier DNA was used with lipofectin. As control in each case, CH0-K1 cells were transfected in an identical manner as those in the test sample, except that no plasmid DNA was included in the transfection mixture. Following either method for transfection, the cells were replated at lower densities in G418 containing medium for 2 - 3 weeks, to select for stable transfectants as described in the Methods section. As control for the neo selection, a parallel batch of CH0-K1 cells was transfected with pSV2BS35 DNA by the calcium phosphate method, and processed for G418 selection. Selection for neoR clones was performed at two G418 concentrations, 400 ug / ml and 500 ug / ml. The lower concentration of G418 had previously been determined to be lethal to all non - transformed CH0-K1 cells. The selection for neoR clones using G418, was carried out for over two to three weeks. After two weeks, no cells survived in the control CH0-K1 cells transfected with pSV2BS35 DNA, as expected. Results of transfection experiments with all three constructs, are presented in Table 6. In pAM1 transfection, 7 stable transfectants were isolated from 16,800 cells screened for G418 resistance. For pAM2 DNA, 32 stable clones were isolated from an equal number of cells used for selection. Thus, lipofectin appeared to be much more efficient compared to calcium phosphate for transfection. This was further confirmed by the transfection results obtained with pAM5 DNA. In this case, starting from 16,800 cells for G418 selection in each case, 47 stable
clones were obtained with lipofectin while only 6 stable clones were obtained with calcium phosphate transfection. This confirmed that the lipofection procedure was highly efficient for DNA transfection. The percent transfection frequency obtained in the three experiments is also compared in Table 6. Lipofectin appeared to be at least 10 fold more efficient compared to the conventional calcium phosphate procedure for pAM5 DNA transfection, while a still higher frequency (more than 50 fold higher compared to that seen with pAM5 DNA) was observed in case of pAM2 DNA.

After selecting the neo<sup>r</sup> clones from each transfection, the selected clones were serially passaged to establish stable cell lines transformed with each construct. The initial passages were performed in a medium containing G418 @ 400 ug / ml, till the stability of each clone was demonstrated. Following this, the stable clone could be propagated even in the absence of any G418 for a few generations, without risking the loss of the integrated plasmid DNA. All subsequent propagation of the stabilised clones could then be performed by only occasional inclusion of G418 in the culture media, after a few successive generations.

4.4.4. DNA analysis of cells transfected with pAM1.

In order to demonstrate the presence of pAM1 DNA in a stably transfected, neo<sup>r</sup> CHO-K1 cell line, Southern blot hybridisation was performed with the genomic DNA from this clone. Total genomic DNA was isolated from the clone 2G1,
Figure 32. Southern blot hybridisation of Kpn I digest of genomic DNA from 2Gl cell line, hybridised with alpha-hCG cDNA as probe.

Genomic DNA was isolated from a stable cell line 2Gl transfected with pAMl. Following digestion with Kpn I, this DNA was electrophoresed on a 0.7 % agarose gel (lane 4). As a negative control, genomic DNA isolated from wild type CHO cells was processed in a similar manner (lane 2). Hind III digest of pAMl DNA was used as the positive control (lane 1). Hind III fragments of lambda DNA were run as the size marker (lane 3). After the electrophoresis run was over, the gel was stained with ethidium bromide and visualised on a UV transilluminator, to mark the position of the marker bands in the gel. Following this, the gel was Southern blot hybridised to a probe consisting of radiolabelled alpha hCG cDNA. An autoradiogram of this filter developed after 72 hrs. exposure is shown. Numbers on the right indicate size in kilobase pairs corresponding to the position of the lambda marker bands.
obtained after transfection with pAM1 DNA. After complete digestion of this DNA with Kpn I, the digest was electrophoresed on a 0.7 % agarose gel. Genomic DNA was also isolated from wild type CHO-K1 cells and processed in an identical manner, as negative control. Also, a Hind III digest of pAM1 DNA, which released the 600 bp alpha hCG fragment, was run as the positive control. This gel was Southern blot hybridised ( using GeneScreen membrane ) to a probe consisting of purified alpha hCG cDNA, nick translated with $^{32}$P-dCTP to a specific activity of $4.9 \times 10^7$ cpm / ug DNA. The hybridisation was carried out in 6 x SSC, 1 % SDS at $65^\circ$C, for 24 hours, followed by washing with a maximum stringency of 2 x SSC, 1 % SDS at $65^\circ$C, for 90 minutes. Fig. 32 shows an autoradiogram of this filter following exposure for 72 hrs. at -70$^\circ$C with an intensifying screen. The probe was seen to hybridise to the 600 bp alpha hCG fragment released from pAM1 DNA, as expected ( lane 1 ). No hybridisation was seen with the wild type CHO DNA digest ( lane 2 ). This confirmed the specificity of hybridisation. In case of 2G1 genomic DNA digest, a single band of approximately 15 kb size exhibited hybridisation to the probe ( lane 4 ). This confirmed that the alpha hCG cDNA present on pAM1 DNA had got integrated into the genome of 2G1 cell line. Further, since there were no Kpn I sites present on the alpha hCG cDNA, and a single site occurred on the vector DNA, presence of a single hybridising band suggested the integration of alpha hCG cDNA into the cell genome had
occurred with little or no gross rearrangement.

4.4.5. DNA analysis of cells transfected with pAM2.

As described above, total genomic DNA was isolated from a stable neoR CHO cell line, AC1, obtained after transfection with pAM2 DNA. This was digested to completion with Hind III and electrophoresed on a 0.7 % agarose gel. The negative control was the same as described above for Fig. 32, while the positive control consisted of a Hind III digest of pAM2 DNA, which released the 500 bp βhCG cDNA insert. This gel was Southern blot hybridised to βhCG cDNA probe, prepared by nick translation with 32P-dCTP to a specific activity of 8 x 10⁷ cpm / ug DNA. The hybridisation, washing and exposure conditions were similar to those described for Fig. 32, and the results are shown in Fig. 33. The probe hybridised specifically to the 500 bp βhCG cDNA fragment ( lane 4 ) as expected, and did not show any hybridisation to the control CHO DNA digest ( lane 3 ), confirming the specificity of hybridisation. A single band was seen to hybridise in case of the AC1 DNA digest at approximately 500 bp position ( lane 2 ). This was expected since Hind III digestion of AC1 genomic DNA would release the βhCG cDNA from the site of integration of pAM2 DNA on the host chromosome. Although this experiment did not reveal the exact number of βhCG cDNA copies that may have got integrated into the host chromosome, it did suggest that no gross rearrangements had occurred in the βhCG cDNA following its integration into the host chromosome. This also indicated
Figure 33. Southern blot hybridisation of Hind III digest of genomic DNA from AC1 cell line, hybridised with βhCG cDNA as probe.

