TOWARDS AN EPITOPE-SPECIFIC VACCINE: EPITOPE-LOSS MUTANTS OF HUMAN CHORIONIC GONADOTROPIN BETA SUBUNIT.

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Thesis submitted for the degree of Doctor of Philosophy
University of London
1996

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ABSTRACT

Human chorionic gonadotropin (hCG) is produced by the trophoblastic cells of the early foetus to ensure the continued production of progesterone which is essential for a successful pregnancy. Due to its well studied biological and immunological characteristics, the hormone-specific beta chain (hCGβ) has been the focus of research to develop immunological contraceptive vaccines. However, the beta-chain has extensive homology with the beta chain of human luteinising hormone (hLHβ). When hCGβ is used as an immunogen antibodies are raised which cross-react with hLHβ. Immunopathology which might arise as a result of this cross-reactivity may limit the acceptability of a contraceptive vaccine based on hCGβ. This project has sought to identify amino acid residues involved in the formation of these shared epitopes and replace them with alternative amino acids so the shared epitopes are eliminated. In this way the concept of producing epitope-specific vaccines is demonstrated, where unwanted B-cell epitopes are removed from a globular protein whilst maintaining the overall conformation in order to maintain desired epitopes. Previous attempts to identify the shared epitopes on hCGβ have been unsuccessful due to the discontinuous nature of these epitopes. Site-directed mutagenesis was applied to residues on hCGβ which were identified from structural information on hCG. Analysis of these mutants with a panel of conformational-dependent monoclonal antibodies allowed the allocation of residues to previously determined epitope regions. The immunogenicity of one hCGβ mutant (Mutant 3) was examined in BALB/c mice by nucleic acid immunisation. This showed that the mutations may have knocked out a major T-cell or B-cell epitope, as it elicited a far lower antibody response than DNA encoding wild-type hCGβ. This may indicate a problem which may be encountered with creating epitope-specific vaccines, where removal of unwanted but immunodominant epitopes leaves a poorly immunogenic molecule.
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ABBREVIATIONS

Ab     antibody
AP     alkaline phosphatase
APC    antigen presenting cell
cAMP   cyclic adenosine 3', 5'-monophosphate
cfu    colony forming units
dghCG  deglycosylated hCG
DMSO   dimethylsulphoxide
E. coli Escherichia coli
EECF   extraembryonic coelomic fluid
ELISA  enzyme linked immunosorbent assay
FITC   fluorescein isothiocyanate
FSH    follicle stimulating hormone
GnRH   gonadotropin releasing hormone
HA     haemagglutinin
hCGβ   human chorionic gonadotropin beta subunit
hCGβ-CTP hCGβ C-terminal peptide
hCGcf hCG core fragment
HEL    hen egg lysozme
hGPH   human glycoprotein hormone
hLHβ   human luteinising hormone beta subunit
HSD    heterospecies dimer
Ig     immunoglobulin
IL     interleukin
IPTG   Isopropyl thiogalactoside
LB     Luria broth
mAbs   monoclonal antibody
MAP    multiple antigen peptide
MBP    maltose binding protein
MHC    major histocompatibility molecule
NA     neuraminidase
NP  nucleoprotein
O.D.  optical density
PBS  phosphate buffered saline
PEG  polyethylene glycol
pfu  plaque forming units
RIA  radioimmunoassay
rfDNA  replicative form DNA
sd H₂O  sterile distilled H₂O
SDS  sodium dodecyl sulphate
ssDNA  single stranded DNA
TBS  Tris buffered saline
TCR  T-cell receptor
Tg  thyroglobulin
TSH  thyroid stimulating hormone
ACKNOWLEDGEMENTS

I would like to thank my supervisors, Torben Lund and Pete Delves, for advice, encouragement and help throughout my PhD. I would like to thank Dr Sarah Ball, my tutor at university, whose enthusiasm for her work eventually rubbed off on me and made me realise a scientific career wasn’t such a bad thing after all. I would also like to thank Professor Ivan Roitt for being very supportive of the project throughout my time in the department. Also much of this work would not have been possible without very successful collaborations with Dr Peter Berger, Professor Neil Isaacs and Dr Adrian Lapthorn.

I send my love and thanks to my family for their support financially and emotionally throughout my student years and who provided a place for me to write my thesis. A huge thanks to Poli who was always ready to listen to a moan and provide words of advice. I would also like to thank Nick and Elise without whose kindness and generosity my thesis writing days would have been much harder.

I am also grateful to my colleagues in the department especially those that were in Lab 513 for always being able to laugh even when there wasn’t much to laugh at.

Last, but never least, thanks to my partner Jeremy with whom I was able to discuss all those things you felt you really ought to know but weren’t sure if you did! Thanks for listening, for reading and providing suggestions for the thesis and simply being there when needed.
Chapter 1

General Introduction
1.1 Introduction

Human chorionic gonadotropin (hCG) has been the subject of several clinical trials for use as a contraceptive vaccine (Talwar et al., 1976; Jones et al., 1988; Talwar et al., 1992; Talwar et al., 1994). This hormone, classically known as the indicator for pregnancy, has a fundamental role in early pregnancy, signalling the presence of the embryo to the mother. When introduced to the non-pregnant female in an immunogenic form, antibodies are produced which will neutralise the native hCG produced at the onset of pregnancy. This will result in the breakdown of the endometrium wall, failure of implantation and an apparently normal menstruation. hCG is a member of a family of glycoprotein hormones which share sequence and structure similarities (Pierce et al., 1981). They all have a common alpha subunit and differ in their hormonal properties by virtue of their unique beta subunits. A second member of this family, luteinising hormone (LH), binds to the same receptor as hCG but is synthesized in the pituitary. The beta subunits of the two hormones are 85% identical in the first 110 amino acids. Therefore when hCG is used as an immunogen antibodies are raised which cross-react onto LH. This may in the long term lead to unwanted side effects.

The approach taken in this investigation is to identify the antigenic areas on the beta-subunit of hCG (hCGβ) which are shared with human LH beta subunit (hLHβ). The residues in these areas may then be substituted so that when the altered hCG is used as an immunogen the cross-reactive antibody response is absent. Thus an epitope-specific vaccine may be developed where the undesired epitopes are removed by protein engineering and the useful, desired epitopes are maintained. This concept of epitope-specific vaccines is explored further in 1.2, an introduction to hCG given in 1.3. Anti-fertility vaccines are discussed in 1.4 with particular relevance given to hCG.

1.2 The concept of an epitope-specific vaccine

There are many instances where protein antigens, intended for use in vaccines, have B-cell epitopes which elicit an undesired immune response. For example there may be epitopes which are similar to a host protein, provoking a potentially damaging auto-immune
response, e.g. *Trypanosoma cruzi* and heart tissue (Van Voorhis et al., 1989). Shared epitopes are of utmost importance in the case of candidate immunological contraceptives based on hCG. These epitopes are shared with hLH, a situation that might in the long term lead to pathogenic effects. Many microbial immunogens possess powerful B-cell epitopes that dominate the immune response but fail to be protective because they are subject to rapid variation in structure (e.g. human immunodeficiency virus; malaria; african trypanosomiasis), competing with and limiting the response to conserved epitopes which could provide an effective vaccine. Thus the concept of creating an epitope-specific vaccine arises (Roitt, 1989). This also applies to T-cell epitopes which have been shown to have deleterious effects, e.g. when the zona pellucida glycoprotein ZP3 is used as an immunogen to elicit anti-fertility effects, it also often destroys the primary follicle population (Paterson et al., 1992). This is believed to be due to a cross-reaction with a self protein displayed on the MHC of the follicle cells with T-cells primed by a T-cell epitope in the ZP3 antigen (Luo et al., 1993). Candidate vaccines may also possess T-cell epitopes which compromise the response to the immunogen through biasing the TH1/TH2 response towards a non-protective subset (Moudgal et al., 1993).

Hence there is a need for new strategies in vaccine development which overcome these problems associated with many protein antigens. These new vaccines should be designed to elicit the appropriate immune response and be devoid of any harmful side effects associated with undesired T- and B-cell epitopes.

### 1.2.1 Vaccination

The concept of vaccination, where non-pathogenic or even small amounts of the pathogenic agents are given to subjects eliciting an immune response which then confers resistance to the virulent disease causing agent, has its roots in centuries of medical practices. Variolation, the practice of introducing the scab from small pox pustules into a healthy human body, was common in China in the Middle ages. Later, in the eighteenth century, Edward Jenner conducted a series of experiments in which he demonstrated that the introduction of the cow-pox virus, which is non-pathogenic in man, protected against a
subsequent challenge with the small-pox virus. Louis Pasteur was the first to show that attenuation (removing the disease causing part) of virulent organisms could also be a basis for making vaccines. Vaccination against diseases has been an accepted part of the world culture for the last 40 years. This has resulted in the virtual eradication of small-pox world-wide and a significant reduction in many other diseases e.g. polio, diphtheria, rubella. In the last two decades there has also been a lot of interest in developing vaccines not for disease but to prevent pregnancy. The need for such anti-fertility vaccines stems from the ever expanding world population. If vaccines could be developed which are safe, effective, reversible, and free of endocrine and metabolic side-effects then they would be a significant addition to current contraceptive methods.

1.2.2 Role of the immune system in a successful vaccine

It is most important to consider which branch of the immune response it is required to activate in order to achieve a state of immunity to the pathogen. Thus, in designing a successful vaccine it is useful to know whether a humoral or cell-mediated response is required, and also whether immunological memory is necessary. If the incubation period of the pathogen is short then the disease symptoms may be underway before the memory cells are activated into antibody production, in which case a high level of circulating antibodies is a requirement of a successful vaccine in addition to memory cell production.

Humoral response

After administration of a vaccine where the antigen remains extracellular, the immune response is initiated with antigen uptake by an antigen presenting cell (APC) through receptor mediated endocytosis (via complement or immunoglobulin (Ig) receptors) or by fluid phase endocytosis. Within the APC the antigen is digested by proteolytic enzymes within endosomes and the processed fragments are bound to class II major histocompatibility molecules (MHC II). This complex of MHC II and peptide is transported to the cell surface of the APC (for review see Germain, 1994). The APCs may be B-lymphocytes (B-cells), macrophages or dendritic cells such as Langerhans cells in the skin. These cells migrate through the lymph system to lymph nodes. B-cells will bind to
the antigen when the paratope on their surface Ig is complementary in structure to an epitope on the antigen. The MHC/peptide complex presented on the APC surface may be recognised by specific T-cell receptors on CD4+ T-helper lymphocytes. The affinity of this reaction is fairly low and other interactions occur between accessory molecules such as LFA-1/ICAM-1, CD2/LFA-3, B7/CD28. T-cells which bind MHC/peptide on dendritic cells, macrophages or activated B-cells (naïve B-cells lack B7) are activated by a costimulatory factor (CD28/B7 interaction). Interleukin-1 (IL-1) is also secreted by the APC and enhances activation of the T-cell. Once activated the T-cell starts to express a range of cytokines and their receptors, the result of which is in part to stimulate clonal proliferation of the T-cell. Meanwhile the B-cell is activated following the TCR recognition of MHC II/peptide complex and costimulatory signal provided by CD40 ligand/CD40 interactions (for review see Lanzavecchia, 1993). This leads to expression of IL-4 receptor on the B-cell, IL-4 is produced by the activated T-helper cell and results in the clonal proliferation of the activated B-cell. These differentiate into plasma and memory cells. The plasma cells secrete antibody which is initially IgM but later may be IgG or other class depending on the subset of cytokines present (Figure 1.1). The secondary immune response occurs on subsequent encounter with the same antigen. The memory cell precursors migrate into follicles and begin to proliferate and form germinal centres. Cell division occurs at a high rate and affinity maturation occurs, with the affinity of the antibody produced increased as much as 100 to 10000 fold. Affinity maturation occurs when the antigen is present at low levels on the closely associated follicular dendritic cells, as a result of somatic mutation. Subsequent competition amongst cells means that only those with the higher affinity Ig will bind to the antigen present on the follicular dendritic cells and be rescued from apoptosis. A secondary signal may also be required to facilitate memory cell differentiation, this is the interaction of CD40 on the developing memory cells with the CD40 ligand which is expressed on T-cells after the TCR has bound to MHC/peptide on the B-cells. The cells may now become memory cells encoding high affinity Ig which are ready to fight a subsequent infection (for review see Sprent, 1994 or Ahmed et al., 1996).

Cell-mediated cytotoxic response

When the vaccine is required to generate an immune response effective against intracellular viruses and other pathogens then the antigen must be delivered to the class I processing
Figure 1.1 Recognition of antigen by the immune system.

A) Dendritic cell (e.g. Langerhans cell in skin) encounters antigen which is ingested by endocytosis, processed and presented on MHC II.

B) Naive T-cell encounters APC, the co-stimulatory signals of the TCR/MHC peptide and CD28/B7 activate the T-cell, aided by IL-1 secreted by the APC.

C) The T-cell secretes IL-2 and then expresses the IL-2 receptor, activation of which causes the T-cell to proliferate. Later the T-cell secretes IL-4, IL-5, IL-6 and IL-10, these act on the B-cell.

D) Naive B-cell encounters antigen which binds surface Ig and receptor mediated endocytosis occurs, antigen is processed and presented on MHC II.

E) MHC/peptide complex on B-cells is recognized by T-cell activated by professional A.P.C. T-cell releases IL-4, 5, 6 and 10 which cause class switching and proliferation of B-cell.
pathway. This may be achieved by the use of live attenuated viruses or defective viral vectors which are able to enter the host cell. Helper T-cells are also required (TH1) and secrete the cytokines which influence the precursor cytotoxic T-cell to proliferate and differentiate. The activated cytotoxic T-cell induces apoptosis in the target cell by the localization of cytoplasmic granules between the nucleus and the target cell and probable subsequent exocytosis of the contents which cause lesions in the target cell membrane.

**T- and B-cell epitopes**

The interaction of Ig with an antigen is dependent on the complementarity of the antigen binding site (paratope) with the B-cell epitope present on the antigen. It does appear that the whole surface area of a protein is potentially antigenic (Davies et al., 1988; Jin et al., 1992). Many of the B-cell epitopes are discontinuous i.e. formed by the juxtaposition of amino acid residues as a result of the tertiary and quaternary structure of the molecule (Barlow et al., 1986; Davies et al., 1988). The area covered appears to between 600 - 900 Å², and typically involves 12 - 15 amino acid residues on both the epitope and the paratope. B-cell epitopes in native proteins are usually hydrophilic residues on the protein surface that are topographically accessible to the antibody. Some epitopes are referred to as immunodominant; these are those which induce a more pronounced immune response than other epitopes in a particular species. T-cell epitopes, in contrast, are linear sequences of the protein which are recognised by a TCR when in a complex with MHC (Hedrick, 1988). The peptides in the MHC are formed by antigen processing. The T-cell epitopes often have amphipathic sequences i.e. possessing both hydrophilic and hydrophobic sequences (DeLisi et al., 1985; Spouge et al., 1987). Immunodominance of T-cell epitopes is determined by the MHC haplotype of the individual.

**Adjuvants**

Proteins usually have to be administered with an adjuvant in order to stimulate an adequate immune response. The adjuvant used can have a major impact on the type of immune response elicited. Many work by prolonging the local persistence of antigen. This will increase the likelihood of the formation of memory cells and selection of high affinity antibodies. Aluminium compounds, which are used as adjuvants in humans, form a
precipitate when mixed with the antigen giving a slower release of the antigen. The precipitate also has an increased size and so the incidence of phagocytosis is increased. These features increase the opportunity for development of a humoral response. Also important is the class of antibody produced. Some antibody classes are more efficient at activating complement, binding to receptors on monocytes, or antibody dependent cell-mediated lysis. Different adjuvants have been observed to produce different classes and subclasses in a particular species, e.g. alum, lipopolysaccharide and Quil A elicit antibodies mainly of the IgG1 isotype in the mouse, whereas Syntex Adjuvant Formulation (SAF) produces IgG2a antibodies (Kennedy et al., 1986). However, despite the evidence from animal models on the importance of the adjuvant for the efficacy of vaccines, alum is so far the only adjuvant approved for routine human use.

1.2.3 Different types of vaccines

Whole organism: attenuated versus inactivated

There are a number of different strategies currently used for vaccine production. The most common are whole organism vaccines. These may be attenuated with the organism having lost its pathogenicity yet retained the capacity to provoke an immune response after transient growth in the host. This may be achieved by culturing the microorganism under abnormal conditions which promote mutations. The alternative is to inactivate the organism by heat or chemical means so that it will no longer replicate in the host. The Salk polio vaccine and pertussis (whooping cough) vaccine are both produced by formaldehyde inactivation. Both forms have advantages and disadvantages (Table 1.1). Attenuated vaccines often only need a single injection as they have a high immunogenicity and efficiently promote memory-cell production due to prolonged exposure to epitopes on the organism during the transient growth. Also a cell-mediated response may be induced with an attenuated organism due to its capacity to replicate within host cells. Inactivated vaccines are more stable in storage, which is an important consideration in third world vaccination programmes. In contrast to attenuated vaccines they will produce a predominantly humoral response and may require multiple boosters. Both types of vaccines have associated risks. The attenuated may revert to the virulent form if there is
not a large mutation induced. The Sabin polio virus has a reversion rate of 1 in four million. However, genetic engineering may be used to irreversibly attenuate a virus by removing the virulent genes. A serious risk associated with inactivated vaccines is inadequate killing, which has occurred with the Salk polio virus resulting in paralytic polio in many of the recipients.

