CHAPTER 6.

SUMMARY AND CONCLUSIONS
1. Full length cDNAs encoding alpha and beta subunits of human chorionic gonadotropin (hCG) were isolated as Hind III fragments from pBR322 based plasmid constructs and were cloned into a eukaryotic expression vector pHAPr-1-neo, under the control of human β-actin promoter.

2. Recombinant plasmids pAM1 and pAM2, carrying cDNAs for alpha hCG and βhCG, respectively, cloned in pHAPr-1-neo, were identified by colony hybridisation using 32P labelled, nick translated hCG subunit cDNAs as probe. These constructs were further characterised by detailed restriction endonuclease cleavage analysis and Southern blot hybridisation, to verify the orientation of the insert with respect to the promoter.

3. A chimaeric gene fusion was engineered by deleting the translation termination codon from βhCG cDNA followed by linking the 3' end of βhCG cDNA to the 32nd codon of HBsAg. This resulted in a single βhCG-HBsAg ORF in which the HBsAg sequence downstream of the 32nd codon, was in frame with the βhCG sequence, constituting a translational fusion. This βhCG-HBsAg cassette was cloned in a eukaryotic expression vector, pSV2S, under the control of SV40 early promoter, to generate the plasmid pSV2BS35. This construct was then analysed by detailed restriction analysis to verify the orientation of the inserted DNA.

4. In another construction, the βhCG-HBsAg ORF was excised from pSV2BS35 by polymerase chain reaction (PCR) mediated
amplification. The PCR was carried out using primers with restriction site overhangs, which introduced Sal I and Hind III sites at the 5' and 3' ends, respectively, of the βhCG-HBsAg ORF.

5. The amplified βhCG-HBsAg cassette was cloned in pUC18 in a forced orientation and a recombinant, pAM3, carrying the correctly cloned insert was identified by blue/white colour selection followed by restriction analysis. This clone was used to isolate the βhCG-HBsAg ORF in bulk as a Sal I - Hind III fragment, which was subsequently cloned into pHBAPr-1-neo. Recombinant plasmid pAM5 carrying the correctly cloned insert DNA, was identified by colony hybridisation using radiolabelled βhCG cDNA as probe, followed by restriction analysis. Southern blot hybridisation with radiolabelled βhCG and HBV DNA probes, also verified the identity of this clone.

6. Transient expression of pSV₂βS35 and pAM5 DNAs was compared by DEAE-dextran mediated transfection into mouse LMtk⁻ cells, followed by assay of the supernate for detection of the cloned gene product, using highly sensitive RIAs for βhCG and HBsAg, respectively. pAM5 was found to exhibit nearly two fold higher expression of βhCG compared to pSV₂βS35. However, no expression of HBsAg was detectable in either construct using RIA for HBsAg. pAM5 was, therefore, selected for performing stable transfections.

7. In order to identify the cell line most suited for stable expression of βhCG-HBsAg fusion protein, pAM5 DNA was
introduced into six mammalian cell lines using DEAE-dextran, and the transient expression of βhCG monitored in the culture supernate by RIA. Of the six cell lines tested, the highest level of βhCG expression was obtained in CHO-K1 cell line. This cell line was selected for generating stable transfectants of pAM1, pAM2 and pAM5 constructs.

8. Stable transfections were carried out using either the conventional calcium phosphate coprecipitation method or by employing a recently developed lipid mediated method (lipofection). Following transfection with either procedure, the cells were selected for neo<sup>R</sup> using G418, to isolate stable transfectants incorporating the cloned DNA. The presence of cloned DNA stably integrated into the genome of these cells, was also demonstrated by Southern blot hybridisation using radiolabelled hCG subunit cDNA probes.

9. Lipofection was found to be much more efficient compared to the calcium phosphate method and worked even with crude preparations of DNA. In addition, as little as one microgram of cloned plasmid DNA was sufficient for a successful transfection using lipofectin and did not require the use of any carrier DNA.

10. Stable clones transfected with alpha hCG or βhCG expression plasmids, were found to express the recombinant alpha and beta subunits of hCG. Using subunit specific antisera, immunocytochemical staining of the stable clones was performed. In each case, the recombinant antigen was
detected in the cytoplasm of these cells, confirming the expression of recombinant gene products.

11. The culture supernates from the stable clones were also tested by RIA to determine whether the cloned gene product was being secreted from these cells. In each case, the supernate was found to contain the cloned gene product. The level of the recombinant hCG subunits secreted into the culture medium was much higher compared to that found intracellularly, confirming the secretory nature of these proteins. Further, expression of alpha hCG was found to be more efficient compared to that observed for βhCG.