Genomic DNA was isolated from a stable cell line AC1 transfected with pAM2. Following digestion with Hind III, this DNA was electrophoresed on a 0.7 % agarose gel (lane 2). As negative control, genomic DNA isolated from wild type CHO cells was processed in a similar manner (lane 3). Hind III digest of pAM2 DNA was used as the positive control (lane 4). Hind III fragments of lambda DNA were run as the size marker (lane 1). After the electrophoresis run was over, the gel was stained with ethidium bromide and visualised on a UV transilluminator, to mark the position of the marker bands in the gel. Following this, the gel was Southern blot hybridised to a probe consisting of radiolabelled βhCG cDNA. An autoradiogram of this filter developed after 48 hrs. exposure is shown. Numbers on the right indicate size in kilobase pairs corresponding to the position of the lambda marker bands.
that the integrated cDNA, if functional in the host cell, should express the intact βhCG subunit rather than an aberrant recombinant protein.

4.4.6. DNA analysis of cells transfected with pAM5.

Total genomic DNA from AB5 cells was digested with different combinations of restriction enzymes and the digests were electrophoresed on a 0.7 % agarose gel. The positive control consisted of pAM5 DNA digested with Hind III and Sal I, to release the βhCG-HBsAg insert (lane 5). The negative control was the same as described for the above experiments. Southern blot hybridisation of this gel was performed using $^{32}$P - labelled βhCG cDNA as probe, with hybridisation and washing conditions being the same as described above. Fig. 34 shows the results obtained in this experiment. The probe hybridised specifically to the 1.1 kb βhCG-HBsAg fragment from pAM5 DNA, as expected (lane 5). No hybridisation was observed with CHO DNA (lane 6) or with the Hind III fragments of lambda DNA marker, affirming the specificity of hybridisation (lane 4). Hind III + Sal I digest of AB5 DNA showed a single band of approximately 1.1 kb (lane 1), indicating that this corresponded to the integrated copies of the βhCG-HBsAg sequence from pAM5 DNA. AB5 DNA digested with Kpn I exhibited two hybridising bands (lane 2). Since there was only one Kpn I site on pAM5 DNA, and there was no Kpn I site interrupting the βhCG-HBsAg gene fusion, the two hybridising bands suggested the presence of at least two copies of this
**Figure 34.** Southern blot hybridisation of restriction endonuclease digests of genomic DNA from AB5 cell line, hybridised with βhCG cDNA as probe.

Genomic DNA was isolated from a stable cell line AB5 transfected with pAM5. Following digestion with Hind III + Sal I (lane 1), Kpn I (lane 2), Hind III (lane 3), the resulting fragments were electrophoresed on a 0.7% agarose gel. As negative control, genomic DNA isolated from wild type CHO cells was digested with Hind III + Sal I and processed in a similar manner (lane 6). Hind III + Sal I digest of pAM5 DNA was used as the positive control (lane 5). Hind III fragments of lambda DNA were run as the size marker (lane 4). After the electrophoresis run was over, the gel was stained with ethidium bromide and visualised on a UV transilluminator, to mark the position of the marker bands in the gel. Following this, the gel was Southern blot hybridised to a probe consisting of radiolabelled βhCG cDNA. An autoradiogram of this filter developed after 48 hrs. exposure is shown. Numbers on the right indicate size in kilobase pairs corresponding to the position of the lambda marker bands.
insert in the AB5 genomic DNA. Similarly, Hind III digest of AB5 genomic DNA exhibited two bands of hybridisation (lane 3). This also confirmed the presence of at least two copies of this insert in the AB5 genomic DNA. The results of Southern blot hybridisation of AB5 genomic DNA thus indicated that at least two copies of \( \beta \text{hCG-HBsAg} \) gene fusion had integrated into this clone. These results also indicated that the \( \beta \text{hCG-HBsAg} \) DNA had not undergone any significant recombination in the genomic DNA, and was maintained intact. Therefore, these integrated copies of \( \beta \text{hCG-HBsAg} \) DNA were expected to be competent for expression.

4.4.7. Northern blot of total RNA from AB5 cells.

Total RNA was isolated from AB5 cells and electrophoresed on a 1.2 % agarose gel containing formaldehyde. As negative control, total RNA isolated from mock transfected CHO cells was also run in the same gel. The positive control consisted of a Hind III + Sal I digest of pAM5 DNA, which released the entire coding region of \( \beta \text{hCG-HBsAg} \) gene fusion of size 1.1 kb. A Taq I digest of phi X 174 RF DNA, filled - in with Klenow polymerase using \( ^{32} \text{P-dCTP} \), was used as the size marker in this gel (Fig. 35 A, lane 1). Northern blot hybridisation of this gel was performed using nick - translated, \( ^{32} \text{P-} \) labelled \( \beta \text{hCG cDNA} \) as probe. The hybridisation was performed as described in the methods section, for 24 hours. The filter was then washed with a stringency upto 1 x SSC, 1 % SDS at 60\(^{0}\)C, for 90 mins. This was followed by autoradiography at -70\(^{0}\)C for 72 hours.
Figure 35. Northern blot of total RNA from AB5 cell line hybridised to \( \beta \)hCG (panel A) and HBV (panel B) DNA probes.

Total RNA was isolated from AB5 cell line stably transfected with pAM5 DNA. Approximately 10 \( \mu \)g of this was electrophoresed on a 1.2% agarose-formaldehyde gel (panel A, lane 3; panel B, lane 2). As negative control, total RNA from wild type CHO cells was used (panel A, lane 2; panel B, lane 1). Hind III + Sal I digest of pAM5 DNA was used as the positive control (panel A, lane 4; panel B, lane 3). A Taq I digest of phiX 174 DNA, filled-in with Klenow polymerase using \( ^{32}P\)-dCTP, was used as the size marker for electrophoresis (panel A, lane 1; panel B, not shown). After the electrophoresis run was over, the gel was used for Northern blot and hybridised to a probe consisting of radiolabelled \( \beta \)hCG cDNA (panel A) or HBV DNA (panel B). Autoradiograms of the two filters are shown. Numbers on the left indicate size in base pairs corresponding to the position of the marker bands.
Fig. 35 A shows the results obtained in this experiment. As expected, the probe hybridised to the βhCG-HBsAg insert at approximately 1.1 kb position (lane 4). No hybridisation was seen with RNA from CHO cells (lane 2), confirming the specificity of hybridisation. In case of AB5 RNA, a single band of approximately 1.4 kb size, was visible at a slightly higher position compared to that seen in the positive control. This indicated that AB5 cells contained a mRNA species of about 1.4 kb, carrying the βhCG sequence. Thus, this 1.4 kb mRNA could correspond to the transcript produced from βhCG-HBsAg gene fusion. The increase in the size of the transcript compared to that of the βhCG-HBsAg coding region was expected due to the presence of 5’ untranslated region from the β-actin promoter, and also due to the presence of the poly A tail on the mRNA.