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<td>Production</td>
<td>virulent pathogen is inactivated (using irradiation or chemicals)</td>
<td>virulent pathogen is grown under abnormal culture conditions to select for avirulent organisms</td>
</tr>
<tr>
<td>Booster requirement</td>
<td>requires multiple boosters</td>
<td>generally requires only a single booster</td>
</tr>
<tr>
<td>Type of immunity induced</td>
<td>mainly humoral immunity</td>
<td>humoral and cell-mediated immunity</td>
</tr>
<tr>
<td>Relative stability</td>
<td>more stable</td>
<td>less stable</td>
</tr>
<tr>
<td>Reversion tendency</td>
<td>cannot revert to virulent form</td>
<td>may revert to virulent form</td>
</tr>
</tbody>
</table>

Table 1.1 Comparison of attenuated (live) and inactivated (killed) vaccines.

Use of recombinant DNA technology in subunit vaccine development

Some of the risks associated with whole organism vaccines can be overcome using recombinant DNA techniques so that only part of the organism is used in a vaccine. The gene encoding a relevant antigen may be expressed in bacterial, yeast or mammalian cells in culture and the protein purified and used as an immunogen. This was first used successfully for the major antigen (VP3) of the foot-and-mouth-disease virus. The VP3 cDNA was inserted into a prokaryotic plasmid and expressed in large quantities in E.coli. The antigen was purified and used to vaccinate animals (Kleid et al., 1981). There is a recombinant antigen vaccine against hepatitis B which has been used successfully in humans (Jilg et al., 1984). This vaccine is produced by harvesting the hepatitis B surface antigen from yeast cells which express the cloned gene. Alternatively recombinant DNA techniques can be used to insert a gene from an organism which is difficult to culture or
which is pathogenic into an attenuated viral or bacterial genome. This would then carry it
to the appropriate site in the body. Studies have been done with vaccinia virus, poliovirus,
adenoviruses and the BCG strain of Mycobacterium tuberculosis. Care must be taken with
this approach when dealing with immunodeficient patients who can develop serious disease
even with attenuated virus (Guleria et al., 1996).

1.2.4 Alternative vaccination strategies

Despite the success with the above types of vaccines there are still many diseases for which
effective vaccines have not yet been developed. This may be due to the nature of the
organism and the immune avoidance strategies it has developed, e.g. African
trypanosomiasis, influenza, human immunodeficiency virus, all of which vary their
antigenic structure. It may also be the result of unwanted side-effects associated with the
proteins used as the immunogen resulting from cross-reactivity with a self protein. More
sophisticated methods are being developed to provide suitable vaccinations for these
diseases. One such method would be to use peptide vaccines. Using synthetic peptides in
vaccines would have many advantages - easy to produce and store, very little biohazard
risk, and by selecting only those epitopes which elicit a protective immune response, those
epitopes on the whole organism or molecule which bring about a deleterious immune
response are excluded.

Peptides suitable for use as vaccines are often identified by examining which peptide
fragments of a molecule are able to cross-react with antibodies raised against the whole
protein. Due to the discontinuous nature of most epitopes very few peptides are usually
identified, most are from the N- and C-termini of the molecule. This is probably since
terminal segments are less constrained by the structural features of the rest of the
polypeptide chain. This has also been observed with hCG (Dirnhofer et al., 1994a;
Dirnhofer et al., 1994a). In the beta subunit only antibodies which recognise the C-
terminus bind to the relevant peptides of the molecule, the C-terminus has a relatively
disordered structure. Some peptides when used as an immunogen, have produced a
specific immune response against the native protein which has in some cases been
neutralizing (Arnon et al., 1971; Rowlands, 1990). Often however, the peptide is unable to mimic the native structure and antibodies produced tend to be of low affinity (Ramakrishnan et al., 1979).

Unlike B-cell epitopes which can be difficult to mimic with synthetic peptides, T-cell epitopes are linear sequences and are more easily identified. A peptide sequence from polio virus VP1 induced little neutralizing antibody, however it did protect against subsequent challenge (Dimarchi et al., 1986; Collen et al., 1991), indicating that the peptide included a T-cell epitope which primed the T-cells to mount an adequate defence upon infection. The concept of MHC restriction is important when considering T-cell peptide vaccines. In an outbred population a vaccine would need to be able to associate with many different polymorphic forms of MHC molecules.

The ideal peptide vaccine would be one which has peptides with both B-cell and T-cell epitopes, and for widespread use the T-cell would have to be universal, i.e. reactive with many different MHC haplotypes. Such sequences have been identified from malaria, influenza and human immunodeficiency viruses (Sinigaglia et al., 1988; Partidos et al., 1990; Roche et al., 1990; Hale et al., 1989). Most synthetic peptides when used as immunogens are linked to a carrier molecule to improve their immunogenicity. The multiple antigen peptide system (MAP) (Tam, 1988) has a small, immunologically inert core matrix of lysine residues which are linked to multiple clusters of peptides, which could be the same peptide or a mixture of peptides. MAP constructs using B and T epitopes from the circumsporozoite of the rodent malaria Plasmodium berghei have been shown to be successful in inducing high levels of circulating antibodies which provide protection against later challenge (Tam et al., 1990; Zavala et al., 1990; Chai et al., 1992).

For situations where a neutralizing antibody response is required, the peptide often has to mimic a complex discontinuous structure. This may not be possible for many B-cell epitopes. An alternative approach to overcoming the problem of the discontinuous nature of B-cell epitopes is to maintain the overall structure of the protein but remove the deleterious B and T-cell epitopes by substitution of the appropriate residues with those which are immunologically inert, e.g. glycine, serine, alanine. Where the structure of the
molecule is unknown, random mutagenesis may be used on the protein followed by an appropriate screening system to identify the areas of the molecule which need to be removed. Mutant molecules would be selected which have lost part or all of the undesirable epitope but retained those which elicit the desired immune response. If the structure of the molecule is known then it is easier to predict the areas to mutate and the substitutions may be modeled on computer to ensure that no gross overall structural change occurs and that the side chains will be accommodated. In this way there is the advantage that the overall structure of the protein is maintained, thus hopefully such an epitope-specific vaccine will be more immunogenic than a peptide vaccine.

1.3 hCG and the Glycoprotein hormones

This family of four hormones consists of follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), LH and CG. All are synthesized in the anterior pituitary except hCG which is produced by trophoblast cells in the placenta in the early stages of pregnancy. hCG is also secreted by many trophoblastic and non-trophoblastic tumours. They are all heterodimeric proteins with non-covalently associated subunits. They share a common alpha subunit transcribed from a single gene, in humans, on chromosome 6. Their differing hormonal properties are conferred upon each by virtue of their unique beta subunit.

1.3.1 hCG biological function

hCG is classically known for rescuing the corpus luteum in the early stages of pregnancy and maintaining progesterone production. In this way the endometrium wall is maintained for the implantation of the developing embryo. hCG has been detected in blastocysts seven days after fertilisation (Hay et al., 1988). After the sixth week of pregnancy the majority of the hCG is produced by the syncytiotrophoblasts (Maruo et al., 1992).
hCG has also been implicated in other ovarian reproductive functions. It may stimulate luteal oestrogen production in early pregnancy, as indicated by experiments where hCG stimulated oestrogen secretion by corpus luteum \textit{in vitro} (Hunter \textit{et al.}, 1981; Fisch \textit{et al.}, 1989). hCG has also been shown to stimulate the synthesis of inhibin from human luteal cells \textit{in vitro} (Wang \textit{et al.}, 1992), and in pregnancy the inhibin concentration rises at the same time that hCG appears in the maternal blood (McLachian \textit{et al.}, 1987).

There have also been contradictory indications of a role for hCG in stimulating placental progesterone and oestrogen in addition to the luteal functions described above. There is evidence for the expression of the LH/CG receptor on trophoblast, decidua and foetal membranes in middle and late pregnancy from immunocytochemical data (Reshef \textit{et al.}, 1990), but the evidence for specific binding of hCG to placental particulate fractions is conflicting (Paul \textit{et al.}, 1982; Chen \textit{et al.}, 1988). The role of hCG on the placenta has not been examined extensively and as indicated much of the work is conflicting.

A third area where hCG may have a regulatory role is in foetal testicular androgen production. It has been observed that the pattern of testosterone concentration in male foetal testes and blood during sexual differentiation, coincides with that of hCG in the foetal serum and amniotic fluid, i.e. both reach peak values between week 11 and 17 of pregnancy. High affinity binding sites for hCG have been identified in foetal testis (Huhtaniemi \textit{et al.}, 1977). hCG has been shown to stimulate testosterone production and thymidine incorporation from Leydig cells in this stage of gestation (Ahluwalia \textit{et al.}, 1974). Therefore proliferation of Leydig cells may also be stimulated by hCG. However, there is a conflicting report where hCG had no effect on testosterone production on foetal testis (Word \textit{et al.}, 1989). hCG is also synthesized by foetal kidney and adrenal gland in the first and second trimester of pregnancy (McGregor \textit{et al.}, 1983; Rothman \textit{et al.}, 1992). Its functions with respect to these findings are as yet unknown.

Patients with hCG-secreting tumours often show symptoms of hyperthyroidism (Nisula, 1989; Kennedy \textit{et al.}, 1991; Hershmann, 1992). Sowers \textit{et al.} (1978) showed that exogenous administration of hCG to normal men can increase thyroid activity. \textit{In vitro} studies using rodent thyroid cell lines have shown that hCG can stimulate adenosine $3', 5'$-
monophosphate (cAMP) production in these cells (Davies et al., 1986; Hershmann, 1988). If the beta subunit of hCG was deglycosylated then this effect was increased (Hoermann et al., 1991). Studies using human thyroid tissues have been conflicting. Some have demonstrated that hCG can bind human thyroid tissues and stimulate cAMP production (but require high levels of hCG) (Carayon et al., 1980; Rothman et al., 1992) whilst others have not (Amir et al., 1985a; Amir et al., 1985b). It may be that there are other factors which influence the thyroid activity in pregnancy. The thyroid-stimulating activity of pregnancy serum is neutralized completely in some individuals with an anti-hCG antiserum and in others only a partial neutralisation or none at all is seen (Kennedy et al., 1992).

Down's syndrome pregnancies have an elevated level of hCG, hCGβ and hCGα (Bogart et al., 1987; Wald et al., 1988) in the serum and hCG is used here as a diagnostic test (Macri et al., 1990). It is not known where in the regulation pathway that this increased production arises.

hCG is a member of the superfamily of cystine knot growth factors (section 1.2.3). This has raised the question whether hCG has any growth factor activity. A number of tumours have been shown to express hCG, as have many tumour cell lines (Acevedo et al., 1992). Whether hCG is just a by-product of these cells or actually has a role is not known, but there is some evidence for growth factor activity. Rivera et al. (1989) observed that hCG appears to be an autocrine growth factor for the ChaGo human lung cancer cell line. ChaGo secretes hCG and hCGα and when transfected with antisense RNA for hCG the growth of the cells is inhibited. Passive immunisation with anti-hCGα antibodies into nude mice transplanted with ChaGo tumour cells, caused necrosis of the tumours (Talwar et al., 1992). More recently, Gillott et al. (1996) have shown the proliferation of bladder cells in the presence of hCGβ. As already mentioned hCG is believed to be responsible for the proliferation of foetal Leydig cells early in ontogeny. Different glycoforms of hCG have been shown to vary in growth promoting properties on rat thyroid cell line FRTL-5 (Hoermann et al., 1991), with hCG having a deglycosylated β subunit being markedly more potent than hCG. hCG secreted from tumour cells often has different glycosylation patterns than that secreted during pregnancy, those from choriocarcinoma are more
fucosylated and less sialylated (Mizuochi et al., 1983), perhaps this is linked to potential
growth factor activity. Thus there is increasing evidence that hCG may have growth factor
activity, this is probably normally used in early stages of foetal development, but has
pathogenic implications for many tumours which secrete hCG.

During pregnancy there is a large amount of free α subunit of hCG secreted from the
placenta. The oligosaccharides on this free α have been shown to be more complex and
have higher amounts of N-acetylglucosamine, galactose, sialic acid and fucose than those
on the combined hCGα (Blithe, 1990). This free α has been shown to stimulate uterine
decidual cell prolactin secretion (Blithe et al., 1991). The extraembryonic coelomic fluid
(EECF) found between the chorionic and amniotic membranes has been shown to have
large amounts of free α and β subunits with twice as much α subunit as β subunit (Blithe
et al., 1995). This compartment is present until the 14th week of pregnancy. The free α is
unable to combine with the beta subunit due to these larger, fucosylated oligosaccharides
(Blithe et al., 1995). These observations suggest that the free α has a role in early
pregnancy and that part of that role is to stimulate prolactin secretion.

1.3.2  hCG gene organisation and regulation of expression

The alpha subunit of hCG is transcribed from a single gene on chromosome 6q21.1-23
(Fiddes et al., 1979). It has four exons and a TATA box sequence 30bp upstream from
the transcriptional initiation site. It is expressed in pituitary as well as placental tissue in
accordance with its co-expression with the other glycoprotein hormones. It is therefore
regulated by a number of different hormone pathways which control the expression of all
the glycoprotein hormones. It has been demonstrated that despite this wide range of
regulation pathways that the transcriptional start site is the same in both pituitary and in
the placenta (Jameson et al., 1986). This suggests that there is only a single promoter
which may have several tissue specific enhancer elements controlling the expression of the
gene.
The beta subunit of hCG is encoded by multiple genes on chromosome 19q13.3 (Boorstein et al., 1982; Policastro et al., 1983). There are six separate hCGβ genes, and a single gene for hLHβ which is at one end of the cluster (Figure 1.2). The genes have a number of sequence differences in the coding region but most are located in the 5' nontranslated region of the first exon. All hCGβ genes are transcribed, albeit with different efficiency. The mRNA levels for each gene were determined as: $\beta_5 > \beta_3 = \beta_8 > \beta_7 > \beta_1,\beta_2$ (Bo et al., 1992) hCGβ genes 1 and 2 have a base change at the donor splice site between exon 1 and intron 1 and an inserted sequence 10bp upstream from the methionine codon in exon 1. This suggested that they were pseudogenes. However, their transcripts were detected in polysomes implying that they are indeed translated. These transcripts would have a different open reading frame and consequently may have different functions.

![Diagram of CG/LH gene cluster on chromosome 19.](image)

The gene cluster of hCGβ/hLHβ has been subject to gene duplication and gene conversion (Talmadge et al., 1984b). It is presumed to be of relatively recent evolutionary origin, which is supported by the presence of CG in so few species, it is only found in humans and primates. The equine CG is in fact transcribed from the LH gene and differs only in its glycosylation pattern (Sherman et al., 1992; Matsui et al., 1994). As LH and CG are very similar in their biological properties, the CGβ gene may have evolved so that
CG could have a different regulation pathway which would allow placental expression (Jameson et al., 1993).

The CGβ and LHβ genes have different promoters. The LHβ gene has a very short 5' non-translated region with a TATA box upstream from the transcription initiation site. The CGβ gene in contrast, has a long 5' untranslated region, there is no TATA box upstream of the transcription initiation site which is located 365 bp upstream of the vestigial LH promoter sequence. The CGβ genes have a TATA box located 38 bp upstream of the methionine translational start codon which appears to be non-functional (Jameson et al., 1986). Not much is known about what regulates the expression of the CG genes in the placenta, though it may be the sequences immediately upstream and surrounding its unique transcriptional start site (Jameson et al., 1993).

The hCGβ and hLHβ genes are 93.3% homologous in the first 330 coding bp (Figure 1.3). hCGβ has a unique carboxyl extension of 24 amino acids. This has been shown to have been derived as the result of a single base deletion giving read through into the LHβ 3' untranslated region (Fiddes et al., 1980).

1.3.3 Structure of hCG

Despite the large number of studies into biological and structural aspects of hCG the tertiary structure has only recently been determined. Two groups independently resolved the x-ray crystallographic structure. One study used hydrogen fluoride treated protein (Lapthorn et al., 1994) and the other used recombinant selenomethionyl protein (Wu et al., 1994). Both groups revealed almost identical structures (Figure 1.4), with residues 2 - 111 in the β and 5 - 89 in the α subunits traced. That the C-terminal extension of hCGβ could not be traced implies that it is of a poorly ordered structure. The α and β subunits each have remarkably similar folds. They are elongated with two adjacent loops at one end and a single long loop projecting in the opposite direction. When the heterodimer is formed the single loop of one subunit is adjacent to the double loops of the other subunit. The crystal structure reveals that the previously defined disulphide bonds could not all be
Figure 1.3 Complete amino acid and nucleotide sequence of the β-subunit of hCG.

Only the hLHβ sequences that are different from the hCGβ sequence are shown, in green underneath the hCGβ sequence. The hCGβ-specific sequence is in blue. At amino acid 114, there has been a deletion in the ancestral LH gene which changed the reading frame giving hCGβ (after this the nucleotide differences between LHβ and hCGβ are not shown).
Figure 1.4 Ribbon diagram of the hCG heterodimer.