12. Recombinant alpha and beta hCG subunits were able to associate in vitro to form a heterodimer which exhibited binding to hCG receptor and was also able to stimulate Leydig cells for steroidogenesis. Thus, recombinant, in vitro reassociated hCG retained the essential biological properties of the native hormone. However, the reconstitution of the biological activity by in vitro reassociation of the recombinant subunits of hCG, did not appear to be very efficient, and could possibly be ascribed to some differences in the glycosylation pattern of recombinant alpha hCG, when expressed in the absence of the beta subunit.

13. Stable transfectants were also obtained with pAM5 DNA. One such clone when analysed by Southern blot hybridisation, appeared to have two copies of the transfected DNA integrated at different sites in the genome of the host cell.
Immunocytochemical and immunofluorescent staining of these cells using antibodies against βhCG as well as those against HBsAg, indicated the presence of immunoreactive cloned gene product in the cytoplasm of these cells.

14. Analysis of the culture supernates from cells transfected with pAM5 DNA also showed BhCG activity when tested by hCG RIA. Approximately 15% of the total βhCG activity was found intracellularly while the rest was recovered in the culture medium, confirming the secretory nature of this protein. The recombinant protein, however, was not detectable by EIA for HBsAg, indicating the lack of some conformational determinants of HBsAg on the βhCG-HBsAg fusion protein. The expression of βhCG-HBsAg fusion protein was more efficient compared to that obtained for hCG subunits alone.

15. The βhCG-HBsAg fusion protein was found to carry B cell epitopes for both βhCG and HBsAg. This was evidenced by Western blot analysis using various antisera against βhCG as well as against HBsAg. This indicated that while the fusion protein retained many of the immunological determinants of βhCG as well as HBsAg, some of the conformational determinants of HBsAg were absent in this protein. This along with some other indirect evidence suggested that the βhCG-HBsAg fusion protein was probably not being assembled into hybrid HBsAg-like particles.

16. In order to derive a possible explanation for the failure of assembly of βhCG-HBsAg fusion protein into hybrid HBsAg-
like particles, the hydropathy profile of this fusion protein was compared with those of βhCG and HBsAg, respectively. It was apparent that the first major hydrophobic domain of HBsAg was absent in the fusion protein. This was substituted by a hydrophilic domain of βhCG, which became contiguous with a hydrophilic domain of HBsAg in the fusion protein. Since the first hydrophobic domain of HBsAg is known to interact with the lipid bilayer in the formation of HBsAg particles, it is speculated that the loss of this hydrophobic domain may be involved in the lack of assembly of the fusion protein into HBsAg-like particles.

17. The βhCG-HBsAg fusion protein was able to associate with recombinant alpha hCG to form a heterodimer which bound the hCG receptor. This heterodimer also exhibited biological activity similar to that of native hCG.

18. The βhCG-HBsAg fusion protein in addition to having immunological determinants of both hCG and HBsAg, thus appeared to closely mimic the native hCG in terms of its biological properties.

19. It was possible to adapt the recombinant cell lines to growth in serum free medium without affecting the yield of the cloned gene product. This opens up the possibility of producing these recombinant gene products more economically and may also render their purification easy.

20. The present study also generated some potential
improvements in the existing methodology, which may be generally useful in recombinant DNA methodology. First, it was seen that PCR can be used as a rapid cloning procedure, by generating appropriate vector-compatible termini on the target DNA, thus circumventing many of the more tedious conventional methods used to accomplish this. Second, use of "in-gel" ligation and transformation procedures was found to allow the rapid creation of recombinant DNA molecules, thus obviating the need to purify the desired DNA fragments from agarose gels. This also can result in saving of time. Third, use of lipofectin for transfection was found to yield a substantially higher efficiency of transfection compared to the conventional calcium phosphate method. This can be especially useful for generating stable transfectants. Fourth, use of lipofectin also allows one to generate stable transfectants even with small quantities of mini-prep plasmid DNA, which is not possible by other conventional methods. This not only obviates the need to prepare large quantities of highly purified plasmid DNA, but can also allow rapid screening of a large number of DNA samples in a transfection assay, to select for a particular gene activity.

21. Finally, although the production of hCG subunits by the recombinant DNA route appears to be feasible using a mammalian cell expression system, the following issues merit careful consideration before any particular system can be selected for this purpose. In the production of alpha subunit, particular attention has to be paid to the
structures of the complex oligosaccharides present on the alpha subunit, in order to produce alpha subunit capable of recombining with the beta subunit. For the production of the beta subunit, stability and consequent yield of the intact beta subunit, is the major concern. Expression of \( \beta \text{hCG} \) as a fusion protein with HBsAg, for producing a carrier-conjugated \( \beta \text{hCG} \) vaccine formulation, appears to not only increase the level of expression of the cloned gene product (probably by imparting some stability to \( \beta \text{hCG} \)), but also retains most of the biological properties of \( \beta \text{hCG} \). This approach can thus have use in the design of improved multivalent \( \beta \text{hCG} \) vaccines for possible use in contraception.