In a parallel experiment, total HBV DNA, nick-translated and labelled with $^{32}$P-dCTP, was used as probe for northern blot hybridisation (panel B). The positive (lane 3) and negative (lane 1) controls were the same as described above. Again, while no hybridisation was observed in the negative control, in case of RNA from AB5 cells, a band corresponding to approximately 1.4 kb was seen to hybridise to the probe (lane 2). This indicated that the 1.4 kb transcript carried HBsAg sequences. These results taken together with those obtained from the above northern blot experiment using βhCG cDNA as probe, confirmed that the 1.4 kb transcript from AB5 cells carried both βhCG and HBsAg.
coding sequences.

4.4.8. **Localisation of recombinant gene products in the mammalian cell lines.**

Following the isolation of neo\(^R\) cell lines transfected in a stable manner with various recombinant plasmid constructs, as described above, the next step was to determine whether the transfected recombinant plasmid was expressing the desired gene product in CHO-K1 cells. In order to demonstrate this, immunocytochemical localisation of the cloned gene product, was performed as described in the Methods section. Cells from clones 2G1 (transfected with pAM1) and AC1 (transfected with pAM2) were reacted with anti-alpha hCG and anti-\(\beta\)hCG antisera, respectively, followed by reaction with a second antibody conjugated to HRP, and finally the colour was developed using DAB. **Fig. 36** shows the localisation of recombinant alpha hCG in 2G1 cells. The staining was seen to be distributed throughout the cytoplasm in 2G1 cells, indicating that the antibodies against alpha hCG bound a protein present throughout the cytoplasm. The negative control in this case, consisted of CHO-K1 cells which were reacted with the same antiserum under identical conditions. However, the cells did not exhibit any staining in the negative control, confirming the specificity of the antibody binding seen in the cytoplasm of 2G1 cells. Another negative control consisted of 2G1 cells reacted only with the second antibody conjugated to HRP, to monitor the background peroxidase activity. However, only a negligible reactivity
Figure 36. Localisation of anti-alpha hCG antibodies on 2G1 cells by immunocytochemical staining with HRP.

Cells from the clone 2G1 stably transfected with pAM1 DNA, were reacted with a polyclonal antiserum against alpha hCG, as described in the methods section. The antibody binding was revealed by a secondary antibody conjugated to HRP, and colour was developed with DAB. Anti-alpha hCG antibody binding was seen to be distributed throughout the cytoplasm of 2G1 cells.
was observed in this case too. Thus, these results indicated that 2Gl cells abundantly expressed a recombinant protein which carried immunoreactive epitopes of alpha hCG. Similarly, in case of AC1 cells, binding of anti-βhCG antibodies was seen throughout the cytoplasm (Fig. 37), while no antibody binding was seen in case of the negative controls as described above for 2Gl cells. Thus, in this case too, the transfected cells were found to express the desired recombinant gene product with immunoreactive epitopes of βhCG.

In case of cells from clone AB5 (transfected with pAM5), the cells were reacted with either anti-βhCG antiserum or with anti-HBsAg antiserum, followed by reaction with the second antibody labelled with peroxidase (Fig. 38). Both anti-βhCG (panel A) and anti-HBsAg (panel B) were found to yield staining distributed throughout the cytoplasm in these cells. In addition, both the antibodies failed to exhibit any staining in the untransfected CHO-K1 cells, used as negative control. Also, AB5 cells did not show any reactivity to the second antibody alone. This suggested that AB5 cells expressed recombinant gene products which carried immunoreactive epitopes of βhCG and HBsAg.

In addition, indirect immunofluorescence was carried out on the AB5 cells, using a rabbit antiserum against HBsAg, followed by reaction with a second antiserum conjugated to fluorescein isothiocyanate (FITC). As seen with peroxidase
Figure 37. Localisation of anti-βhCG antibodies on AC1 cells by immunocytochemical staining with HRP.

Cells from the clone AC1 stably transfected with pAM2 DNA, were reacted with a polyclonal antiserum against βhCG, as described in the methods section. The antibody binding was revealed by a secondary antibody conjugated to HRP, and colour was developed with DAB. Anti-βhCG antibody binding was seen to be distributed throughout the cytoplasm of AC1 cells.
Figure 38. Localisation of anti-βhCG (panel A) and anti-HBsAg (panel B) antibodies on AB5 cells by immunocytochemical staining with HRP.

Cells from the clone AB5 stably transfected with pAM5 DNA, were reacted with either a polyclonal antiserum against βhCG (panel A) or a polyclonal antiserum against HBsAg (panel B), as described in the methods section. The antibody binding was revealed by a secondary antibody conjugated to HRP, and colour was developed with DAB. Primary antibody binding, in each case, was seen to be distributed throughout the cytoplasm of AB5 cells.
staining of the cells, the reactivity to this antiserum was uniformly distributed all over the cytoplasm in AB5 cells (Fig. 39). No fluorescence was observed in the control CHO-K1 cells treated with the same antiserum, or in the conjugate control. This again indicated the presence of recombinant antigen with HBsAg immunoreactive epitopes, being expressed in these cells.

The results of immunofluorescence taken together with those obtained from immunocytochemistry experiments, confirmed that AB5 cells transfected with pAM5 DNA, expressed cloned gene products bearing both $\beta$hCG and HBsAg epitopes. These results, however, did not indicate whether the immunodeterminants of both $\beta$hCG and HBsAg were present on a single gene product that was expressed by AB5 cells.

From the results of immunocytochemical staining of CHO-K1 cells transfected with recombinant plasmid constructs, it was clear that the stable transfectants expressed the cloned gene products, which were detectable in the cytoplasm of these cells, using appropriate antibodies. The next step was to determine whether these proteins were being secreted outside the cell, and to quantitate the levels of the expressed gene product in each case.
Figure 39. Localisation of anti-HBsAg antibodies on AB5 cells by immunofluorescence.

Cells from the clone AB5 stably transfected with pAM5 DNA, were reacted with a rabbit polyclonal antiserum against HBsAg, as described in the methods section. The antibody binding was revealed by a secondary antibody conjugated to FITC. Anti-HBsAg antibody binding was seen to be distributed throughout the cytoplasm of AB5 cells.
4.5. **Analysis of recombinant gene products expressed in mammalian cells.**

4.5.1. **RIA of recombinant hCG subunits secreted by stable cell lines.**

Supernates from stable clones grown in culture, were analysed by subunit specific RIAs, to measure the activity of the cloned gene products. Culture supernates from stable clones transfected with pAM1 DNA were assayed for alpha hCG activity by RIA, using a monoclonal antibody against alpha hCG. Fig. 40 shows RIA results of representative stable cell lines transfected with pAM1 DNA. Positive control consisted of purified total hCG, ranging in concentration from 0.03 - 1.0 ug / ml, while the negative control consisted of a supernate from CHO-K1 cells grown in culture. The clones were found to express variable levels of alpha hCG. The highest level of alpha hCG was expressed in clone 2G1 (Fig. 40 inset). Precise quantitation of the gene product was determined by taking into account the cell number and the time in culture, and based on these, this clone was found to secrete 371 ng alpha hCG / $10^6$ cells / 24 hours.