The hCGα subunit is in blue, the hCGβ subunit in green and the extent of the carbohydrates is illustrated in a stick representation, coloured by atom type (C white, N blue, O red). The hCGβ C-terminus, for which the structure is unknown, is illustrated by a series of points with a box indicating the residues implicated in the OT3A binding site. The areas on the loops 1 and 3 targeted for mutation are circled. This figure was provided by Dr A Lapthorn produced using SETOR (Evans 1993).
made (Mise and Bahl, 1980; Mise and Bahl, 1981), there is a cystine knot motif at the core of the molecule from which the loops project out. The cystine knot motif, also found in neural growth factor, platelet derived growth factor and tumour growth factor β, is formed by two adjacent parallel strands joined by two disulphide bonds forming a ring, through which another disulphide bond is formed joining a second pair of strands. The subunit similarity is at its highest in the area of the cystine knot. With the elongated structure of hCG there is a high ratio of protein surface to hydrophobic core and the subunits associate to a large extent over the whole surface. The majority of the structural motifs in the loops are β-sheet and β-turns. In the long loop of the α chain there are, however, two turns of an α-helix. The loop from Cys 90 to Cys 110 in the β subunit is wrapped over and through the long loop of the α-subunit. This ‘seat belt’ is held in place by the cystine bond β26 - 110, which is the last disulphide to form in the folding pathway of hCG (1.3.7) (Bedows et al., 1992b; Huth et al., 1992b).

1.3.4 Epitope analysis of hCG

As shown very comprehensively in the works of Dirnhofer et al. (1994a and 1994b) the antigenic determinants on the surface of hCG are largely non-contiguous, especially those on hCGβ. This meant that previous attempts to map hCG in terms of binding areas for monoclonal antibodies (mAbs) had proved difficult. Bahl et al. (1976) first noted the importance of the secondary and tertiary structure of both hCG and its β-subunit for antibody recognition. Swaminathan and Braubstein (1978) demonstrated that only peptides with intact disulphide bonds (derived from proteolytic digestion of hCG) would compete with 125I-hCG for binding to an anti-hCG sera. Several groups have produced large panels of mAbs against hCG and its subunits which were used to analyse antigenic areas (Bidart et al., 1985; Bidart et al., 1987; Berger et al., 1990) these have been reviewed in Bidart et al. 1993. These mAbs may be placed in broad groups depending on their reactivity with hCG: a) those which only recognise the heterodimer, b) those which only recognise either of the uncombined subunits, or c) those which recognize both heterodimer and a subunit. These mapping studies have been based on sandwich enzyme
linked immunosorbent assays (ELISAs) (Berger et al., 1990), competitive inhibition using labeled antigen (Bidart et al., 1987b), chimeras of hLHβ and hCGβ (Moyle et al., 1990), and peptide mapping (Dirnhofer et al., 1994b). Berger et al. (1990) have extensively mapped the antigenic areas of hCG and used this data to plot a cylindrical Mercator's projection (Figure 1.5). The mAbs used have been used in this present study and the epitope regions they defined are referred to throughout (Table 2.1). Therefore in the following discussion on the epitope regions on hCG the work of Berger’s group is mainly considered.

**Antibodies which only recognise the heterodimer**

Some mAbs will only recognise a quaternary structure produced when the α and β subunits combine. These epitopes are referred to as “c” epitopes. Some mAbs in this group do show a weak interaction with free subunit (e.g. B101 cross-reacts about 10% with hCGβ (Krichevsky et al., 1988)).

**Free subunit specific mAbs**

Of seven epitope regions defined on the α-subunit (Berger et al., 1990), two regions, α6 and α7, were specific only to the alpha chain. Using overlapping peptides α6 was shown to involve residues α33 - 42, also α7 was the only epitope region not destroyed by reducing and alkylating hCGα (Dirnhofer et al., 1994a). The β-subunit has two epitope regions which are chain-specific, these are the β6 and β7 regions.

**Antibodies which recognise both free subunit and heterodimer**

Most mAbs produced in mice appear to fit into this category. There are five such epitope regions on the alpha subunit and seven on the beta subunit.

**hCG-specific epitopes**

More important in terms of producing an hCG-specific contraceptive vaccine is whether the epitope regions are specific to hCG or shared with hLH. A small proportion of mAbs raised against hCG do not cross-react with hLH. mAbs produced by immunisation with the C-terminus are highly specific for hCG. Berger et al. (1990) defined one epitope region...
Figure 1.5 Epitope map of free human chorionic gonadotropin beta subunit.

In the cylindrical Mercator's projection the epitope regions are represented by concentric circles, some of which confluence to larger domains. Adjacent structures with common outer circumference are not compatible for simultaneous antigen detection. Two epitopes (β6 and β7) disappear upon subunit assembly. The hCG-specific epitope regions are depicted in cyan and the hCG/hLH shared epitopes are depicted in green.

Reproduced and modified according to Berger et al (1990) and Schwarz et al (1986).
Moyle et al. (1990) used hLH/hCG chimeras to define the position for a number of hCG specific antibodies. When hCG is used as an immunogen there are usually few mAbs produced which recognise the C-terminus (Bidart et al., 1993). Most mAbs specific for this region have been produced by immunising with synthetic peptide linked to a carrier protein (Matsuura et al., 1978; Bidart et al., 1985). The vast majority of mAbs specific for the C-terminus of hCG are of poor affinity suggesting the low immunogenicity of this region of hCG (Bidart et al., 1993) though there are exceptions to this (e.g. mAb OT3A used in this study, Stevens W. personal communication). Different groups have reported that there are two main regions of antigenicity on the C-terminus. These are regions β110-116 (epitope region β9) and β137 - 144 (epitope region β8) (Bidart et al., 1987a; Dirnhofer et al., 1994b) with region 109 - 119 being the more immunogenic sequence in the C-terminus (Iyer et al., 1992).

**Shared epitopes**

The alpha chain is identical in all the human glycoprotein hormones and consequently all the epitope regions on hCGα are shared with the other members of the family (Berger et al., 1990). The beta subunits are specific to each hormone, however there is extensive homology between hLHβ and hCGβ (85% in first 110 amino acids). Therefore it is not surprising that the majority of the epitope regions determined on the β-subunit are shared with hLH (Berger et al., 1990). This is summarized in table 1.2. At the time this work commenced there was limited information on the residues involved in the shared epitopes. mAb INN-hCG-58 bound slightly to a peptide representing β45-52 and mAb B108, which is only weakly cross-reactive was mapped to β76-78 (Moyle et al., 1990; Dirnhofer et al., 1994).

**1.3.5 Receptor interactions**

The receptor of hCG is shared with LH and is found on testicular Leydig cells and ovarian theca, granulosa, luteal and interstitial cells (McFarland et al., 1989). It is a member of a family of G protein-coupled receptors which have seven transmembrane segments with a
large glycosylated extracellular domain. Binding of the receptor by its ligand results in the increase of cAMP which leads ultimately to an increase in steroid synthesis and secretion.

<table>
<thead>
<tr>
<th>epitope region</th>
<th>specificity</th>
<th>residues known to be involved at start of this work</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1</td>
<td>hGPH, hGPHα</td>
<td></td>
</tr>
<tr>
<td>α2</td>
<td>hGPH, hGPHα</td>
<td>α 13 - 18</td>
</tr>
<tr>
<td>α3</td>
<td>hGPH, hGPHα</td>
<td></td>
</tr>
<tr>
<td>α4</td>
<td>hGPH, hGPHα, oLHα</td>
<td>α 17 - 22</td>
</tr>
<tr>
<td>α5</td>
<td>hGPH, hGPHα, oLHα</td>
<td></td>
</tr>
<tr>
<td>α6</td>
<td>hGPH, oLHα</td>
<td>α 33 - 42</td>
</tr>
<tr>
<td>α7</td>
<td>hGPH</td>
<td></td>
</tr>
<tr>
<td>β1</td>
<td>hCG, hCGβ</td>
<td></td>
</tr>
<tr>
<td>β2</td>
<td>hCG, hCGβ, hLH, hLHβ</td>
<td></td>
</tr>
<tr>
<td>β3</td>
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</tr>
<tr>
<td>β4</td>
<td>hCG, hCGβ, hLH, hLHβ</td>
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</tr>
<tr>
<td>β5</td>
<td>hCG, hCGβ, hLH, hLHβ</td>
<td></td>
</tr>
<tr>
<td>β6</td>
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<tr>
<td>β9</td>
<td>hCG, hCGβ</td>
<td>β 110 - 116</td>
</tr>
</tbody>
</table>

Table 1.2 Epitope regions defined on hCG, their specificity and any residues demonstrated to be involved in the binding of mAbs (Berger et al., 1990a; Dimhofer et al., 1994a).

hGPH = human glycoprotein hormone, which all share common α-subunit, oLHα = ovine luteinising hormone α subunit.

To examine the relationship of hCG with its receptor, mAbs have been used by several groups, and their ability to either neutralise or bind to hormone/receptor complex established. It appears that there is a group of mAbs that very efficiently block binding of hCG to its receptor and hence neutralise the action of the hormone. These mAbs all react strongly with hCG heterodimer. There is another group of mAbs which do not have any effect on receptor binding of hCG, these include some mAbs directed to the C-terminus. In between these groups there is a gradation of effect (Norman et al., 1985; Krichevsky et
Neutralisation of hCG may be one method by which an hCG vaccine may prevent fertility.

In the absence of neutralisation data, information on which mAbs bind to the hormone receptor complex may also give information regarding the importance of certain epitope regions in eliciting an effective immune response. The mAbs (and other mAbs which share the same epitope regions) used in this study have only been examined for their ability to bind the hormone/receptor complex. Only those mAbs mapped to epitope regions β3, β5 and β8 react with hCG when it is bound to the receptor (Schwarz et al., 1991b).

There is also growing evidence for the presence of the hCG receptor on non-gonadal tissue. It has been found in the female uterus (Reshef et al., 1990), rat prostates (Tao et al., 1995), the brain (Lei et al., 1993), human myometrial smooth muscle cells (Kornyei et al., 1993) and also lymphocytes of pregnant women (Lin et al., 1995). It is as yet unknown what the function of the receptor is in these tissues.

1.3.6 Glycosylation

hCG is heavily glycosylated with 30% of its weight derived from the oligosaccharides. There are two N-linked and four O-linked glycosylation sites on the beta chain, and two N-linked sites on the alpha chain. It has been shown that removal of the sugars allows mAbs raised against glycosylated hCG to react with higher affinity to the deglycosylated hCG (Schwarz et al., 1991a; Sairam and Jiang, 1992). The Asn 52 - linked oligosaccharide does appear to be important in signal transduction with the CG/LH receptor. Deglycosylated hCG (dghCG) has been shown to bind the receptor with higher affinity, but it fails to activate the receptor (Chen et al., 1982; Sairam et al., 1992). This has been shown to be due to the Asn 52 linked oligosaccharide on the alpha chain (Matzuk et al., 1989). The O-linked oligosaccharides extend the life of the hormone in circulation and it has been shown that the unique C-terminal extension of hCGβ bearing these oligosaccharides may be added to FSHβ, and the in vivo half life of the hormone is increased (Fares et al., 1992). The Asn- linked carbohydrates may also be of importance.
in the folding and correct disulphide bond arrangement of hCG (Feng et al., 1995b). The
glycosylation on the free α-subunit and hCG vary through pregnancy, hCGα becomes
more highly branched and both hCGα and hCG become more fucosylated as gestation
progresses (Skarulis et al., 1992). The more complex carbohydrates on free α found in
the EECF prevent its association with the β-subunit, this EECF free α is thought to be
linked with prolactin secretion (Blithe. 1990; Blithe et al., 1991)

1.3.7 Folding pathways of hCG

It has been shown that hCG has a number of partially disulphide bonded intermediates in
its folding pathway (Bedows et al., 1992; Huth et al., 1992) which occurs in the
endoplasmic reticulum. hCGβ was the first mammalian protein to have its folding pathway
elucidated. The folding pathway is illustrated in figure 1.6.

\[ \text{pβ1 early} \rightarrow \text{pβ1 late} \rightarrow \text{pβ2 free} \rightarrow \text{pβ2 combined} \rightarrow \text{native hCG} \]
\[ 34 - 88 \ 9 - 90 \ 93 \rightarrow 100 \]
\[ 38 - 57 \ 23 - 72 \ 26 \rightarrow 110 \]

Figure 1.6 Folding pathway of hCG in the lumen of the endoplasmic reticulum.

The disulphide bonds 34 - 88 and 38 - 57 are formed by the time pre β1 late (pβ1 late) is detected, 9 - 90
and 23 - 72 are formed when pβ1 late converts to pβ2 free. The final two disulphide bonds are important
in subunit association. (Bedows et al., 1993). N.B. The disulphide bonds assigned in this study are not
those as determined by the crystal structure, therefore either there is an exchange in part of the pathway or
else 9 - 57 must form before 38 - 90 or vice versa.

1.4 Anti-fertility Vaccines

The increased understanding of the immune system and vaccinology over the last twenty
years had led to a heightened interest in the concept of contraceptive vaccines. The
majority of the research has been done on hCG, with the results of the first clinical trials
published twenty years ago (Talwar et al., 1976). The World Health Organisation (WHO)
established a task force for fertility regulation in 1973 to investigate the possibility of
developing birth control vaccines, the major objective of which has been the development
of anti-hCG vaccines. However, there are several areas of the reproductive system that
are also being investigated as potential targets for immuno-contraception.

General criteria for developing contraceptive vaccines have to be considered where
vaccines are to be used in healthy subjects to prevent an otherwise natural occurrence.
The target antigen should be specific to the targeted reproductive tissue so that there are
no unacceptable metabolic or hormonal disturbances nor immunopathology at a distant
site. To be effective, the target antigen must be accessible to the immune system,
therefore it must either be secreted or present on the surface of the cell. The reversibility
of an immune response must be considered. It is more desirable to have a vaccine where
the effects are long lasting so repeated immunisations are not necessary, this may be more
a question of the administration of the immunogen rather than just the choice of the target
antigen. It may not always be necessary to have a reversible contraceptive, and some
future vaccines could be regarded as alternatives to surgical sterilisation. However, it does
often occur that the recipient may change their mind about their fertility status and in these
cases it is more desirable to have a vaccine whose effects may be reversed.

1.4.1 Gamete antigen vaccines

Anti-sperm
Several groups have identified antigens on sperm which have a contraceptive potential.
This group of antigens has the advantage in terms of general acceptance, in that a few
patients, both male and female, presenting at infertility clinics are shown to have naturally
occurring antibodies to sperm antigens (Boettcher. 1979, Paradisi et al., 1996). Anti-
sperm antibodies may immobilise, agglutinate or kill sperm. Animal models have been
used very successfully for one particular sperm antigen PH-20 (Primakoff et al., 1988).
This produces a very effective and reversible contraceptive. However there is as yet no
homologous sperm antigen discovered in humans. Other antigens identified for
contraceptive development in human sperm are a 90kD protein (Aitken et al., 1987),
SP10 (Herr et al., 1990), FA-1 (Naz et al., 1991) and the sperm-specific isozyme of lactate dehydrogenase (LDH)-C4 (Goldberg, 1990).

Anti-zona pellucida vaccines
A glycoprotein component of the zona pellucida, ZP3 is involved in the sperm-egg recognition (Dean, 1992). Despite the species specificity of this interaction ZP3 is highly homologous in many mammalian species. After binding to receptors on ZP3 the spermatozoa are induced to undergo the acrosome reaction and eventually penetrate the zona to reach the oocyte. ZP3 antigens are therefore attractive candidates for contraceptive vaccine development and antibodies raised against ZP3 from one species are shown to inhibit in vitro fertilisation of other species. Also in view of the small number of mature eggs (and therefore ZP3) present at any one time, presumably, in vivo low levels of antibodies would be effective. However, despite the promising anti-fertility effect of anti-ZP3 antibodies, active immunisation studies have shown a long-term problem. Immunisation of squirrel monkeys with porcine ZP3 gives reversible and prolonged infertility, but also disrupts ovarian cyclicity (Sacco et al., 1983). This has been shown to be due to loss of the primordial follicle population. This is believed to be due to a cell-mediated immune response derived from a deleterious T-cell epitope in the ZP3 sequence (Luo et al., 1993). Investigations are currently underway to construct vaccines using only B-cell epitopes, these were identified by screening ZP3 fragments with mAbs against ZP3. A peptide thus identified has been conjugated to a carrier and used successfully to immunise mice without development of the ovarian pathogenesis previously observed (Millar et al., 1989).

1.4.2 Hormone based vaccines

Gonadotropin releasing hormone
Gonadotropin releasing hormone (GnRH) regulates the synthesis of gonadotropins in both males and females. Immunisation against GnRH will produce an anti-fertility effect by reducing levels of gonadotropin secretion which will prevent egg, sperm and steroid hormone production. This may also lead to a loss of secondary sexual characteristics and
decline in libido as a result of major endocrine disturbances (Giri et al., 1990). GnRH is also constantly present and there may be long-term immunopathological effects when immunising against such a molecule. Nevertheless Talwar's group is exploring the possibility of using GnRH vaccines in post-partum women to extend lactational amenorrhoea and also for treatment of men with prostrate cancer (Talwar et al., 1992).

**Follicle stimulating hormone**

FSH binds to receptors on Sertoli cells and influences germ cell maintenance and spermatazoa production. In the female, FSH binds to granulosa cells in the ovarian follicles stimulating the production of both FSH and LH receptors thus increasing steroid production. The steroids exert a feedback regulation of FSH and LH secretion and trigger a surge of LH production which induces ovulation. The use of an FSH vaccine in males has been investigated by long term studies in bonnet monkeys where ovine FSH (oFSH) was delivered with an adjuvant accepted for human use (Moudgal et al., 1992). All the animals produced antibodies and were rendered infertile until cessation of booster injections, then nine out of ten animals regained fertility. There was no cross-reactivity of the antibodies produced with hLH and testosterone levels were unaffected. Therefore oFSH could be developed as an anti-fertility vaccine for use in man.

**Human chorionic gonadotropin**

As already described hCG has been the subject of much work in the development of contraceptive vaccines. It has the advantage in being a hormone which has been extensively studied and thus much is known about its biological activity and more recently structure. There have been clinical trials with two different forms of hCG. Talwar's group has opted for using an ovine alpha chain with the human beta subunit. Previously they had used hCGβ conjugated to tetanus toxoid (TT) (Talwar et al., 1976). This induced antibodies against both hCG and TT in Phase I clinical trials. However, not all recipients produced adequate titres to protect against pregnancy. They then sought to improve the immunogenicity of hCGβ by associating it with the ovine alpha chain (conjugated to tetanus toxoid or diphereria toxoid) to form a heterospecies dimer (HSD). This has been used in Phase II clinical trials and is found to be more immunogenic than hCGβ and also the antibodies induced by the HSD appeared to have a greater bioneutralizing activity than
those induced by hCGβ (Pal et al., 1990). Despite extensive homology between the ovine alpha and human alpha chain they did not report any antibodies which cross-reacted with hFSH or hTSH (Singh et al., 1989). The Phase II trials involved multiple injections and raised contraceptive levels of antibodies in 80% of the women. The effect was reversible, and one pregnancy occurred in 1224 cycles where contraceptive levels of antibodies were present. Despite the presence of antibodies which cross-reacted with hLH, ovulation was not impaired. This is probably due to a previously observed phenomena where a low level of hLH, due to partial neutralization of cross-reactive antibodies, is sufficient to induce ovulation (Stevens et al., 1973).

Despite the early evidence that the cross-reaction with hLH does not seem to cause any immunopathology or disrupt the ovarian cycle, others have taken a more cautious approach, as the question of long-term effects of such cross-reactivity in humans is still not answered. The WHO are undergoing Phase II clinical trials on a peptide vaccine consisting of the unique C-terminal extension of hCGβ (hCGβ-CTP) coupled to a carrier protein. Antibodies raised to this peptide should be completely devoid of cross-reactivity with the other glycoprotein hormones. A prototype vaccine of hCG-CTP administered to baboons lowered fertility from 70% to 7.5% (Stevens et al., 1981). However, the hCGβ-CTP may not be as effective as hCGβ in controlling fertility. Antibodies raised against hCGβ-CTP do not inhibit binding of the hormone to its receptor (Dirnhofer et al., 1993), therefore the only route open for efficacy is the removal of antibody antigen complexes. Also as the antibodies raised against hCG-CTP can still bind the hormone when complexed with the receptor there is the potential for initiation of an auto-immune attack on the ovary (Dirnhofer et al., 1994c). The hCGβ-CTP in comparison to hCG or hCGβ is also not particularly immunogenic (Ramakrishnan et al., 1979b; Dirnhofer et al., 1994b) though it may be possible to overcome this limitation using suitable delivery and adjuvant systems.

There is a need to develop more sophisticated vaccines for the many situations where the whole native molecule may not be used due to antigenic variation, inappropriate response and side-effects associated with part(s) of the molecule. It is proposed that a means of overcoming these difficulties is to selectively alter the molecule to remove undesirable
properties, whilst retaining the overall conformation. Hence the required immune response may still be elicited by the desired epitopes which are retained in the correctly folded native molecule. It may also be possible to augment the immune response by introducing suitable T-cell epitopes. A model for this approach is the design of an anti-fertility vaccine using hCG. The likelihood of developing pituitary immunopathology with the long-term use of such a vaccine will be greatly reduced if the epitopes shared with hLHβ are removed from hCGβ.
1.5 Aim

To remove discontinuous epitopes shared between hCGβ and hLHβ, whilst maintaining the overall structure of the molecule.
Chapter 2

Materials and Methods
2.1 Materials

2.1.1 hCG monoclonal and polyclonal antibodies

<table>
<thead>
<tr>
<th>mAb</th>
<th>epitope region</th>
<th>specificity</th>
<th>isotype</th>
</tr>
</thead>
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<td>β1</td>
<td>hCGβ, hCG</td>
<td>IgG1</td>
</tr>
<tr>
<td>INN-hCG-32</td>
<td>β</td>
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<td>IgG1</td>
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<td>hCGβ</td>
<td>N/D</td>
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<td>hCGβ</td>
<td>N/D</td>
</tr>
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<td>hCGβ, hCG</td>
<td>IgG1</td>
</tr>
<tr>
<td>CD34</td>
<td>N/A</td>
<td>CD34</td>
<td>IgG1</td>
</tr>
</tbody>
</table>

Table 2.1 mAbs used to analyse surface expressed hCGβ mutants.

All are conformationally-dependant except OT3A which is specific for a linear determinant in the C-terminal extension of hCGβ (hCGβ 133 - 139). All mAbs are produced in BALB/c mice and except OT3A have been described previously (Berger et al., 1990) and are a gift from Dr P Berger (Institute of Biomedical Aging, Innsbruck, Austria), except OT3A which was a gift from Dr Wim Stevens (Organon Technika, Netherlands). The negative isotype control mAb for flow cytometry, QB-END/10 anti CD34 is also described (Quantum Biosystems Ltd., Cambridge). N/D = not done.

<table>
<thead>
<tr>
<th>Polyclonal antiserum used for mutant analysis</th>
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</thead>
<tbody>
<tr>
<td>specificity</td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>MS</td>
</tr>
<tr>
<td>P3</td>
</tr>
<tr>
<td>238</td>
</tr>
<tr>
<td>SighCG</td>
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</tbody>
</table>

Table 2.2 Polyclonal antibodies used for mutant analysis.
2.1.2 Oligonucleotides

Oligonucleotides were ordered either from Oswell (Edinburgh, UK) or from Genosys (Cambridge, UK) and are listed in table 2.3.

<table>
<thead>
<tr>
<th>Use</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>S hCGβ subcloning</td>
<td>ACCGGAATTCCAGGGGCTCCTGTGTTG</td>
</tr>
<tr>
<td>AS hCGβ subcloning</td>
<td>TTGGTCGACTTGTGGAGGATCGGGGTTGTC</td>
</tr>
<tr>
<td>S hCGβ sequencing</td>
<td>GTCTGCGCCGCCGCCCTGCC</td>
</tr>
<tr>
<td>AS hCGβ sequencing</td>
<td>GTTCACCCCGGCGGCG</td>
</tr>
<tr>
<td>S sequencing primer</td>
<td>CTGTCATGTGCACTCTGCGC</td>
</tr>
<tr>
<td>S sequencing</td>
<td>GCCCCCACCATTGCCGCCG</td>
</tr>
<tr>
<td>AS sequencing</td>
<td>GGATGGAATCTCAAGCCGACCATC</td>
</tr>
<tr>
<td>CDM8 sequencing</td>
<td>GTGGGAGGTCTATATAAGCAG</td>
</tr>
<tr>
<td>pHEN S cloning</td>
<td>GAACCATGCTCCAGGGGCTGGCTGTCG</td>
</tr>
<tr>
<td>pHEN S cloning (Ass)</td>
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</tr>
<tr>
<td>MHC S cloning</td>
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</tr>
<tr>
<td>MHC AS cloning</td>
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</tr>
<tr>
<td>Mutant C9 S</td>
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<tr>
<td>Mutant C9 AS</td>
<td>GGGACCGGAGCCGCCGGAGGACCCCTGAGGCTCCCTGGGAGGCTGAGGCTGCCTTCTGTGTCG</td>
</tr>
<tr>
<td>Mutant C23 S</td>
<td>GGGATGAGTCGGCAGTGGCCGGGTACCAGGCTGCCCTGAGGCTGAGGCTGCCTTCTGTGTCG</td>
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<tr>
<td>Mutant C23 AS</td>
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<td>GGCTCCAGGGCTGGCCGGCGGCTGGAGGCTGCCCTGGGAGGCTGAGGCTGCCTTCTGTGTCG</td>
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<td>Mutant C57 AS</td>
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<tr>
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<td>GCCGAGGCGAGGGGTGAGGGCTCTCGGGGCGAGGCTGCCCTGGGAGGCTGAGGCTGCCTTCTGTGTCG</td>
</tr>
<tr>
<td>Mutant C72 AS</td>
<td>CCCGCGCTCCGGCTCGCCGCGGAGGGCAGCCAGGTAAACCGGATGGACTCAAGAGC</td>
</tr>
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<td>Mutant 1 S</td>
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</tr>
<tr>
<td>Mutant 1 AS</td>
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</tr>
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<td>Mutant 2 S</td>
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</tr>
<tr>
<td>Mutant 2 AS</td>
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</tr>
<tr>
<td>Mutant 3 S</td>
<td>GGGTCCCGCTCCAGGGTAAAACCCAGCTCTCAGCGGCTG</td>
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<tr>
<td>Mutant 3AS</td>
<td>CGTGGAGAGCCAGGAGACAGCTGAGGACTGCAAGAGC</td>
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<tr>
<td>Mutant 4 S</td>
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<tr>
<td>Mutant 5 S</td>
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<td>Mutant 5 AS</td>
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<tr>
<td>Mutant 6 S</td>
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<td>Mutant 6 AS</td>
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<td>Mutant 7 AS</td>
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<td>Mutant 8/20 S</td>
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<tr>
<td>Mutant 9 AS</td>
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</tr>
</tbody>
</table>
Table 2.3 Primers used in this study.

2.1.3 *Eschericia coli* (*E*. *coli*) strains

801-H*: *ara* Δ(lac-pro) *nalA* argJΔam *rif* thi1 (tsp):*:kan* *eda*-51::*Tn10* / F’ *lacPl*

801-I*: *ara*14 *leuB*6 *azi6* lacY1 *proC* 14 *tsx* 67 Δ(ompT-fepC) 226 *entA* 403

TB1*: *F* *ara* Δ(lac-pro *AB*) *rpsL* (strr) [φ80 *lacΔ(lacZ)* H15] *thi* *hsdR* (rE*’mK*’)

NF1829*: MC1000/F[lacIq1, *Tn5(KanR)*]

TG1*: Δ(lac-pro) *supE* *thi* *hsdD5/F*’ *traD36* proA*B*’ lacPl *lacZΔM15

HB2151*: *ara* Δ(lac-pro) *thi/F*’ proA*B*’ lacPl *lacZΔM15

XL1-Blue*: recA1 endA1 gyrA96 (NaI) *thi* *hsdR17* (rE*’mK*’) supE44 *relA1* lac / F’ *::Tn10*

JM101*: supE *thi* Δ(lac-pro*AB*) / F’ *traD36* lacPl Δ(lacZ) M15 *pro AB*

* From New England Biolabs (MA, USA);  † General Laboratory stocks;  ‡ Kindly donated by Professor Jan Engberg (Department of Biological Sciences, The Royal School of Pharmacy, Copenhagen Denmark);  § From Stratagene (California, USA).
2.1.4 Mice

Six week old female BALB/c mice were purchased from Olac (Bicester, UK).

2.2 Methods

2.2.1 E.coli techniques

*E.coli* general growth techniques

*E.coli* liquid cultures were grown at 37°C with orbital shaking at 180 r.p.m. (Gallenkamp Orbital incubator) in Luria Broth (LB: 1% NaCl; 1% Bactotryptone; 0.5% yeast extract [culture media from Difco laboratories, ] ) or 2 x TY medium (5% NaCl; 1.6% Bactotryptone; 1% yeast extract), in universal tubes (for 3-10 ml), Falcon tubes (for 10-20 ml) or autoclaved baffled conical flasks (for 50 ml - 1 litre). Cultures were always started from a single well-defined colony grown on an agar (LB or 2 x TY agar) plate. Strains used varied depending on the expression vector. A full list and their genotypes is given in section 2.1.3.

Preparation of competent *E.coli* cells and transformation

*E.coli* was routinely transformed using a method based on the CaCl₂ heat shock method (T.Lund personal communication). 400 µl of an overnight culture was used to inoculate 100 ml of LB containing 2.5 mM KCl; 10 mM MgCl₂; 10 mM MgSO₄. The culture was grown at 37°C 180 r.p.m. until the A₆₀₀ was 0.4 - 0.55. Following incubation on ice for 15 minutes the culture was spun at 3000g for 15 minutes at 4°C. The cell pellet was resuspended into 40 ml of ice-cold buffer 1 (16 mM MES; 100 mM RbCl; 10 mM CaCl₂; 50 mM MnCl₂ pH 5.8) and incubated on ice for a further 15 minutes before being spun at 3000g for 15 minutes at 4°C. The cells were resuspended into 2 mls ice cold buffer 2 (10 mM Pipes; 10 mM RbCl; 75 mM CaCl₂; 15% glycerol v/v pH 6.5) and
aliquoted in lots of 200 µl into a 1.5 ml tube. The cells were either used immediately for transformation or stored at -70°. Frozen competent cells were defrosted for 5 minutes on ice. DNA was added to the cells which were incubated a further 30 minutes on ice before being heat shocked at 42°C for 2 minutes. After 5 minutes on ice, 800 µl LB was added and the bacteria incubated at 37°C for at least 40 minutes. The cells were finally plated onto selective LB agar plates and incubated overnight in a 37°C oven.

**Preparation of E.coli lysate**

The bacteria in a 100 ml overnight *E.coli* culture were pelleted at 3000g for 10 minutes at 4°C, then resuspended in 3 ml TE buffer (10 mM Tris-HCl pH 7.4; 1 mM EDTA). Samples were freeze/thawed four times and then sonicated in 6 x 20 second bursts. Cell debris was pelleted at 18200g for 10 minutes at 4°C, and the translucent yellow supernatant stored in aliquots at -20°C.

**Induction of pMAL fusion proteins**

**Liquid culture**

A single colony was grown in 2 ml LB plus 100 µg/ ml ampicillin for 2 hours, then transferred to 100 ml LB plus ampicillin and grown to an optical density (O.D.) of $A_{600}$ of 0.5. An uninduced sample was taken, spun in at 18200g for 10 minutes at room temperature and frozen at -20°C. Isopropylthiogalactoside (IPTG) was added to a final concentration of 0.3 mM and the culture grown for a further 2 hours. Samples were then analysed by SDS PAGE (see 2.2.4).

**Nitrocellulose filters**

Cultures were grown overnight in a 96 multiwell plate. Nitrocellulose filters were placed on LB agar plates, pre-warmed to 37°C. Using a pinned lid (Nunc /Gibco, Paisley, UK) bacteria were transferred from the plate onto the filters. The filters were left at 37°C until a glassy sheen was just visible, indicating bacterial growth. The filters were transferred to blotting paper soaked in 0.3 mM IPTG. After 6 minutes they were put on LB agar plates with 40 µg/ ml IPTG. They were left to grow for 3 hours. Colonies were analysed by lysing on blotting paper soaked in 5% sodium dodecyl sulphate (SDS) and placed at 95°C for 30 minutes. The bacterial protein was fixed by electrophoresis onto the nitrocellulose
membrane, in the same manner as proteins are transferred from SDS PAGE gels onto nitrocellulose (See 2.2.4). The filters were washed in the wash buffer (PBS [0.1M phosphate buffer; 0.0027 M KCl; 0.137 M NaCl, pH 7.4], 0.1% TritonX100, 0.5% gelatin) for 10 minutes, then in wash buffer plus 10 μg/ml DNAase for 10 minutes, and finally for 10 minutes in wash buffer. The filters were blocked in TBS-T; 5% milk powder (TBS-T: 20 mM Tris-HCl; 137 mM NaCl, pH 7.6; 0.1% Tween 20) and analysed as for western blot.

**Induction of pFAB and pHEN constructs**

A single colony was grown in 2 x TY medium, 100 μg/ml ampicillin, 1% glucose until the O.D. at A600 was 0.5 to 1.0. The cells were then pelleted at 3000g for 5 minutes at 4°C, and resuspended into 2 x TY medium, 100 μg/ml ampicillin, 1 mM IPTG and grown for 16 hours. The protein was then analysed by SDS PAGE or western blot.

2.2.2 Bacteriophage

**M13 filamentous bacteriophage system for producing ssDNA for sequencing**

*Subcloning into M13 phagemids*

Single stranded DNA (ssDNA) was prepared for sequencing the hCGβ cDNA, using the M13mp18 and M13mp19 phagemids (Stratagene). hCGβ cDNA was ligated into the BamH I and Hind III sites of the phagemids from pMALcRIhCGβ clones. The ligations were transformed into JM101 as in section 2.2.1, except that after final incubation on ice, 40 μl 0.1M IPTG was added to a 5 ml bijou to which 200 μl JM101 were added followed by 40 μl of 4% X-gal (in dimethylformamide). 4 ml of top agar kept at 42°C was added to the bacterial mixture which was then plated onto LB agar plates. Plaques were allowed to develop overnight at 37°C.