Similarly, culture supernates from stable clones transfected with pAM2 DNA, were assayed for beta hCG activity in a RIA using an anti-beta hCG monoclonal antibody. The positive control consisted of purified hCG, ranging in concentration from 1 - 40 ng / ml, while the negative control consisted of culture supernate from CHO-K1 cells. Fig. 41 shows the RIA results of a few representative cell lines transfected with
Figure 40. Radioimmunoassay for alpha hCG expression in CHO-K1 cells transfected with pAM1 DNA.

Culture supernates were collected from stable cell lines obtained after transfection with pAM1 DNA, and selection with G418. Aliquots from the culture supernates were tested in duplicate in a RIA using a monoclonal antibody against alpha hCG. In a typical experiment, 13,000 dpm of $^{125}$I-labelled hCG was allowed to bind with the monoclonal antibody to produce a $B_0$ of 4330 dpm, with non-specific binding of 490 dpm. The addition of known amounts of non-radioactive standard hCG (in the range of 1.5 ng - 50 ng) as positive control (+ve control), resulted in the inhibition of binding to give 519 dpm at the highest hCG concentration. Percent inhibition was expressed as $\left(\frac{100 - \text{bound counts}}{B_0} \times 100\right)$. Negative control (-ve control) consisted of culture media from CHO-K1 cells, and exhibited only a low background value. Inset shows the precise quantities of the recombinant alpha hCG expressed by various clones.
**Concentration of alpha hCG**

(ng / $10^6$ cells / 24 hrs.)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>2G1</td>
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</tr>
<tr>
<td>1E11</td>
<td>352</td>
</tr>
<tr>
<td>1F2</td>
<td>202</td>
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<tr>
<td>IG3</td>
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</tr>
<tr>
<td>BC6</td>
<td>178</td>
</tr>
<tr>
<td>BD1</td>
<td>154</td>
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pAM2 DNA. The clones were found to express variable levels of βhCG as estimated in the culture supernate by RIA (Fig. 41 inset). However, the expression level of βhCG was very low in all the clones, the highest level being 80 ng / 10^6 cells / 24 hours (clone AC1). The reason for low level expression of βhCG in these clones was not clear.

From the RIA results of culture supernates from the stable clones for hCG subunits, it was evident that the stable clones were secreting the recombinant gene product into the culture medium. In order to determine whether the total amount of the expressed gene product was secreted into the culture medium, or whether some fraction of the cloned gene product failed to get secreted outside the cell, intracellular concentrations of each subunit were determined in the above clones. For this, the cells were first pelleted, washed twice with PBS to completely get rid of the culture medium, and sonicated for 2 mins. The sonicate was cleared by centrifugation at 10,000g for 10 mins. and the supernate was used for RIA. In each case, approximately 85 % of the total amount of the cloned gene product expressed from a given clone, was found to be secreted outside the cell. The fraction remaining inside the cell probably represented the molecules of expressed gene product at an intermediate stage in the secretory pathway. Thus, it could be concluded that there was no major block in the secretion of the above cloned gene products from CHO-K1 cells. This also eliminated the possibility of a defect in the secretory pathway of pAM2.
Figure 41. Radioimmunoassay for βhCG expression in CHO-K1 cells transfected with pAM2 DNA.

Culture supernates were collected from stable cell lines obtained after transfection with pAM2 DNA, and selection with G418. Aliquots from the culture supernates were tested in duplicate in a RIA using a monoclonal antibody against βhCG. In a typical experiment, 10,000 dpm of $^{125}$I-labelled hCG was allowed to bind with the monoclonal antibody to produce a $B_0$ of 5201 dpm, with non-specific binding of 550 dpm. The addition of known amounts of non-radioactive standard hCG (in the range of 50 pg - 2 ng) as positive control (+ve control), resulted in the inhibition of binding to give 1153 dpm at the highest hCG concentration. Percent inhibition was expressed as \[100 - \left( \frac{\text{bound counts}}{B_0} \times 100 \right)\]. Negative control (-ve control) consisted of culture media from CHO-K1 cells, and exhibited only a low background value. Inset shows the precise quantities of the recombinant βhCG expressed by various clones.
Concentration of BHCG
(ng/10^6 cells/24 hrs.)

<table>
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<tr>
<th>Cell Line</th>
<th>Concentration</th>
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<tbody>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>AC4</td>
<td>26</td>
</tr>
<tr>
<td>AD2</td>
<td>&lt;15</td>
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**Percent Inhibition**

**Cell Lines**

CONTROL

CELL LINES
transfected cell lines, as being responsible for the low level of \( \beta \)hCG detected in the culture supernates from these clones. Thus, there was an obvious differential expression of the two subunits of hCG in the above clones, though the precise reason for this differential in the expression levels of the alpha and beta subunits remains unclear.

4.5.2. Receptor binding activity of the recombinant alpha and beta subunits of hCG.

Further characterisation of the recombinant subunits of hCG was done by determining whether the two subunits could associate to form a heterodimer proficient in binding hCG receptor. For this, culture supernate from 2G1 cells secreting alpha hCG was mixed with that from AC1 cells, which secreted \( \beta \)hCG. The product of association between these was then tested for binding to hCG receptor, in a competitive RIA. As shown in Fig. 4, the in vitro associated alpha and beta subunits of hCG, bound the hCG receptor, though only low levels of the receptor binding activity was seen. The two subunits, per se, did not show any receptor binding activity. This indicated that the two recombinant subunits were able to associate correctly, such that the resulting dimer retained the ability to bind hCG receptor. The low level of receptor binding activity seen in the above experiment also suggested that recombination of the two subunits was probably inefficient.

4.5.3. Biological activity of the recombinant subunits.

The dimer resulting from the in vitro reassociation of
Figure 42. Receptor binding activity of in vitro associated recombinant subunits of hCG.

Culture supernate from the cell line 2G1 (secreting recombinant alpha hCG) was mixed with an equal volume of culture supernate from the cell line ACL (secreting recombinant βhCG), as described in the methods section. The mixture as well as the individual supernates were then assayed in a competitive radioreceptor assay, for binding to the LH/hCG receptor. 50 ng of standard hCG (Std. hCG) was used as positive control for the receptor assay.
SOURCE OF hCG

PERCENT INHIBITION

Std. hCG 50 ng

2G1

AC1

2G1 + AC1
recombinant alpha and beta subunits, as described above, was tested for biological activity as measured by testosterone production following binding to the LH receptor on Leydig cells. As shown in Fig. 43, the recombinant dimer bound the receptor causing testosterone production from the Leydig cells. The biological activity of the dimer, however, was not very high. This suggested some disturbance in the structure of the dimer, probably induced by a slightly altered subunit conformation.