*ssDNA preparation*

From an overnight culture of JM101 (from a colony on a minimal agar plate selecting for F' episome) grown in LB, 1.5 ml was taken and transferred to 150 ml LB and grown for a further 1 hour. A plaque of M13mp18hCGβ or M13mp19hCGβ phage was transferred
into 1 ml LB and phage allowed to diffuse for 5 minutes before adding 5 ml of the JM101 culture. The infected culture was grown for 8 hours before the cells were pelleted and the supernatant spun again to remove any residual bacteria. A quarter volume of 20% polyethyleneglycol (PEG); 3.5M ammonium acetate was added to the supernatant and the phages precipitated by incubation at 4°C overnight. The phage suspension was then spun at 28000g for 20 minutes at 4°C. The supernatant was removed and the pellet resuspended in TE buffer. Phenol chloroform (25:24:1 phenol : chloroform : isoamyl-alcohol, saturated with TE pH 8.0) extractions were then performed followed by a chloroform extraction (using water saturated chloroform), i.e. an equal volume of phenol/chloroform was added and the sample inverted to mix then spun at 18200g for 15 minutes at room temperature. The upper aqueous layer was transferred to a clean 1.5 ml tube and the extraction repeated until a clean interface was observed. This was repeated with the chloroform. The ssDNA was precipitated with 1/10 vol 3 M sodium acetate and 2.5 volumes ethanol, and pelleted at 18200g, for 10 minutes at room temperature. The ssDNA was then reuspended in 50 μl TE buffer. An equal volume of phenol chloroform was added and the sample inverted to mix then spun at 18200g for 15 minutes at room temperature.

**M13 filamentous bacteriophage system for random mutagenesis**

**Subcloning into M13 phagemids**

hCGβ cDNA was excised from pHENhCGβ via the Hind III and Not I sites. This fragment was then ligated into pBluescript (Stratagene). A Hind III / Sst I fragment from the pBluescript clone was then ligated into M13mp18 and M13 mp19 and transformed. This resulted in hCGβ cDNA in the M13 phagemids with extra DNA sequence 5’ and 3’. This was to provide a larger stretch of double stranded DNA for the enzymes Nco I and Not I to bind when the mutated ssDNA annealed.

**M13 mp18/19hCGβ clones analysed by rDNA preparation**

Replicative form DNA (rDNA) was prepared from the plaques of M13mp18hCGβ and M13mp19hCGβ by a method in Kadonaga *et al.* (1985). An overnight culture of JM101 grown in LB from a colony on a minimal agar plate, was added to 2 ml LB and grown for 1 hour. Single plaques were picked into 500 μl LB and left to diffuse for 1 hour. The
plaque mixture was then added to the JM101 culture and grown for a further 6 hours. The bacteria were then spun at 18200g for 10 minutes at room temperature and the supernatant used to inoculate 200 ml JM101 grown in LB to an O.D. at A<sub>600</sub> of 0.1 - 0.2, then allowed to grow for a further 6 hours. The culture was spun at 8000g for 10 minutes at 4°C. The bacteria pellet were used to prepare rfDNA following a scaled up general mini prep method (section 2.2.3) and the supernatant was stored as stock phage solution at 4°C.

**ssDNA preparation**

The method used is based on Kadonaga et al. (1985). A 1 litre culture of JM101 grown to A<sub>600</sub> 0.1 to 0.2 was inoculated with the M13 stock solution (30 ml) and grown for a further 6.5 hours. The cultures were spun at 8000g for 10 minutes at 4°C and the supernatant transferred to clean tubes. A quarter volume of 20% PEG (6000); 2.5M NaCl was added. Phage were precipitated at 4°C on ice for 30 minutes then pelleted at 11000g for 20 minutes at 4°C, then resuspended in 25 ml TE buffer, pH 8.0. The phage were re-precipitated by adding a quarter volume 20% PEG (6000); 2.5M NaCl and incubating for 15 minutes on ice before pelleting at 17000g, 4°C, 20 minutes. The phage were resuspended in 80 ml of a TE/CsCl solution with a final density of 1.4g/ ml (5.5g CsCl added to 87 ml TE pH 8.0). The phage were spun in a Beckman 70 Ti rotor at 48000 r.p.m for 36 hours at 20 °C. The phage band was collected and dialysed extensively at 4°C against TE, pH 8.0. ssDNA was then isolated from the purified phage preparation by repeated phenol chloroform extractions. The ssDNA was then ethanol precipitated and resuspended in TE, pH 8.0 and stored at 4°C.

**Bacteriophage expression system**

**Preparation of helper phage**

20 mls (approx 4 x 10<sup>12</sup> phage) of helper phage (either R408 or VCSM13) were added to a log phase (A<sub>600</sub> = 0.3), 1 litre of E.coli strain harbouring the F' episome (grown under episome selecting conditions). This gave a multiplicity of infection of approximately 20:1 (phage to cells). The culture was grown for a further 8 hours. Then phage were harvested from the supernatant by spinning at 10000g for 30 minutes at 4°C. Helper phage were then titrated and stored at 4°C.
Recombinant phage production

pHEN 1 constructs: An overnight culture of E.coli strain TG1 with the pHEN 1 construct was grown in 2 x TY, 100 µg/ml ampicillin and 1% glucose. 180 mls of 2 x TY, 100 µg/ml ampicillin, 1% glucose was inoculated with 20 mls of the overnight culture and grown until log phase (A600 = 0.3) was reached. The culture was then spun at 3000g for 20 minutes at 4°C, and the bacteria resuspended in 2 x TY (100 µg/ml ampicillin). Helper phage VCSM13 was added at a multiplicity of infection 20:1. After 1 hour at 37°C, kanamycin was added to 50 µg/ml. The culture was either grown for a further 8 hours or left overnight. The phage were harvested from the supernatant by pelleting the bacteria at 10000g for 20 minutes at 4°C, then adding 1/5 volume of 2.5 M NaCl, 20% PEG (8000), leaving for 1 hour at 4°C then centrifuging at 10000g for 10 minutes at 4°C. The phage pellet was resuspended in 1/100th the original volume of TE pH8.0, and re-spun at 18200g for 5 minutes at room temperature to remove any residual bacteria. The concentrated phage were stored at 4°C.

pFABS constructs: An overnight culture of E.coli strain NF1829 with the pFAB3 construct was grown in LB (with 100 µg/ml ampicillin and 50 µg/ml kanamycin). 180 mls of LB (with 100 µg/ml ampicillin, 50 µg/ml kanamycin) was inoculated with 20 mls of the overnight culture and grown until log phase (A600 = 0.3) was reached. IPTG was added to the appropriate concentration and the culture was grown for a further 30 minutes. Helper phage VCSM13 or R408 was then added at a multiplicity of infection 20:1. The culture was grown for a further 8 hours or left overnight. The phage were harvested from the supernatant as described.

Titration of phage

A single colony of XL-1 Blue E.coli strain was grown in LB for 4 - 5 hours. Dilutions of the phage were made in LB. 100 µl of diluted phage were added to 200 µl of the XL-1 Blue cells. This was left for 30 minutes at 37°C, then 300 µl LB was added and it was grown for a further hour. The bacteria were then pelleted at 18200g for 10 minutes at room temperature and resuspended in 100 µl LB. They were then either plated onto LB agar plates with ampicillin and tetracycline to select for transducing units or had 5 ml top agar added and were then plated on LB agar plates for plaque forming unit titration.
2.2.3 Handling of DNA

**Plasmid Preparation**

Depending on the yield and quality of plasmid DNA required, one of a variety of preparative methods was used:

*General mini prep*

For yields of 10 - 30 μg DNA for restriction digests, transformations and sequencing.

A 5 ml overnight culture was spun at 3000g for 15 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 300 μl Maxi buffer (50 mM glucose; 25 mM Tris-HCl pH 7.5; 10 mM EDTA). The cells were transferred to a 1.5 ml tube. 600 μl Lysis buffer (0.2M NaOH; 1% SDS) was added and 300 μl 3M potassium acetate. The tube was inverted until a clear lysate was observed. The lysate was spun at 18200g in a microfuge for 6 minutes at room temperature. The clear supernatant was transferred to a clean tube. RNaseA was added to a final concentration of 50 μg/ml and incubated at 37°C for 30 minutes. Phenol chloroform and chloroform extractions were carried out as described then the DNA was precipitated by the addition of 1/10 v/v 3 M sodium acetate and 2.5 volumes ethanol. The prep was spun at 18200g for 10 minutes at room temperature. The supernatant was poured off and the pellet washed in 70% ethanol and resuspended in 50 μl TE buffer.

*Promega Wizard mini prep columns*

For yields of 10 - 50 μg DNA for restriction digests, transformations, sequencing, this method was used on occasions for convenience. DNA preparation from a 3 ml overnight *E.coli* culture carried out as per manufacturer’s instructions. Briefly the culture was spun at 3000g for 10 minutes at 4°C and the cell pellet resuspended in 200 μl resuspension buffer (50 mM Tris-HCl, pH 7.5; 10 mM EDTA; 100 μg/ml Rnase A) and transferred to a 1.5 ml tube. 200 μl of Lysis buffer was added to the cell suspension and the tube inverted until a clear lysate was observed. 200 μl 1.32 M potassium acetate, pH 4.8 was added to the lysate and the tube again inverted to mix. The lysate was spun in at 18200g for 6 minutes at room temperature and the clear supernatant transferred to a fresh tube. 1 ml of resin was added to the supernatant and the tube inverted repeatedly for 20 seconds.
This resin/DNA mix was then passed through a mini column which binds the DNA. 2 ml wash buffer (83 mM NaCl; 8.3 mM Tris-HCl, pH 7.5; 2.08 mM EDTA; 58% ethanol) was passed through the column, and the DNA eluted with 50 μl TE.

Maxi Prep

CsCl Method: A 1 litre *E. coli* culture was set up from a smaller 5 ml culture and grown overnight at 37°C 180 r.p.m. The culture was spun at 3000g for 30 minutes at 4°C. The bacterial pellet was resuspended in 40 ml MAXI buffer, then lysed with 80 ml Lysis buffer and 40 ml 3M potassium acetate added. This was then spun at 3000g for 20 minutes at 4°C. The supernatant was filtered through nylon into a clean centrifuge bucket and 100 ml isopropanol added. After a brief mix the prep was spun at 3000g for 30 minutes at 4°C. The pellet was washed with 70% ethanol and resuspended in 5 ml TE buffer. This was transferred to a universal and TE buffer added until a weight of 9.0g was reached. 10.0g CsCl was added and 1.0 ml ethidium bromide (5 mg / ml). This was then transferred to a Beckman quick-seal tube (Cat No 342413) and spun in a Beckman ultra centrifuge in Ti 70 rotor at 35000 r.p.m. for 48 hours at 20°C. The bottom band containing supercoiled plasmid DNA was removed with a needle and syringe into a universal which already contained 5 ml sterile distilled H₂O (sd H₂O). 2.5 volumes of ethanol was added and spun at 3000g for 15 minutes at 4°C, the pellet was washed with 70% ethanol and resuspended into 800 μl TE buffer. Phenol / chloroform extractions were carried out as described until a clear interface was obtained, followed by a chloroform extraction. The DNA was precipitated with 1/10 v/v 3M sodium acetate and 0.6 volume isopropanol, spun at 18200g for 10 minutes at room temperature, the pellet washed and resuspended into 360 μl TE buffer and then an ethanol precipitation carried out as previously described. The final DNA pellet was washed in 70% ethanol and then resuspended into 1 ml TE buffer.

PEG method: Carried out as for CsCl method until the first isopropanol precipitation. The resulting pellet was washed in 70% ethanol and resuspended in 3 ml TE buffer. The solution was transferred to a 12 ml polyallomer tube (Beckmann Cat no. 331372) and an equal volume of 5 M LiCl added. The tube was inverted to mix and spun at 18200g for 10 minutes at 4°C. The DNA was precipitated by mixing the supernatant with an equal volume of isopropanol and pelleting at 18200g for 10 minutes at 4°C. The DNA pellet
was resuspended into 400μl TE buffer; 20 μg/ ml RNaseA and left at room temperature for 30 minutes. A PEG precipitation was carried out by adding an equal volume of 13% PEG; 1.6 M NaCl. The tube was inverted to mix and then spun at 18200g for 10 minutes at 4°C. The pellet was washed in 70% ethanol and then resuspended into 400μl TE buffer. Phenol chloroform extractions were then carried out as described until a clear interface was obtained and followed by one chloroform extraction. Finally an ethanol precipitation was carried out as described and the pellet washed in 70% ethanol and resuspended into 1 ml TE.

**Qiagen Columns:** For nucleic acid immunisation DNA was prepared on Qiagen columns (Qiagen, CA, USA). Cultures were grown and bacterial cells pelleted as for other maxi prep methods. The bacterial cells were resuspended in 5 ml of buffer P1 for every 100 ml of LB culture (P1: 50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 μg/ ml RNase A). An equal volume of buffer P2 was added to the cell suspension (P2: 200 mM NaOH; 1% SDS), the sample was mixed gently and incubated at room temperature for 5 minutes. The same volume of pre-chilled buffer P3 was added and the sample immediately mixed gently and incubated on ice for 20 minutes (P3: 3 M potassium acetate, pH 5.5). The sample was mixed again before centrifuging at 3000g for 30 minutes at 4°C. The supernatant was removed to fresh tubes and re-centrifuged under the same conditions. The supernatant was then transferred to clean tubes and 0.7 volumes of isopropanol was added and the sample was mixed gently and spun at 3000g for 30 minutes at 4°C. The DNA pellet was then resuspended in a small volume of TE buffer, pH 7.0 and buffer QBT added to the appropriate volume for the column size (QBT: 750 mM NaCl; 50 mM MOPS, pH 7.0; 15% ethanol; 0.15% Triton X-100). The sample was then added to the column which had previously been equilibrated with the appropriate volume of buffer QBT for the column size. After all the sample had passed through, the column was washed with an appropriate volume of wash buffer QC (QC: 1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% ethanol). The DNA was eluted with buffer QF (1.25 M NaCl; 50 mM Tris-HCl, pH 8.5; 15% ethanol). The eluate was precipitated with 0.7 volumes of isopropanol as before, and the pellet washed with 5 ml of 70% ethanol. The air-dried pellet was finally resuspended in normal saline at a concentration of 1 mg/ ml. The DNA sample was frequently collected after it had passed through the column and re-applied to the same column which was re-
equilibrated. This often resulted in the same amount of DNA as was obtained after the first run through the column.

**Restriction enzyme digests**

Typically 0.5 - 1μg plasmid DNA was digested with 5 units of the specific enzyme in the presence of the appropriate buffer (provided by manufacturer) and 0.1 mg/ml bovine serum albumin where necessary, normally to a final volume of 20μl. Digests were carried out overnight in a 37°C oven or for 3 hours in a 37°C oven unless the manufacturer stated otherwise.

**Electrophoresis**

DNA size analysis was achieved by running the DNA sample in agarose gels alongside DNA size markers. For general analysis the buffer used in the gel and for running was 1x TBE buffer (100m Tris; 100 mM Boric acid; 1.25 mM EDTA) with ethidium bromide at 0.5µg/ml in the gel. Typically the size marker would be 1 μg λ DNA Hind III / EcoRI, with 20% Ficoll / Orange G used as a loading dye. Gels were run at 5 V/cm. Visualization was achieved using an UV transilluminator and photography using Polaroid film type 667 and an orange filter. When DNA was to be eluted from the gel, the buffer used was 1x TAE (40 mM Tris; 1 mM EDTA, pH 8.0 adjusted with glacial acetic acid). The band was visualized under U.V. and cut out with a clean scalpel. It was then melted at 70°C for 5 - 10 minutes. Then the Promega Wizard PCR purification kit was used (Promega, Madison, USA), following manufacturer’s instructions for purifying DNA samples run on agarose gels. Briefly, the excised gel band was melted at 70°C for 5 minutes, then 1 ml of PCR purification resin was added. This was mixed for 20 seconds and then pushed through a wizard mini column 2 ml of 80% isopropanol was used to wash through the column and then the DNA was eluted using 50 μl of TE.

**Subcloning**

Genes were subcloned into vectors either by direct excision from one plasmid into another, or by PCR amplifying with primers to include restriction sites. Cloning vectors were made as described below and were phosphatased when necessary. PCR fragments were kinased if they were to be blunt cloned into a plasmid. After ligation of insert to plasmid, the
plasmids were transformed into bacteria, and mini preps performed on the selected clones. These were analysed by restriction digest and all constructs were fully sequenced using the sequencing primers listed in table 2.3.

Cloning vectors

5 - 10 μg of the vector was digested with the appropriate restriction enzyme(s). Phosphatasing was done at this stage. The reaction mix was then run on a 1% TAE agarose gel. The appropriate band was eluted from the gel. Cloning vectors were stored at -20°C.