4.5.4. Estimation of βhCG-HBsAg fusion protein by hCG RIA.

Culture supernates from clones stably transfected with pAM5 DNA were assayed for βhCG activity by RIA. The positive and negative controls in this assay were the same as described above for βhCG RIA. As seen in Fig. 44, stable transfectants of pAM5 DNA exhibited a wide range of βhCG activity. The highest amount of βhCG activity was observed in the clone AB5 and this was estimated to be 529 ng βhCG / 10^6 cells / 24 hours (Fig. 44 inset). The βhCG activity in these clones was thus much higher compared to the levels seen in cells transfected with pAM2 DNA, and expressing βhCG alone. The βhCG activity expressed by AB5 cells (transfected with pAM5 DNA), in particular, was more than 6.5 fold higher compared to that observed in AC1 cells (transfected with pAM2 DNA). Further, to estimate the intracellular level of the cloned gene product, the sonicate supernate from AB5 cells was prepared and used for βhCG estimation by RIA. About 84 % of the total βhCG activity expressed by AB5 cells
Figure 43. Biological activity of the recombinant hCG.

Culture supernate from the cell line 2G1 (secreting recombinant alpha hCG) was mixed with an equal volume of culture supernate from the cell line AC1 (secreting recombinant βhCG), as described in the methods section. The mixture as well as the individual supernates were then assayed in a mouse Leydig cell system, and testosterone production was monitored. Standard hCG (Std. hCG) in varying amounts ranging from 1 - 50 pg, was used as positive control. Testosterone production corresponding to 50 pg hCG is indicated as positive control. The recombinant subunits of hCG upon in vitro reassociation, were biologically active, albeit the reconstitution of biologically active hCG appeared to be somewhat inefficient.
Figure 44. Radioimmunoassay for βhCG expression in CHO-K1 cells transfected with pAM5 DNA.

Culture supernates were collected from stable cell lines obtained after transfection with pAM5 DNA, and selection with G418. Aliquots from the culture supernates were tested in duplicate in a RIA using a monoclonal antibody against βhCG. In a typical experiment, 11,000 dpm of $^{125}$I-labelled hCG was allowed to bind with the monoclonal antibody to produce a $B_0$ of 5000 dpm, with non-specific binding of 460 dpm. The addition of known amounts of non-radioactive standard hCG (in the range of 50 pg - 2 ng) as positive control, resulted in the inhibition of binding to give 950 dpm at the highest hCG concentration. Percent inhibition was expressed as \[ \frac{100 - (\text{bound counts} / B_0 \times 100)}{} \]. Negative control consisted of culture media from CHO-K1 cells, and exhibited only a low background value. Negative control consisted of culture media from CHO-K1 cells. Inset shows the precise quantities of the recombinant βhCG expressed as βhCG-HBsAg fusion protein, by various clones.
Concentration of BHCG in BHCG-HBsAg fusion protein
(ng / 10^6 cells / 24 hrs.)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>AB5</td>
<td>529</td>
</tr>
<tr>
<td>AB4</td>
<td>527</td>
</tr>
<tr>
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</tr>
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<tr>
<td>AB6</td>
<td>508</td>
</tr>
</tbody>
</table>

CELL LINES

+ve Control

-ve Control

PERCENT INHIBITION
was secreted into the culture medium while the rest was intracellular. These results were quite similar to those obtained for transfectants of pAM1 and pAM2 DNAs, thereby confirming that the signal sequences of the recombinant hCG subunits were being recognised in the secretory machinery of CHO-K1 cells.

4.5.5. Screening for hybrid HBsAg particles.

The evidence collected so far for the expression of cloned gene products from pAM5 DNA in CHO-K1 transfectants, only indicated the presence of βhCG activity in these supernates, although immunocytochemical staining studies indicated the presence of both anti-hCG and anti-HBsAg reactive proteins in the cytoplasm of AB5 cells. It was not clear, however, whether the immunoreactive proteins seen in the immunocytochemistry experiments, represented a single species of βhCG-HBsAg fusion protein, with antigenic epitopes of βhCG as well as HBsAg. The next step, therefore, was to determine the presence of HBsAg activity in these supernates.

To determine whether the βhCG-HBsAg fusion protein was secreted from the cells as hybrid HBsAg-like particles, and for a quantitative estimation of the amount of HBsAg being secreted from AB5 cells as βhCG-HBsAg fusion protein, the culture supernate was tested in EIA for HBsAg using a commercially available kit (Auszyme HBsAg Diagnostic Kit, Abbott Laboratories, USA). No HBsAg activity could be detected in the supernate using this assay. However, since this assay was based on a monoclonal antibody directed
against the conformational epitopes of HBsAg, the lack of reactivity could be due to the lack of proper conformation of the βhCG-HBsAg fusion protein, or due to lack of particle formation. Alternately, the concentration of HBsAg in the AB5 supernate could be too low to be detected by this assay. Therefore, failure to detect HBsAg activity by this EIA did not conclusively indicate the absence of the HBsAg portion on the secreted cloned gene product, anticipated to be βhCG-HBsAg fusion protein. To resolve this situation, an alternate approach was used. The culture supernate was processed to attempt the purification of hybrid HBsAg particles by the density gradient centrifugation procedure employed routinely for purifying HBsAg particles. Such a procedure was expected to concentrate the hybrid HBsAg particles, making these amenable to detection by the HBsAg EIA. The culture supernate was centrifuged on a 4 - 20 % sucrose density gradient at 78,000 x g, for 5 hours at 4°C, and 20 fractions of 0.6 ml each were collected. All these fractions when tested by the above EIA for HBsAg, were found to be negative. Further, representative fractions from the above 20 fractions were tested for βhCG by RIA. βhCG activity was found to be distributed equally over almost all of the fractions. This indicated that the density gradient centrifugation did not cause any sedimentation of the βhCG-HBsAg fusion protein, probably because the fusion protein was not packaged into any assembled structure of a higher density, but rather existed as free polypeptide
chains. This also indicated that the lack of HBsAg like conformation in the gene product expressed from pAM5, was more likely to be the reason for the lack of detection by EIA of this protein in the culture supernate.

Confirmation of the presence of HBsAg protein sequence in the pAM5 cloned gene product, was then sought by determining the presence of antigenic epitopes of HBsAg origin on the expected fusion protein as also the size of this protein, by Western blot analysis, as described below.

4.5.6. Western blot analysis.

AB5 cells which had stably integrated pAM5 DNA, secreted into the culture medium, a cloned gene product exhibiting immunoreactivity to the anti-βhCG monoclonal antibody as evidenced by the hCG RIA. While this confirmed the presence of the immunoreactive epitopes of βhCG origin on the gene product being expressed (and secreted) by these cells, this did not reflect the size of the recombinant protein. Also, the lack of detection of HBsAg epitopes on the secreted gene product from AB5 cells, was not in accordance with the results obtained with immunocytochemistry and indirect immunofluorescence studies using anti-HBsAg antisera, which suggested the presence of such immunoreactive proteins inside the cells. To resolve these issues, Western blot analysis of the culture supernate was performed.

Culture supernate from AB5 cells, was resolved on 12.5% SDS-PAGE, followed by electroblotting of the proteins on to nitrocellulose, which was reacted with different antisera.
Figure 45. Western blot analysis of AB5 culture supernate using anti-βhCG polyclonal (panel A) and monoclonal (panel B) antisera.

Culture supernate collected from AB5 cells grown in culture for 24 - 72 hrs., was resolved on 12.5% SDS-PAGE (panel A, lane 2; panel B, lane 3). As negative control, culture supernate from wild type CHO cells was also used (panel A, lane 1; panel B, lane 2). Standard hCG was used as positive control (panel A, lane 3; panel B, lane 1). Panel A shows reactivity with a polyclonal anti-βhCG antiserum and panel B shows the reactivity with a monoclonal antibody against βhCG. Position of standard molecular weight markers is indicated on the right. Numbers indicate the molecular weight in kilodaltons (kDa).
Western blot of AB5 supernate using polyclonal anti-βhCG antibodies, revealed the presence of four immunoreactive bands (Fig. 45 A). Standard βhCG purified from pregnant urine was used as the positive control while the negative control consisted of supernate from untransfected CHO-K1 cells grown in culture. The positive control showed several immunoreactive bands, including a major band with Mr approximately 23 kDa (lane 3), which would correspond to the size of βhCG. The additional immunoreactive bands seen in the positive control probably represented associated forms of hCG subunits, and also reflected the heterogeneity in the extent of glycosylation of different hCG molecules. No immunoreactive bands were observed in the negative control (lane 1), thereby confirming the specificity of the immunoreactivity obtained with AB5 supernate. Of the four immunoreactive bands observed in the AB5 supernate (lane 2), the largest protein was estimated to have Mr approximately 37 kDa (indicated by an arrow), while the next immunoreactive band had Mr of 31 kDa, approximately. This largest immunoreactive band was thus close to the expected size of the cloned gene product anticipated from βhCG-HBsAg gene fusion. The other three immunoreactive bands of smaller sizes probably corresponded to proteolytic cleavage products from the larger fusion protein of Mr 37 kDa. Similar results were obtained when the Western blot was reacted with a monoclonal antibody directed against βhCG (Fig. 45 B). In this case too, no immunoreactive bands were obtained in the negative
control (lane 2) while the positive control showed at least three diffuse, immunoreactive bands (lane 1). In case of AB5 culture supernate, four immunoreactive bands were seen, with the largest immunoreactive protein exhibiting approximate $M_r$ of 37 kDa (lane 3), again confirming the results obtained earlier using polyclonal antiserum against $\beta$hCG. The immunoreactivity of the 37 kDa protein seen with anti-$\beta$hCG antibodies was reproducibly seen in all Western blots performed with different batches of the AB5 culture supernate. Occasionally, however, AB5 supernate exhibited only the two larger immunoreactive bands, while the smaller immunoreactive bands of $M_r$ less than 30 kDa, were not seen in the Western blots. This indicated that the smaller immunoreactive bands were quite likely to be products of proteolytic cleavage from the bigger protein, since the cleavage appeared to be non-uniform.

In order to confirm the presence of HBsAg epitopes on the cloned gene product, a Western blot of AB5 supernate was performed, using a polyclonal anti-HBsAg antiserum, raised in goats, as the primary antibody. The positive control in this case consisted of culture supernate from a recombinant cell line secreting HBsAg particles. The negative control was the same as described above for the other Western blots. As an additional control, HBsAg was added to the supernate from CHO-K1 cells (negative control) and run in parallel on the same gel. As seen in Fig. 46, the positive control showed a major immunoreactive band of $M_r$ 24 kDa, as expected (lane 1).
Figure 46. Western blot analysis of AB5 culture supernate using anti-HBsAg polyclonal antiserum.

Culture supernate collected from AB5 cells grown in culture for 24 - 72 hrs., was resolved on 12.5% SDS-PAGE (lane 2). As negative control, culture supernate from wild type CHO cells was also used (lane 3). Standard HBsAg from the culture supernate of a recombinant cell line, was used as positive control (lane 1). An additional control consisted of CHO supernate to which HBsAg had been added (lane 4). After electroblotting, the filter was reacted with anti-HBsAg polyclonal antiserum. Standard HBsAg gave a strong band corresponding to P24 while the band corresponding to GP24 was visible only faintly and has not reproduced well in the photograph. Position of standard molecular weight markers is indicated on the right. Numbers indicate the molecular weight in kilodaltons (kDa).
for HBsAg polypeptide. In addition, a band of Mr 27 kDa, corresponding to the glycosylated form of HBsAg P24, was also expected to be seen in this sample. However, this band was not distinct in the Western blot. As earlier, no immunoreactive bands were observed in the negative control (lane 3). The specificity of the immunoreactivity was further verified by the presence of an immunoreactive band in the other negative control, to which HBsAg supernate had been added (lane 4). The AB5 supernate showed two immunoreactive bands of approximate Mr 37 kDa and 31 kDa, respectively (lane 2). The presence of immunoreactive bands in the AB5 supernate thus confirmed the presence of a cloned gene product of Mr 37 kDa, with antigenic determinants of HBsAg origin. Further, these immunoreactive bands were very close to those seen in Western blots with anti-βhCG antisera. Above results of Western blot were reproducible. Moreover, using a different polyclonal anti-HBsAg antiserum raised in rabbits, identical results were obtained, thereby confirming the above deductions about the presence of HBsAg determinants on the cloned gene product secreted in AB5 culture supernate.

The evidence from the above Western blot experiments using anti-βhCG as well as anti-HBsAg antisera, taken together, indicated that AB5 culture supernate contained recombinant gene products carrying antigenic epitopes of βhCG as well as HBsAg. Further, the size of the immunoreactive protein in each case was determined to be approximately 37 kDa, and was close to the expected size of cloned βhCG-HBsAg.
Figure 47. Western blot analysis of AB5 culture supernate using anti-βhCG (panels A and B) and anti-HBsAg (panels C and D) antisera.