Ligations

Restriction fragments were joined using T4 DNA ligase. Fragments were incubated in ligase buffer (50 mM Tris-Cl, pH 7.8; 10 mM MgCl₂; 10 mM dithiothreitol; 1 mM ATP; 25 μg/ml BSA), at a DNA concentration 0.1 to 1 μM in 5' termini. Ligation mixes were routinely left overnight at 16°C. When subcloning, positive and negative ligation controls were set up. The positive ligation control included cloning vector, buffer, and ligase but no insert. This would indicate the level of self-ligation of the vector. The negative ligation control included cloning vector and buffer only. This indicated the level of any contaminating undigested cloning vector in the preparation.

Phosphatasing

To remove 5' phosphate groups from DNA to prevent ligation. Calf intestinal phosphatase (CIP) was added to the DNA at 0.1 unit per 0.1 pmol DNA ends in either CIP buffer (50 mM NaCl; 10 mM Tris-Cl, pH 7.9; 10 mM MgCl₂) or in a restriction enzyme buffer, and incubated at 37°C for 30 minutes. The CIP was inactivated by heating to 70°C for 10 minutes.

Phosphorylation

To add phosphate groups to the 5' ends of oligonucleotides or PCR products prior to ligation. DNA was incubated in phosphorylation buffer (70 mM Tris-Cl, pH 7.6; 10 mM MgCl₂, 5 mM dithiothreitol) at 37°C for 30 minutes in the presence of T4 polynucleotide kinase. The enzyme was heat-inactivated at 65°C for 20 minutes.
DNA sequencing - single stranded and double stranded

All sequencing reactions were based on the dideoxynucleotide chain termination method, using the Sequanase Kit Version 2.0 (USB, Ohio, USA). The methods followed were based on those described in the manufacturer’s instructions:

**Single stranded DNA sequencing reactions**

3 μg single stranded DNA was mixed with 2 μl of Sequanase® 5 x reaction buffer (200 mM Tris-HCl pH 7.5; 100 mM MgCl₂; 250 mM NaCl) and 3 pmol primer to a final volume of 10 μl. The mix was then heated to 65 °C for 2 minutes and allowed to cool for 10 minutes at room temperature. Then 1 μl of 0.1 M dithiothreitol, 2 μl of the diluted labeling mix (7.5 μM dGTP; 7.5 μM dCTP; 7.5 μM dTTP), 0.5 μl (0.185MBq) S³⁵ dATP (Deoxyadenosine 5’-[α-Thio]triphosphate,[³⁵S]. NEN DuPont, Dreieich, Germany) and 2 μl (1.2 units) of the diluted Sequanase enzyme were added. This mix was incubated at room temperature for 5 minutes and then 3.5 μl of the mix was added to each of four tubes containing 2.5 μl (8 μM of dideoxy and 80 μM of four deoxynucleotides) of each of the four dideoxynucleotides, previously incubated at 37°C. The four mixes were incubated at 37 °C for 5 minutes then the reaction was stopped with 4 μl of Stop mix (95% formamide; 20 mM EDTA; 0.05% bromophenol blue; 0.05% xylene cyanol).

**Double stranded DNA sequencing reactions**

3 - 5 μg double stranded DNA was mixed with 2 μl 2 M NaOH in a final volume of 10 μl. This was heated at 65 °C for 5 minutes and then allowed to cool at room temperature for 10 minutes. Then 3 pmol primer, 3 μl 3 M sodium acetate, and 75 μl ethanol were added. 75 μl ethanol was added and the mix incubated on a dry ice/ethanol bath for 20 minutes. The precipitated DNA was then spun at 18200g for 10 minutes at room temperature. The pellet was washed with 70% ethanol and then resuspended in 7.5 μl sd H₂O. 2 μl of the Sequanase 5 x reaction buffer, 1 μl 0.1M DTT, 2 μl diluted labeling mix, 0.5 μl S³⁵ dATP and 2 μl diluted Sequanase was then added. This was incubated at room temperature for 5 minutes and then the reaction was continued as described for ssDNA sequencing after the room temperature incubation.

The reactions were run on a 6% acrylamide gel with either 1 x TBE or 0.5 x TBE as the buffer. the gels were fixed in 10% acetic acid, 10% methanol before being vacuum dried at 80°C for 15 minutes. Gels were then exposed to Fuji RX X-ray film for 1 - 5 days.
Polymerase Chain Reaction

Based on original method of Saiki et al. (1985). The conditions for each amplification reaction were calculated separately, all were based on the following general method. In a sterile 0.5 ml microfuge tube these reagents were added:

- **template DNA**: 50ng-2 μg
- **primer 1 (sense)**: 100pmoles
- **primer 2 (antisense)**: 100pmoles
- **Taq or Pfu DNA polymerase**: 1 unit (Taq - Promega, Pfu - Stratagene)
- **10 x buffer**: 5 μl
- **MgCl₂ (if not included in buffer)**: 2.5 mM (titrated for optimum)
- **dNTPs**: 0.2 mM
- **sd H₂O**: to a final volume of 50 μl.

The reaction mixture was overlaid with 100 μl light mineral oil. A typical amplification would be:

94°C, 4 minutes, x 1 cycle; 94°C, 1 minute, (denaturing), 55°C, 1 minute (annealing), 72°C, 2 minutes, (polymerisation), x 25 cycles; 72°C, 4 minutes, x 1 cycle; 4°C soak until samples analysed.

Typically 5 μl of the final reaction was analysed on an agarose gel. For purification a low melting point TAE gel was used in conjunction with Promega Wizard PCR prep kit.

Using PCR to subclone into a different plasmid

PCR was used to facilitate subcloning into different plasmids. Where restriction sites needed to be introduced into the 5’ and 3’ ends of the DNA being subcloned, these sites would be included in the 5’ end of the sense and antisense PCR primers. Sufficient bases would be added after the restriction site to allow for efficient cleavage of the restriction endonuclease. A list of the primers used for different subclonings is given in table 2.3.

RT PCR

cDNA produced from a suitable mRNA source was used as a template in a PCR reaction. Gene-specific primers were used and an optimized annealing temperature in order to specifically amplify the gene of interest. The primers used to amplify specific genes from total cDNA are shown in table 2.3.
**Site directed mutagenesis by overlap PCR**

Based on a method by Horton and Pease (1991). Two primer sets were designed. In set A the sense primer was specific for a region 5' of the polylinker in the plasmid containing the gene to be mutated. Similarly in set B the antisense primer was specific for a region outside of the polylinker but to the 3' end. The antisense of set A and the sense of set B were designed to cover the region where the mutation was introduced and to have a complementary overlap of at least 16 nucleotides (Figure 2.1). Two PCR reactions were set up, with one using primer set A and the other using primer set B. The resulting products were then mixed and used as the templates in another PCR reaction for the overlap extension where the external primers were included to amplify the product. The products were cleaned on a low melting point gel and the Promega PCR purification kit.

**Random mutagenesis**

Based on Kadonaga et al. (1985). The mutagenesis buffer pH 5.0 was freshly made by adding 0.084g methoxylamine hydrochloride (Sigma, Dorset, UK) to 1 ml of 1M sodium acetate buffer pH 5.5. 150 μl of this mutagenesis buffer was immediately added to 10 μg M13mp18hCGβ ssDNA (in 50 μl of 2 mM Tris-HCl buffer, pH 8.0; 0.2 mM EDTA) giving a final volume of 200 μl and a final methoxylamine concentration of 0.75M. M13mp19hCGβ was prepared in the same way and the two solutions were incubated in the dark at 50°C under mineral oil for 105 minutes. The majority of the oil was removed with a cut yellow tip and then with a chloroform extraction. The aqueous phase was transferred to a clean tube and left at 37°C to allow the remainder of the chloroform to evaporate. The two samples were mixed together and ethanol precipitated. The DNA was resuspended into 150 μl TE and digested with 100 units Nco I and Not I. The resulting fragment was subcloned into pHEN1.

**cDNA production**

The hCGβ cDNA was synthesized from a mRNA of third trimester placenta (kind gift of Dr Sally Pemble, Molecular Toxicology, Dept. of Biochemistry and Molecular Biology, UCL). 5 μg mRNA was incubated at 37°C for 90 minutes in a solution containing: 50 mM Tris-HCl, pH8.3), 8 mM MgCl2, 10 mM dithiothreitol, 0.4 mM oligo(dT), 0.9 mM dNTPs, and 50 units of moloney murine leukemia virus reverse transcriptase.
The wild-type hCGβ sequence is indicated by the blue lines. Two separate PCR reactions are performed, with one using the green primers (set A) and the other the orange primers (set B). The mutant sequence is incorporated into the primers and hence into the two products (indicated by red area). These two products (marked A from primer set A, and B from primer set B) are then both used in a third PCR reaction where together with the outer primers of set A and B, they prime the synthesis of the complete mutated hCGβ sequence.
The MHC transmembrane fragment was made in a similar manner, but using a liver RNA preparation from C57BL/10 mouse (prepared by Julius Kieskiwicz, Department of Immunology, UCL, London).

### 2.2.4 Analysis of proteins

#### SDS Page gels

Polyacrylamide gels were made using Protogel solution (30% w/v acrylamide, 2.7% crosslinked with bisacrylamide (37.5:1 ratio), National Diagnostics, Hull, UK). The appropriate percentage separating gel was made by varying the amount of Protogel used (separating gel was 0.375M Tris-HCl pH 8.8; 0.1% SDS, stacking gel was 5% acrylamide, 0.126M Tris-HCl pH 6.8, 0.1% SDS), using a bottom running buffer of 20 mM Tris-HCl, pH 8.8 and the top buffer (25 mM Tris; 192 mM glycine; 0.2% SDS). Samples were diluted to appropriate concentration and 2 x loading buffer added (125mM Tris-HCl, pH 6.8; 20% glycerol v/v; 2% β-mercaptoethanol v/v; 0.1% bromophenol blue w/v 0.1% SDS w/v). Samples were incubated at 100 °C for 5 minutes before loading. Appropriate protein size markers were included (e.g. Amersham, Rainbow protein markers, molecular weights 14,300 - 200,000). Gels were run until the dye front was at the bottom and were then either stained for 30 minutes in Coomassie stain (0.2% Coomassie in 40% methanol 7% acetic acid) or transferred onto nitrocellulose by electrophoresis for western blot. The stained gel was destained for several hours in destain buffer (45% methanol; 10% acetic acid).

#### Western Blots - ECL and Alkaline phosphatase

The protein gel was equilibrated in transfer buffer (24.7 mM Tris, 246 mM NaCl, 20% methanol) for half an hour before being transferred by electrophoresis onto nitrocellulose (Hybond-C, Amersham, UK). Transfer was confirmed by staining in 0.1% ponceau red (in 5% acetic acid) and by staining the marker lane in 0.1% amido black.

Filters were blocked in 5% milk powder in TBS-T for 1 hour with shaking at room temperature. Then filters were washed with three 10 minute incubations in TBS-T buffer.
Antibody incubations were done in TBS-T buffer for 1 hour each, washing in between with three 10 minute washes. Visualization was achieved using the Amersham ECL Western blotting kit. Equal volumes of solution 1 and solution 2 were mixed and the filter immersed in the mixed solution for 1 minute. The filter was then exposed to Kodak X-OMAT-AR film for differing lengths of time.

Radio immunoassays to detect hCGβ on the surface of recombinant phage

mAb INN-hCG-58 was diluted to 1:250000 in RIA buffer (PBS; 1% BSA; 0.05% Tween 20) and 50 µl aliquoted per reaction tube. 250 µl of sample was then added and 100 µl 1²²hCG (diluted to 25000 counts / 100 µl, NEN DuPont, Dreieich, Germany). 150 µl anti-mouse IgG beads (Tachisorb R Immunoadsorbent S.aureus beads, coated with rabbit anti mouse IgG, Calbiochem) were added to the reaction which was then slowly mixed for 2 hours. The mix was then briefly spun (2 minutes 18200g, room temperature), the supernatant was removed and the beads were washed twice in RIA buffer and finally the radioactivity of the pellet measured.

ELISA to detect hCGβ on the surface of recombinant phage

ELISA plates (Elisa plates maxisorp Nunc Gibco (Paisley, UK)) were coated with 100 µl/well of the capture antibody at 5 µg/ml in carbonate-bicarbonate buffer, pH 9.6 (0.286% w/v NaHCO₃; 0.17% w/v Na₂CO₃) at 4°C overnight or 37°C for 1 hour. The wells were washed five times with PBS; 0.05% Tween 20. The wells were blocked with 200 µl of 4% milk powder in PBS; 0.05% Tween 20 for 1 hour at room temperature. The wells were washed as before and 100 µl of the phage sample added and incubated at room temperature for 1 hour. After washing 100 µl of the sheep anti M13 polyclonal antiserum (5Prime 3Prime Inc, CO, USA) diluted in the blocking buffer (1/1000 - 6 µg/ml) was added for 1 hour at room temperature. After washing, there was a final incubation of peroxidase conjugated donkey anti-sheep IgG (whole molecule, affinity isolated, Sigma) for 1 hour room temperature and after the final washes the 100 µl of the substrate (3,3',5,5'-Tetramethylbenzidine : 5mg in 500 µl dimethylsulphoxide (DMSO) added to 50 ml 1 x acetate citrate buffer (citrate buffer: 0.5 M sodium acetate adjusted to pH 6.0 with 0.5 M citric acid) to which 37 µl of H₂O₂ was added) was added. After the reaction had
proceeded sufficiently it was stopped by the addition of 50 µl 12% H₂SO₄. The optical density (O.D.) was measured at A₄₁₀.

**Analysis of mouse sera**

Before testing the mouse sera, all the conformationally-dependant mAbs used in this study were assessed for their ability to recognise direct coated hCG and hLH protein, as the protein may partially denature on contact with the ELISA plate. All the mAbs strongly recognised the direct coated hCG at both 1 µg/ml and at 5 µg/ml (data not shown). This implies that amongst the coated population of hCG molecules a wide range of antigenic areas were accessible. mAb INN-hCG-32 was titrated to 1/32000 for use as an internal control when analysing the sera. mAbs INN-hCG-64 and INN-hCG-68 (which, being hCGβ-specific, should not recognise heterodimer) also recognised the hCG to a lesser extent. This implies that there is free beta-subunit in the preparation. The mAbs tested with the directly coated hLH protein also gave a strong signal (data not shown).

ELISA plates were coated with 100 µl/well of either hCG or hLH (hCG from pregnancy urine, hLH from pituitary extracts, Sigma) at 1 µg/ml in carbonate-bicarbonate buffer, pH 9.6, and left overnight at 4°C. In between each incubation the wells were washed 3 times with PBS; 0.05% Tween 20. After blocking with 2% milk powder in PBS: 0.05% Tween 20 for 1 hour at 37°C, 100 µl of 1/100 dilution of the mouse sera was added per well and incubated at 37°C for 2 hours. The detection antibody was a 1:6000 dilution in block buffer of alkaline phosphatase conjugated goat anti-mouse IgG (Fc-specific, Sigma), 100 µl was added and incubated for 1 hour at 37°C. Finally the substrate (p-Nitrophenyl Phosphate 1.0 mg/ml in carbonate-bicarbonate buffer containing 0.5 mM MgCl₂) was added and the O.D. measured at A₄₁₀.

2.2.5 Transfections

**Calcium chloride**

COS7 cells were transfected using a CaCl₂ method (based on Gorman, 1988). Briefly, 1.5 x 10⁶ cells were seeded into an 80cm² flask on the day before transfection. The following
day the culture medium was replaced with fresh medium. The DNA mixture was prepared using stock solutions warmed up to room temperature. For each flask transfected a solution consisting of 10 μg DNA, 93 μl 2 M CaCl₂ and 50 μl H₂O to 750 μl was added dropwise to a tube containing 750 μl 2 x HBS pH 7.1. The translucent precipitate which forms was added to the medium in the flask of cells and left on the cells for either 4 hours or 48 hours. 48 hours was found to result in better transfection efficiency. The medium was then removed and the cells washed in serum-free medium. A glycerol shock was then performed by adding 1.5 ml 15% glycerol/HBS per flask for 2 minutes at 37°C (HBS: 0.82% NaCl; 5.94% Hepes; 0.2% Na₂HPO₄). The cells were washed in serum-free medium and fed with complete medium. The cells were harvested 72 hours post-transfection. The transfection efficiency varied between 5 and 20%. This method was used for the glycine/serine mutants.