Culture supernate collected from AB5 cells grown in culture for 24 - 72 hrs., was resolved on 12.5% SDS-PAGE (lanes 2, 6, 8, 12). As negative control, culture supernate from wild type CHO cells was also used (lanes 1, 5, 9, 11). Standard hCG (lanes 3 & 4) and HBsAg (lanes 7 and 10) were used as positive controls. An additional control consisted of CHO supernate to which both HBsAg and hCG had been added (lane 13). After electroblotting, the filter was cut into four parts, and reacted with anti-βhCG polyclonal (lanes 1 - 3), anti-βhCG monoclonal (lanes 4 - 6), goat anti-HBsAg polyclonal (lanes 7 - 9), and rabbit anti-HBsAg polyclonal (lanes 10 - 13) antisera, respectively. In each case, an immunoreactive protein of approximately 37 kDa (see arrow) was seen, besides other smaller immunoreactive proteins. Position of standard molecular weight markers is indicated in the center. Numbers indicate the molecular weight in kilodaltons (kDa).
fusion protein. Since in each case, the size of the recombinant immunoreactive product was much bigger than that expected either for βhCG or HBsAg alone, this strongly indicated that the immunoreactive recombinant protein observed in Western blots with anti-βhCG as well as anti-HBsAg antisera, did indeed represent a single species of cloned βhCG-HBsAg fusion protein.

Conclusive evidence to demonstrate that the antigenic epitopes of HBsAg as well as βhCG, were indeed present on the same protein was provided by the following Western blot experiment (Fig. 47). AB5 supernate was electrophoresed in multiple lanes, with appropriate positive controls, and following transfer of proteins on to nitrocellulose, the nitrocellulose was cut into four portions (panels A - D). The filter shown in panel A was reacted with anti-βhCG polyclonal antiserum as the primary antibody, while that in panel B was reacted with a monoclonal anti-βhCG antibody. The other two portions of this blot were reacted with a goat anti-HBsAg polyclonal antiserum (panel C) and a rabbit anti-HBsAg polyclonal antiserum (panel D), respectively. AB5 supernate exhibited four immunoreactive bands with each of the anti-βhCG antisera (lanes 2 and 6), with the largest immunoreactive protein band corresponding to Mr of approximately 37 kDa (indicated by an arrow). The positive control in this consisted of purified standard hCG (lanes 3 and 4). The negative control consisted of supernate from CHO-K1 cells and did not show any immunoreactive
bands (lanes 1 and 5). In case of the other two filters which were reacted with anti-HBsAg antibodies (panels C and D), AB5 supernate exhibited only two immunoreactive bands of approximate Mr 37 kDa and 31 kDa, respectively (lanes 8 and 12). The positive control in this case, consisted of HBsAg containing culture supernate (lanes 7 and 10). Culture supernate from CHO-K1 cells was used as the negative control, and did not show any immunoreactive bands (lanes 9 and 11).

Further, in order to ascertain that the immunoreactivity seen in AB5 supernate with anti-HBsAg antibodies, resulted from the presence of HBsAg epitopes in the βhCG-HBsAg fusion protein, and was not caused by the presence of βhCG in the recombinant protein, an additional control was included. This consisted of CHO-K1 supernate, to which HBsAg supernate as well as purified βhCG had been added prior to electrophoresis. Only the 24 kDa immunoreactive band corresponding to the positive control (lane 10) was observed in this case, while no additional immunoreactive bands which might correspond to βhCG, were seen (lane 13). This confirmed that the anti-HBsAg antiserum was specific to HBsAg, and did not give any immunoreactivity to βhCG, which in turn, indicated that the immunoreactivity observed in AB5 supernate with anti-HBsAg antisera, was caused by the presence of antigenic determinants of HBsAg rather than those of βhCG, on this protein.

From the above results of immunoblotting experiments, it was evident that both anti-βhCG and anti-HBsAg antisera
reacted to a protein of $M_r$ 37 kDa contained in the AB5 supernate. In addition, a protein of $M_r$ 31 kDa also appeared to be immunoreactive to both anti-$\beta$hCG and anti-HBsAg antisera. Thus, the two bands of the expressed $\beta$hCG-HBsAg fusion cassette reacted with both anti-$\beta$hCG and anti-HBsAg antisera, indicating that immuno-determinants of both proteins were present in a common product. This confirmed that the $\beta$hCG-HBsAg ORF expressed a fusion protein of approximate $M_r$ 37 kDa, being secreted from the AB5 cells, and exhibiting immunoreactivity to anti-$\beta$hCG as well as anti-HBsAg antisera. The additional 31 kDa immunoreactive protein appeared to be a limited cleavage product of the 37 kDa $\beta$hCG-HBsAg fusion protein, such that some of the epitopes of HBsAg as well as BhCG, were retained on the resulting product. Occasionally, Western blot of AB5 supernate also revealed other much smaller proteins, immunoreactive to anti-$\beta$hCG antisera alone. These could be the products of more extensive cleavage of the larger fusion protein.

4.5.7. **Receptor binding activity of $\beta$hCG-HBsAg fusion protein.**

In order to determine whether the $\beta$hCG-HBsAg fusion protein retained the biological properties of $\beta$hCG, the ability of this chimaeric protein to associate correctly with the alpha subunit of hCG, and bind the hCG receptor, was tested.

Culture supernate from AB5 cells secreting $\beta$hCG-HBsAg
fusion protein was mixed with an equal volume of culture supernate from 2Gl cells secreting recombinant alpha hCG subunit. The product of association between these was then tested for hCG receptor binding activity, by a radioreceptor assay. As shown in Fig. 48, the in vitro associated recombinant alpha hCG and βhCG-HBsAg fusion protein, exhibited binding to hCG receptor. The two components, per se, did not show any receptor binding. This indicated that βhCG even after linkage to HBsAg, retained the ability to associate correctly with the alpha subunit of hCG, such that the resulting dimer could bind the hCG receptor. In addition, receptor binding activity of this heterogeneous dimer appeared to be higher compared to that seen earlier in case of the in vitro associated recombinant hCG subunits. The precise reason for this was not clear.

4.5.8. Biological activity of the recombinant chimaeric βhCG-HBsAg / alpha hCG heterodimer.

The heterodimer resulting from the in vitro association of the chimaeric βhCG-HBsAg fusion protein and the recombinant alpha hCG, as described above, was tested for its biological activity, to stimulate testosterone production following its binding to the LH receptors on Leydig cells. As seen in Fig. 49, this associated product was biologically active and caused testosterone production from stimulated Leydig cells. This indicated that βhCG-HBsAg fusion protein associated correctly with alpha hCG subunit to form a chimaeric heterodimer, which not only bound hCG / LH
Figure 48. Receptor binding activity of \textit{in vitro} associated recombinant alpha subunit of hCG and \textit{BHCG-HBsAg} fusion protein.