**DEAE Dextran transfection**

The transfection method using a combination of dextran and chloroquine is very toxic to the cells. The protocol followed (Aruffo, 1994) was optimised to get the highest efficiency but with an acceptable viability. Briefly, 1.5 x 10⁶ cells were seeded into an 80cm² flask on the day before transfection. Six ml of the transfection mixture, (10% NuSerum (Becton Dickinson, MA, USA), 1-2 μg/ml supercoiled DNA [CsCl or PEG prepared] and 250 μg/ml DEAE dextran [average molecular weight - 500000]) was added to the washed monolayer and left in a 37°C incubator for 60 minutes. Chloroquine was then added to a final concentration of 200 μM and the cells incubated for a further 120 minutes. The transfection mixture was then removed, the monolayer washed with PBS and 3 ml of 10% DMSO (in PBS) added for 2 minutes. The cells were washed again and complete medium added (Dulbecco’s Modified Eagle Medium [Gibco BRL, Paisley, UK], 10% foetal calf serum [Sigma]). The cells were split 1:1 24 hours later and harvested 65 - 72 hours after transfection. A transfection efficiency of 20 - 50% was routinely obtained. N.B. the method was altered slightly for Mutants 11 - 21, the DNA, DEAE dextran and chloroquine were all added at the same time for 120 minutes. Also as explained below, the incubation period varied with different transfections.
Optimising chloroquine concentration and exposure time

Various concentrations of chloroquine, and exposure times to the dextran/chloroquine/DNA mixture, were used (Figure 2.2). Increasing the chloroquine concentration and exposure time both increased the transfection efficiency but lowered cell viability. The best compromise was established as 200 μM chloroquine and 90 minutes exposure. With this method an efficiency of 20 - 50% was routinely obtained. It was noticed that the transfection efficiency seemed to decrease with the passage of the cells. It was essential that the cells were continually monitored for viability and that the exposure time to the transformation mixture was altered accordingly to maintain transfection efficiency.

2.2.6 Tissue culture techniques

Maintenance of cell line

COS7 cells were cultured in Dulbecco’s Modified Eagle Medium supplemented with 10% Foetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine. Cells were grown in a humidified 37°C incubator with 5% CO₂. Cells were routinely grown in 80cm² flasks and were split when the cells were at a density of 3 x 10⁶/flask or greater.

Freezing down and defrosting cells

Cells were spun down at 700g for 5 minutes at 4°C, and resuspended at 1 x 10⁶ cells/ml in cold foetal calf serum, 10% DMSO, then aliquoted into cryotubes. These were then put overnight at -70°C then transferred to a liquid nitrogen tank. Cells to be recovered were removed from liquid nitrogen and placed in a 37°C water bath to thaw quickly. When thawed the cells were transferred to a 20 ml universal container and 9 ml of medium added dropwise to the cells. The cells were then spun (room temperature at 700g for 5 minutes), and the cells resuspended into complete medium and transferred into a flask.

Counting cells and determining viability

A 10 μl aliquot of cells was added to 10 μl of a 0.4% trypan blue solution. The cells were then placed on a haemocytometer and at least 100 cells counted. Those cells which
Figure 2.2 Optimising chloroquine concentration and exposure time in DEAE dextran transfection of COS7 cells.

COS7 cells were transfected with pCDM8hCGB with either 200μM or 400μM chloroquine by the DEAE dextran method. The dextran/chloroquine mixture was left on the cells for either 45 min, 90 min, 120 min or 150 min. The cells were analysed 72 hours post transfection by staining with anti CD34 as negative control or with mAb INN-bLH-1 and analysed by flow cytometry. The number of viable cells for each of the transfection conditions per 80 cm² flask is written above the bars.
excluded the trypan blue were counted as live cells and those which took up the dye were counted as dead cells

2.2.7 Facs analysis

**Single indirect staining of cells for flow cytometry**

Cells were stained in duplicates of $2 \times 10^5$ viable cells for each mAb tested. Harvested cells were washed with 10% foetal calf serum, 0.02% NaN₃ in PBS. They were incubated with 100 μl of mAb for 30 minutes on ice, washed twice in PBS; 0.02% NaN₃ and then incubated with 100 μl of fluorescein isothiocyanate (FITC) conjugated F(ab')₂ rabbit anti-mouse Ig (Dako, High Wycombe, UK). Following washing the cells were fixed in 1% formaldehyde in PBS, and Facs analysis performed using a Becton-Dickinson Facscan. Markers were set on the negative control which was routinely an anti-CD34 IgG1. All cells to the right of this marker were deemed to be positively transfected. All mAbs were titrated in order to give roughly the same staining intensity.

**Double staining**

As for single staining except that after incubating the cells with the mAb, the second incubation was with a FITC conjugated F(ab')₂ goat anti-mouse IgG (Fc specific) polyclonal serum. The cells were then incubated with the polyclonal antibody and then with a R-phycoerythrin conjugated goat anti-rabbit IgG affinity isolated antibody (Sigma).

2.2.8 Nucleic acid immunisation

DNA was prepared with Qiagen columns and resuspended in normal saline (0.9% NaCl) at 100 μg/ml. A week prior to the commencement of DNA vaccinations the 6 week old female BALB/c mice were given 50 μl cardiotoxin in the tibia anterialis muscle of each leg. The cardiotoxin causes a local necrosis of the muscle tissue, when the cells divide to repair the tissue the uptake of the DNA is presumed to be facilitated (Davis et al., 1993a). All DNA immunisations were given in the same muscle, with 50 μl DNA injected into each
leg. Four immunisations were given, 2 weeks apart. Blood samples were taken by tail bleeding the day before each injection. The animals were culled by terminal anaesthesia two weeks after the final injection and the mice exanguinated by cardiac puncture. Serum samples were prepared by clotting at 4°C for 3 hours, followed by centrifuging at 18200g for 10 minutes at room temperature. Serum was removed to a fresh tube and if necessary the spin was repeated to remove any red blood cells carried over.

N.B. The injections and tail bleeds were performed by Dr Brian De Souza (Department of Immunology, UCL, London) or the personnel of the Biological Services division (UCL, London).
Chapter 3

Prokaryotic expression of hCGβ
3.1 Introduction

The prokaryotic expression of hCGβ was investigated to determine a suitable system for the analysis of mutant hCGβ molecules. First a mutagenesis method had to be established to generate molecules which may be analysed for the loss of B-cell epitopes. At the start of this investigation the three-dimensional structure of hCGβ was not known. Random mutagenesis was therefore employed to analyse residues important in epitope formation. Random mutagenesis using degenerate PCR primers has been used previously to successfully identify epitope-loss mutants of CD2 (Peterson et al., 1987). Random mutagenesis has also been achieved by UV light (Drobetsky et al., 1993; Poirier et al., 1993), DNA polymerases which lack proof-reading activity (Liao et al., 1990), PCR with nucleotide derivatives (Ueda et al., 1995) or chemicals such as N-methyl-N'-nitro-N-nitrosoguanidine (Potvin et al., 1991), hydroxylamine (Kang et al., 1990) or methoxylamine (Kadonaga et al., 1985). In this investigation methoxylamine was used and its suitability for random mutagenesis of hCGβ assessed.

The analysis of mutant hCGβ molecules has certain requirements. The hCGβ must be expressed in a system that allows, 1) correct folding of the native molecule, 2) ease of production and expression of mutants, 3) simple and rapid screening of mutants with a panel of mAbs. Prokaryotic expression of foreign protein has been used for over 20 years (Cohen et al., 1973) and the use of E.coli as a host species is well established. The mechanisms for controlling gene expression in this well characterised organism are reasonably understood. There are many laboratory strains which are known to stably maintain plasmids. Several systems are commercially available, catering for a variety of expression requirements and E.coli does tend to very efficiently over-express cloned genes. E.coli also has a short doubling time and thus experiments can be done relatively quickly. The different types of expression systems for foreign proteins cater for different needs. Some produce a chimeric recombinant protein with the desired protein fragment fused with subunits of other proteins. The resulting fusion proteins have particular properties which for example may facilitate its purification, or allow isolation of the mutant
gene with the mutant protein. Two different prokaryotic expression systems were explored here, pMAL and phage display.

In the pMAL system the cloned gene is inserted downstream of the malE gene in a translational reading frame. The malE gene encodes maltose binding protein (MBP). When expression is induced from the efficient P_{lac} promoter, a fusion protein results consisting of the cloned gene product with MBP at its amino terminus (Maina, 1988). This allows the recombinant protein to be purified from E.coli proteins using a maltose column. There is a cleavage site for Factor Xa between the MBP gene and the cloned gene so that the MBP can in principle be removed from the protein of interest (see figure 3.4).

By using different vectors, the fusion protein may be directed to the bacterial periplasm or be retained in the cytoplasm. The pMALp vector has the full length signal sequence of the malE gene which directs the fusion protein to the periplasm. In pMALcRI this signal sequence is deleted so that fusion proteins are retained in the cytoplasm. Exporting the fusion protein to the periplasm may be advantageous for proteins which require the formation of disulphide bridges for correct folding, as these may not be formed in the reducing environment of the cytoplasm. hCGβ, which has six disulphide bonds, may be one such molecule.

The M13 filamentous bacteriophage system was also examined. This system was first used in 1985 (Smith, 1985). The protein of interest is cloned into a phagemid between the phage pelB leader sequence and the phage gene III. Gene III encodes a phage coat protein gIIIp, present in three to five copies at the tip of the bacteriophage (Goldsmith et al., 1977). The gIIIp/cloned gene fusion protein is directed to the periplasm by the pelB leader sequence. Upon superinfection with helper phage, the fusion protein is assembled into the phage coat along with the native gIIIp encoded by the helper phage and a single stranded copy of the phagemid may be packaged into some of the phage particles (Figure 3.1). The secreted phage have on their surface a mixture of normal gIIIp and gIIIp fusion protein. The bacteriophage can then be enriched for phage expressing the fusion protein by panning against specific antibodies. This system could be adapted for screening
After growth of E.coli culture containing the phagemid to log phase, there are slight differences in the two systems. In the pHEN system the glucose repression is removed and helper phage are added. These superinfect the bacteria via the sex pilus and ssDNA replication is initiated. The gIIIP fusion protein is produced and transported to the periplasm where it is incorporated with native phage proteins encoded on the helper phage. Recombinant and wild type phage are assembled and secreted. The pFAB system differs only in that IPTG is added to induce gIIIP fusion protein production before helper phage are added.
mutants to isolate those which had lost an epitope. If a mixture of randomly mutated DNA is sub-cloned into the phagemid, the mutated protein will be expressed on the surface of the bacteriophage. By panning the bacteriophage with mAbs it will be possible to select those which express hCGβ. Those which have lost an epitope and hence no longer bind a mAb may then be selected from this population. This has the potential to be a very powerful screening system since bacteriophage are generally found at concentrations of $1 \times 10^{11}$ ml. It also has the advantage that when selecting the mutant protein, the DNA encoding it is also obtained.

Two bacteriophage expression plasmids were examined, pHEN1 and pFAB3. pHEN1 (Hoogenboom et al., 1991) has an amber stop codon before gene III, giving two possible products. A truncated fusion product is produced excluding gIIIp if grown in a non-suppressor *E.coli* strain such as HB2151 which supports the amber codon. A full length fusion protein is produced if expressed in an *E.coli* strain having the phenotype supE, such as TG1, which suppresses the amber stop codon. The HB2151 strain, which lacks the amber suppressor, is used for making soluble product. pFAB3 is an alternative phagemid. It contains a truncated form of gene III, where the amino-terminus is deleted. It has been proposed that large fusion partners exceeding 110 amino acids may lead to excessive breakdown of the fusion protein or impair gIIIp function (Parmley et al., 1988). Using a truncated gene III may therefore facilitate expression of larger inserts. Another reason for deleting the N-terminus is that this is the part of gIIIp which renders the bacteria resistant to phage infection (Crissman et al., 1984).

The various expression systems were examined in order to assess their suitability for screening large populations of randomly mutated hCGβ clones with the aim of identifying epitope-loss mutants.
3.2 Results

3.2.1 Cloning of the hCGβ gene

The cDNA for full length hCGβ was cloned using RT-PCR (Figure 3.2). Total cDNA was made from a third trimester placental total RNA sample and was used as the template for PCR amplification using primers based on the published sequence (Fiddes et al., 1980b; Talmadge et al., 1984b). The cloned hCGβ gene was shown to be identical to the published sequence. The presence of Asp at residue 117 and the Pro at residue 126 encoded by CCC identified the cDNA as that of Gene 3 in the hCGβ/LHB gene cluster. Gene 5, the most frequently expressed gene has CCG at residue 126 (Talmadge et al., 1984a).

3.2.2 Random mutagenesis

Random chemical mutagenesis was performed using methoxylamine (Kadonaga et al., 1985). Methoxylamine being a nonalkylating agent is more reactive on ssDNA (Singer et al., 1996) therefore the reactions were done on ssDNA prepared from M13 vectors containing hCGβ cDNA. Attempts to anneal the two mutated ssDNAs from mp18hCGβ and mp19hCGβ and digest the resulting dsDNA were unsuccessful, so the pooled ssDNAs were used as the template in a PCR reaction. and subsequently subcloned into the appropriate expression vector. dsDNA sequencing was performed on 24 mutant clones (Figure 3.3) and a mutation frequency of 2.3 substitutions per clone was observed from a total of 4380 bases sequenced, 26% of which were silent mutations, 9% introduced stop codons and 65% were useful changes. This was a suitable starting point for analysis of mutant clones. If the number of mutations needed to be altered the mutagenesis conditions could be changed (Budowsky, 1976).
Figure 3.2 RT-PCR to clone hCGβ cDNA.

cDNA was reverse transcribed from placental total RNA and used as the template in a PCR reaction. The reaction was optimised for $\text{Mg}^{2+}$ concentration.

Lane 1  control - no DNA  
Lane 2  5mM Mg Cl$_2$  
Lane 3  10 mM Mg Cl$_2$  
Lane 4  15 mM Mg Cl$_2$  
Lane 5  20 mM Mg Cl$_2$  
Lane 6  control no DNA no MgCl$_2$  
Lane 7  φx 174 Hae III marker
Figure 3.3 DNA sequence analysis of 4 hCGβ clones which have been subjected to random mutagenesis.

The dideoxy mixes for each clone have been loaded so that the same bases from each clone are adjacent, i.e. All T mixes together etc. This allow easier identification of mutated regions. The mutagen, methoxylamine, induces GC to AT transitions which can be seen in clones 14, 16 and 17 where indicated.
3.2.3 Expression of hCGβ/malE fusion protein in pMALp and pMALcRI

Using appropriate PCR primers (Table 2.3) the hCGβ cDNA was subcloned into pMALp and pMALcRI (Figure 3.4) to generate pMALphCGβ and pMALcRIhCGβ respectively. A Coomassie stain on SDS PAGE gel (not shown) of cell lysates from induced cultures of pMALcRIhCGβ and pMALphCGβ showed there was a fusion protein of molecular weight 72 kD produced by the pMALcRIhCGβ culture. The pMALphCGβ culture did not produce the expected fusion protein but there were induced proteins of smaller sizes. The signal sequence of the malE gene in this plasmid routes the fusion protein to the periplasm. It is possible that proteolysis may occur in the periplasm resulting in the smaller molecular weight bands seen in the induced pMALphCGβ cultures. However, these cultures were not examined in a western blot with hCG-specific antisera where binding of the sera to the low molecular weight bands may have confirmed them as degradation products.

Western blot analysis on the pMALcRIhCGβ cultures confirmed the 72kD band as hCGβ fusion protein, with soluble hCG able to compete for binding to the specific antibody (Figure 3.5). The primary Ab (SighCG) also bound non-specifically to E.coli proteins, this could be reduced by the addition of E.coli lysate.

Screening bacterial colonies with antibodies

In order to screen hCG mutants for those with an epitope-loss, E.coli colonies were induced and lysed on nitrocellulose filters. These may then be analysed with a panel of mAbs.

A weak signal was seen from induced pMALcRIhCGβ colonies grown and induced on nitrocellulose filters, this was often indistinguishable from that of the uninduced colonies. Once again pMALphCGβ induced colonies showed no fusion protein. E.coli lysate had to be added to reduce non-specific binding of the primary Ab (SighCG). The filters often gave a high background. A typical result is shown in figure 3.6. These results suggest that this system may not be the most appropriate for screening mutant hCGβ clones.
Figure 3.4 pMAL constructs.

a) hCGβ cDNA was subcloned into pMALp and pMALcRI as an EcoR I and Hind III fragment. In pMALp the signal sequence of the malE gene is intact (white box) allowing export of fusion protein to the periplasm, this is deleted in pMALcRI. Encoded at the 3' end of the malE gene is a Factor Xa cleavage site which allows for purification of hCGβ from maltose binding protein.

b) A restriction digest analysis of MAXI prep DNA of both constructs is illustrated. 1µg DNA was digested overnight at 37°C with EcoR I and Sal I and run on 1% agarose gels. λHind III/EcoR I is run as a size marker (M). Lane I 1 is pMALp, lane I 2 is pMALphCGβ, lane II 1 is pMALcRI, lane II 2 is pMALcRlhCGβ.
Figure 3.5 Induction of hCGβ/MBP fusion protein in *E.coli.*

Cell lysates of pMALcRI and pMALcRIhCGβ cultures were run on 12% SDS-PAGE gels. Approximately $1 \times 10^7$ cells of both induced and uninduced cultures were loaded per lane with from left to right on each gel: uninduced pMALcRIhCGβ, induced pMALcRIhCGβ, uninduced pMALcRI, induced pMALcRI. The 2 different gels in A and B ran slightly differently as seen in the band pattern. The gels were transferred onto nitrocellulose and blocked with 5% milk powder. The filters were then incubated in SighCG, a polyclonal antiserum raised against hCG. Filter B had 1μg/ml hCG protein included in the incubation. After washing, the filters were incubated with a peroxidase conjugated anti-rabbit polyclonal antiserum and the bands visualized using ECL with a 10 second exposure.
Colonies containing pMALcRIhCGβ and pMALphCGβ were grown on nitrocellulose filters and half were induced with 0.3 mM IPTG (e, f, g, h). The colonies were grown for a further 3 hours and the cells lysed with SDS and the proteins electrophoretically immobilised onto the membranes. The membranes were blocked with 5% milk powder and then incubated with SighCG both in the presence (b, d, f, h) and absence of E. coli lysate (a, c, e, g). hCG protein was added to b, d, f and h. Detection was with a peroxidase conjugated anti-rabbit polyclonal antiserum and visualization with ECL using a 15 second exposure.
3.2.4 Bacteriophage expression system

This system enables proteins to be expressed on the surface of bacteriophage. This would then enable an efficient screening process to be set up, where the DNA of selected mutants could be recovered.