Culture supernate from the cell line 2G1 (secreting recombinant alpha hCG) was mixed with an equal volume of culture supernate from the cell line AB5 (secreting recombinant \textit{BHCG-HBsAg} fusion protein), as described in the methods section. The mixture as well as the individual supernates were then assayed in a competitive radioreceptor assay, for binding to the LH/hCG receptor. 50 ng of standard hCG (Std. hCG) was used as positive control for the receptor assay.
Biological activity of the \( \beta \text{hCG-HBsAg} \) fusion protein recombined with recombinant alpha hCG.

Culture supernate from the cell line 2G1 ( secreting recombinant alpha hCG ) was mixed with an equal volume of culture supernate from the cell line AB5 ( secreting \( \beta \text{hCG-HBsAg} \) fusion protein ), as described in the methods section. The mixture as well as the individual supernates were then assayed in a mouse Leydig cell system, and testosterone production was monitored. Standard hCG ( Std. hCG ) in varying amounts ranging from 1 - 50 pg, was used as positive control. Testosterone production corresponding to 50 pg hCG is indicated as positive control. The \( \beta \text{hCG-HBsAg} \) fusion protein upon \textit{in vitro} reassociation with recombinant alpha hCG subunit, resulted in a biologically active chimaeric hCG species, and the reconstitution of this biologically active chimaeric hCG appeared to be more efficient than that observed for the unmodified recombinant hCG subunits ( see figure 43 ).
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Gene Product</th>
<th>DMEM</th>
<th>DMEM + Proline</th>
<th>DMEM + Proline</th>
<th>HAM'S F-10</th>
<th>HAM'S F-12 (Serum Free)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2G1</td>
<td>hCG</td>
<td>33</td>
<td>181</td>
<td>360</td>
<td>371</td>
<td>367</td>
</tr>
<tr>
<td>AB5</td>
<td>BhCG-HBsAg</td>
<td>52</td>
<td>120</td>
<td>504</td>
<td>529</td>
<td>540</td>
</tr>
</tbody>
</table>

* hCG subunit activity expressed as ng / 10⁶ cells / 24 hrs.
receptor, but also caused testosterone production, thereby closely mimicking the native hCG hormone molecule.

4.5.9. **Serum free culture of stable cell lines.**

Preliminary studies were conducted to test different culture media formulations, to examine the possibility of growing the stable clones in a cost effective media formulation, without compromising on the level of expression of the recombinant gene product. These experiments were carried out with AB5 and 2G1 cell lines as representative cases. These cell lines were grown in different culture media formulations, and the effect of culture medium was monitored by testing the supernate from different culture conditions, for βhCG or alpha hCG activity by RIA. Following media were used for culturing the cell lines: [a] DMEM + 10 % FCS ; [b] DMEM + L-Proline (35 mg / L) + 10 % FCS ; [c] DMEM + L-Proline + Ham’s F-10 ( 1 : 1 ) + 10 % FCS ; [d] Ham’s F-12 + 10 % FCS.

The comparative expression levels of the cloned gene products obtained with the different media formulations are shown in Table 7. Maximal expression of cloned gene products was obtained when the cells were cultured in Ham’s F-12 medium or in a 1:1 mixture ( V / V ) of DMEM supplemented with proline and Ham’s F-10 media. All the above media were supplemented with 10 % FCS. Since the constructs were transfected into CHO-K1 cells, which lacked the genes for dihydrofolate reductase ( dhfr ) and for proline biosynthesis, these cells were dependent on external
supplements into the culture medium, to overcome these deficiencies. Thus, while addition of proline into the medium appeared to be vital for the health of these cells, it also led to a better level of expression of the cloned gene product.

The cell culture was then tried using a serum free medium (Opti MEM, Gibco Laboratories, USA). Following seeding, the cells were maintained in Ham’s F-12 medium supplemented with 10% FCS, for 24-48 hours to allow optimal cell growth and achieve a high cell density. This media was then replaced with the serum free medium for cloned gene product accumulation. Finally, the media was harvested after 48 hours and assayed for βhCG or alpha hCG by RIA. AB5 cells were found to secrete nearly 540 ng βhCG / 10^6 cells / 24 hours. This was about the same level as that obtained with the cells grown in a serum supplemented medium. In case of 2G1 cells, approximately 340 ng alpha hCG / 10^6 cells / 24 hours, was found to be secreted. Thus, in each case, there was little or no reduction in the level of expression of the cloned gene product, when the cells were cultured in a serum free medium. Further, purification of the recombinant gene product would be easier from the serum free culture system.

The above preliminary evidence, thus suggested that serum free culture could possibly be utilised for the large scale purification of these cloned gene products, without significantly compromising the yield obtained otherwise from serum supplemented culture conditions.
4.5.10. **Computer analysis.**

Computer analysis for protein structure prediction of the βhCG-HBsAg fusion protein was undertaken based on the known a.a. sequences of the component proteins. Hydropathy profile of the βhCG-HBsAg fusion protein was plotted using the algorithm of Kyte and Doolittle (1982) with a window size of 6 residues (**Fig. 50**). This was compared with the hydropathy profiles of βhCG and HBsAg proteins, plotted using similar parameters. By comparing the regions around the site of gene fusion in the three sequences, it was seen that the first major hydrophobic domain of HBsAg (shaded area) had been replaced in the fusion protein with a largely hydrophilic region of βhCG. Thus, in the fusion protein, a hydrophilic domain of βhCG became contiguous with the first hydrophilic domain of HBsAg.

Further, using the algorithm of Margalit et al. (1985), amphipathy scores of all the three proteins were obtained and the predictions of helper T cell epitopes based on these scores, were studied. As expected, the βhCG-HBsAg fusion protein was predicted to recruit some of the additional helper T cell epitopes of HBsAg besides retaining those present on the βhCG moiety itself. A closer examination of the predicted epitopes with a high amphipathic score, on the HBsAg sequence, revealed that the protein fusion also led to a slightly altered amphipathy profile of the HBsAg moiety, reflected by a change in the size and number of the predicted helper T cell epitopes. Specifically, there was a reduction...
Figure 50. Comparison of hydropathy profiles of $\beta$hCG, HBsAg and $\beta$hCG-HBsAg fusion protein.

Hydropathy plots of $\beta$hCG (top), HBsAg (middle) and $\beta$hCG-HBsAg fusion protein (bottom), were obtained by using the algorithm and hydropathy values of Kyte and Doolittle (1982), with a window of six residues. Hydrophobic residues plot above the centre line while the hydrophilic residues plot below the centre line. The dotted line indicates the site of fusion between $\beta$hCG and HBsAg sequences. Shaded area indicates the first major hydrophobic domain of HBsAg sequence, which is absent in the $\beta$hCG-HBsAg fusion protein.
in the amphipathy score of the segment between a.a. 192 - 204 of HBsAg.

Although the direct effects of these alterations in the hydropathy profile of HBsAg were not determined experimentally, however, a few speculations have been noted in the next chapter (Discussion).