The cDNA for hCGβ was subcloned into pHEN1 (Figure 3.7) using PCR and suitable primers.

Western blot analysis of pHENhCGβ in HB2151 with a mAb against the c-myc tag showed a strong induced band of 26.3 kD (Figure 3.8a) in six out of seven clones. There was no c-myc fusion protein detectable on western blot when pHENhCGβ was induced in E.coli strain TG1 (data not shown). One clone positive for c-myc production in HB2151 was analysed on western blot with hCG-specific antibody (SighCG). A protein of the same size was identified and was therefore confirmed as the hCGβ/c-myc fusion protein showing maximal induction after one hour (Figure 3.8b). This clone when grown in TG1 again showed no induced band. Therefore there is no detectable expression of a fusion protein when the gIIIp suppression is removed.

The induction and western blot presented in figure 3.8 was done by Miss Nicky Peet.

Deletions in the plasmid as a possible cause of non-expression

Some foreign proteins expressed in E.coli are toxic to the host cell. If the fusion product of gene III/hCGβ was toxic to E.coli the gene would be under a selection pressure to be eliminated. To test this, phage were made by superinfecting two cultures, one harbouring pHEN1 and the other pHENhCGβ and protein production induced. TG1 cells were then superinfected by the harvested phage. The cells were then plated onto ampicillin plates so that only those rescued by the phagemid DNA would survive. Mini preps showed that both the wild type pHEN1 and the PHENhCGβ DNA were of the correct size with no deletions. Therefore it did not appear that deletions of the plasmid were responsible for the lack of expression.

Periplasmic isolation of expressed proteins

The routing to the periplasm of the hCGβ/c-myc fusion protein produced in HB2151 was investigated. Whole cell lysates, protoplasts and protoplast supernatant from both pHEN1
a) hCG cDNA was subcloned into pHEN 1 as a Nco I/Not I fragment, both with and without the hCG signal sequence, so that it was at the N-terminus of the gene III.

b) A restriction digest analysis of plasmid DNA of both constructs. 1 μg was digested with Not I and NcoI and run on a 1% agarose gel. Lambda Hind III/EcoR I digest was run as a size marker (M).
Figure 3.8 Induction of pHENhCGβ in HB2151.

pHEN 1 and pHENhCGβ clones were grown in *E.coli* HB2151 in presence of glucose to O.D. 0.5. Then washed and resuspended in LB and 1mM IPTG, and grown O/N. Cell lysates were run on 12% (A) and 12.5% (B) SDS PAGE gels (10⁷ cells were loaded per lane), and transferred to nitrocellulose and blocked with 5% milk powder. A) Western blot using mAb 9E10 specific for c-myc. Lane 1 is pHEN 1 and lanes 2 - 9 different clones of pHENhCGβ. Detection was with a peroxidase conjugated anti-mouse polyclonal antiserum and visualization with ECL for 20 second. A protein of 26.3 kD was recognized in 6/7 of the pHENhCGβ clones. B) pHENhCGβ clone from lane 6 in A was further analysed, with pHEN 1 as negative control. Lanes 1 - 5 are pHEN 1 and 6 - 10 are pHENhCGβ. Samples were taken 50 min (lane 2 and 7), 120 min (lane 3 and 8), 189 min (lane 4 and 9) after induction and also after overnight (Lane 5 and 10). Uninduced samples were run in Lanes 1 and 6. 2 µg hCG protein was run in Lane 11. SighCG (purified on *E.coli* column) incubated with filter and detected with peroxidase conjugated anti-rabbit polyclonal antiserum and visualized with ECL using a 30 second exposure. A band of the same size as in A was identified.
and pHEnhCGβ grown in HB2151 were analysed by western blot (data not shown). All the induced protein seen in HB2151 cells was retained in the cytoplasm, no fusion protein was seen in the protoplast supernatant.

This suggests that either the fusion protein is not being routed to the periplasm, or that it is degraded in the periplasm, though no low molecular weight bands were seen to be reactive with the polyclonal Ab in the periplasmic protein lanes on the western blot.

**Removal of hCG signal sequence and use of truncated gIIIP**

hCGβ was subcloned without its signal sequence into the pHEn1 phagemid to test whether the inclusion of the hCG signal sequence disrupts the transport pathway to the periplasm. This signal sequence normally directs hCG to be secreted from placental cells and is cleaved from the final protein. This construct was designated pHEnhCGβΔss (Figure 3.7).

Both hCGβ and hCGβΔss were subcloned into pFAB3 (Figure 3.9) to test whether the large size of the fusion protein could be leading to increased proteolysis in the pHEN constructs (Parmley et al., 1988). pFAB3 has a truncated gene III, thus a smaller fusion protein will be produced.

Despite removal of the signal sequence there was still no expression of an hCGβ/gIIIP fusion protein with pHEnhCGβ in *E. coli* TG1. Western blot analysis of pFAB constructs in *E. coli* NF1829 revealed only a possible faint band in the pFABhCGβΔss lane of the approximate expected size 48kD (data not shown). The lack of expression of fusion protein from the pHEN constructs suggested that degradation may have occurred, possibly due either to the large size of the fusion protein or toxic effects of the gIIIP.

**Optimising expression in pFABhCGβ**

With the aim of reducing possible degradation of the gIIIP/hCGβ fusion product, two protease deficient *E. coli* strains, 801-I and 801-H were tested for expression of
Figure 3.9 pFAB 3 constructs.

a) hCGβ cDNA was subcloned into pFAB 3 as a Nco I / Not I fragment, both with and without the hCGβ signal sequence, so that it was at the N-terminus of the gene III.

b) A restriction digest analysis of plasmid DNA of both constructs is illustrated. 1μg was digested with Not I and NcoI and run on a 1% agarose gel. Lambda Hind III / EcoR I digest was run as a size marker (M). Lane I 1 is pFABhCGβ and Lane II 1 is pFABhCGβAss.
hCGβ/gIIp. Only pFAB constructs were tested since the pFABhCGβΔss had shown a faint induced protein. The growth and induction conditions were investigated.

Cultures of pFABhCGβ induced in *E.coli* strain 801-I revealed a strong band of 43kD in a western blot (Figure 3.10). The expected size of the truncated gIIIp/hCGβ is 47kD. This band was present at all time points and addition of IPTG did not increase the amount of fusion protein. This constitutive expression is expected as *E.coli* strain 801-I does not have the strong lac repressor lacIq.

In addition to the absence of lacIq, *E.coli* strain 801-I also has no F episome. There can therefore be no control over the expression of the fusion protein, and without the F episome encoding the sex pili, bacteriophage would not be able to infect the cells. The *E.coli* strain 801-H has both the lacIq and an F episome, and was examined for fusion protein expression.

Cultures of pFABhCGβ and pFAB3 in *E.coli* strain 801-H were grown and induced under different conditions (Figure 3.11). The western blot from these cells showed a very strongly induced band of 43kD in all the pFABhCGβ and pFABhCGβΔss post-induction lanes, though this varied in amount under the different conditions. The pFABhCGβ cultures produced more gIIIp/hCGβ when higher amounts of IPTG were used for induction until a maximum was produced with 2 mM. Growing the cultures for longer than one hour post-induction only increased gIIIp/hCGβ production when 2 mM IPTG was used. The pFABhCGβ cultures grown at 30°C and induced with 2 mM IPTG produced the most fusion protein.

The pFABhCGβΔss cultures did not show such a variation with the different culture conditions. There was little difference between the cultures induced with 2 mM IPTG and with 1 mM, in both production increased slightly after two hours. The culture grown at 30°C produced the same amount of gIIIp/hCGβ as the 2 mM culture, though it was produced maximally after only one hour.
Therefore it appears that 2 mM IPTG is sufficient for maximal induction and it is better to grow the cultures at 30°C. This probably further reduces proteolysis of the foreign fusion protein.

**Phage preparation of pFABhCGβ and pFABhCGΔss in E.coli strain 801-H and analysis by Radioimmunoassay**

Recombinant phage were prepared from pFABhCGβ and pFABhCGΔss cultures to investigate whether the hCGβ was expressed on the phage surface. The phage supernatant were analysed by RIA (Figure 3.12). None of the phage preparations competed with \(^{125}\text{I}\text{hCG}\) for binding to a rabbit polyclonal anti-hCG (P3). Therefore hCGβ was not detectable on the surface of the phage.

**Panning of phage**

In view of the absence of detectable levels of surface hCGβ in the total phage preparation, attempts were made at enriching the phage population for any phage which might be expressing hCGβ.

Phage preparations were panned on tubes coated with P3 or with a rabbit polyclonal raised against thyroglobulin (Tg) as a negative control. Separate control tubes were coated with P3 or Tg antisera and incubated with \(^{125}\text{I}\text{hCG}\). The \(^{125}\text{I}\text{hCG}\) control tubes had 148 counts for the anti-Tg coated tube and 12244 counts for the P3 coated tubes. This shows that the P3 antibodies were specific for hCG and were coated adequately.

Eluted phage were titrated. \(2 \times 10^{12}\) cfu/ml (colony forming units, i.e. phage with pFAB DNA and thus able to rescue untransformed *E.coli* on ampicillin plates) of pFABhCGβΔss had been added initially. The titration of the phage eluate as pfu (plaque forming units i.e. total number of phage) showed no difference between the control tubes and the hCG coated tubes (Table 3.1).

No phage had specifically bound to the hCG antiserum, therefore it appears that there is no hCG on the surface of the phage.
pFABhCGβΔss on anti-hCG coated tube | on anti-Tg coated tube
---|---
1.5 x 10^5/ml cfu | 2.2 x 10^5/ml cfu
4 x 10^8/ml pfu | 5 x 10^8/ml pfu

Table 3.1 Titres of eluted phage expressed as both cfu and pfu.
The same number of phage were eluted from the anti-hCG coated tube as from the anti-Tg coated tube.
There were > 1 x 10^11/ml pfu in the unbound supernatant from 2 x 10^12 cfu added.

**Commercial helper phage used in preparation of recombinant phage**
The pHEN system has been used successfully in many laboratories. To establish if there was a fault in our protocol we prepared bacteriophage following guidelines from Dr Greg Winter, who developed pHEN1. The main difference in the method is that it uses helper phage M13KO7 which are purchased (Stratagene, California, USA) rather than grown in the laboratory.

These bacteriophage were concentrated 10 x by PEG precipitation and tested in an ELISA. They were captured with a polyclonal anti-hCG (P3) and detected with a polyclonal anti-M13. If concentrated phage were used there was a high background signal (data not shown). This background was minimised if the concentrated phage were diluted 10 x (Figure 3.13).

When the mAb OT3A was used for detection of bound phage, no signal was observed. This could be explained in two ways: a) The C-terminus is next to gIIIp, perhaps the epitope is not accessible to the antibody due to obstruction by the gIIIp (Lowman *et al.*, 1991) b) The anti-M13 is raised against phage coat proteins III and VIII and so there are a large number of potential binding sites on each phage giving an amplification not possible with mAb OT3A. OT3A can only bind to a maximum of three to five fusion proteins per phage. This implies that there was only a low percentage of phage expressing hCGβ in the population.
Figure 3.10 Induction of pIII/hCG fusion protein in 801-I.

pFAB 3 and pFABhCGβ in E. coli 801-I were grown until O D. 0.5, then samples harvested after 60 min (lanes 2 and 7), 95 min (lanes 3 and 8), 135 min (lanes 4 and 9), 240 min (lanes 5 and 10) and 305 min (lanes 6 and 11). 10^7 cells were run per lane on a 12% SDS PAGE gel, 2µg hCG protein was run in lane 1. The gel was transferred to nitrocellulose and blocked with 5% milk powder. SighCG (pre-adsorbed on an E. coli column) was incubated in the presence of E. coli lysate. After washing a peroxidase conjugated anti-rabbit polyclonal antisera was used for detection and visualization was achieved with ECL and a 2 minute exposure. A band of 43 kD was observed at all time points in the pFABhCGβ, this band was absent in pFAB 3.
Figure 3.11 Expression of hCGβ from pFAB3 in 801-H.

Cultures of pFAB3, pFABhCGβ and pFABhCGβ ss in *E. coli* 801-H were grown to O.D. 0.4 and induced with different amounts of IPTG. $10^7$ cells were lysed and loaded per lane on 12% SDS PAGE gels from time 0 (uninduced), 1 (60 minutes) and 2 (120 minutes after induction). Samples of pFABhCGβ and pFABhCGβ ss grown at 30°C were also run. The gels were transferred to nitrocellulose and blocked with 5% milk powder. The filters were incubated with SighCG (pre-adsorbed on *E. coli* column) in the presence of *E. coli* lysate and detection was with an HRP conjugated anti-rabbit polyclonal antiserum. The results were visualized with ECL (3 minute exposure). H = hCG protein
Figure 3.12 RIA to detect hCGβ on the surface of recombinant phage.

In each case differing amounts of phage supernatant or hCG protein were added to beads coated with anti-rabbit Ig which had been incubated with 1:5000 P3 polyclonal anti-hCG and 20000 cpm $^{125}$I-hCG. The counts remaining after 2 hour incubation and washing are recorded.

a) Phage supernatant from pFAB, pFABhCGβ and pFABhCGβss- cultures (grown in E.coli 801-H).

b) Standard curve drawn from increasing amounts of hCG protein.

All samples were done in duplicate and standard errors are shown.
Testing conformation of gIIIp/hCGβ fusion protein

mAbs shown previously to be specific for discontinuous epitopes on hCGβ (Dirnhofer et al., 1994b) were used to examine if the hCGβ fusion protein has folded correctly (see Table 2.1).

Phage preparations were tested in an ELISA where the capture antibody was either P3 or a mAb raised against hCG. The mAbs used included a representative from almost all of the previously defined epitope regions (Berger et al., 1990). All the mAbs tested did recognise hCG on the surface of phage though the two mAbs from the β1 epitope region only bound weakly (Figure 3.13).

pHENhCGβ is therefore able to express at least partially folded hCGβ on its surface when detected in an ELISA system, although perhaps at a low level detectable only when there is an amplification step through the anti-M13 Ab.
Figure 3.13 ELISA on phage supernatants from pHEN and pHENhCGβ cultures.

Plates were coated with capture antibody (for A, B and C this is P3 polyclonal anti-hCG, in D a panel of mAbs is used). Detection in A is with mAb OT3A, in B and D with a sheep polyclonal anti-M13 and in C mAb OT3A is used.

A) Positive controls using hCG protein with OT3A mAb as the detection antibody.
B) Phage supernatant captured with P3 polyclonal and detected with anti-M13 polyclonal.
C) Phage supernatant captured with P3 polyclonal and detected with OT3A mAb.
D) Comparison of capture antibodies. A panel of mAbs is used as well as P3 polyclonal to capture the phage supernatant and detection is with the sheep polyclonal anti-M13.
3.3 Discussion

The conditions for random mutagenesis were established. Methoxylamine is a simple nonalkylating agent and induces GC to AT transitions in DNA (Budowsky, 1976). Its reaction is illustrated in figure 3.14. The restriction of the mutagen only attacking cytosine residues does limit the number of amino acids in hCGβ open to mutation. 22 out of 145 residues may not be mutated by methoxylamine. Therefore a combination with other mutagens which attack other residues may be advisable.

![Figure 3.14 Action of methoxylamine on cytosine.](image)

Methoxylamine reacts with cytosine (only reacts very slowly with adenine) to put a hydroxyl group on the N3 and a methoxyamino group (C-NH-OCH₃) on the C4. The product is no longer able to base-pair with guanine, and will base-pair with adenine instead, resulting in GC → AT transitions.

Two prokaryotic systems were examined for their suitability for expression of hCGβ, and for their use in screening large numbers of randomly mutated clones. The pMAL system showed very high expression of hCGβ in one of the two vectors analysed. No hCGβ was seen when the protein was exported to the periplasm. There may be proteolytic enzymes present in the periplasm to which hCGβ is particularly susceptible. This phenomena was also seen in the phagemid expression systems and was only resolved when protease
deficient *E. coli* strains were used. This could have consequences for the folding of hCGβ as the reducing environment of the cytoplasm does not favour disulphide bond formation. hCGβ has six disulphide bonds which have been shown to be essential for correct folding, assembly and secretion in eukaryotic systems (Suganuma *et al.*, 1989; Huth *et al.*, 1992; Bedows *et al.*, 1993; Furuhashi *et al.*, 1994). The absence of detectable hCGβ in the pMALp expression vector meant that only the pMALcRI system was available for mutant analysis. This was unlikely to produce correctly folded hCGβ as all fusion protein was formed in the reducing environment of the bacterial cytoplasm. It may have been possible to further optimise the pMAL system in order to reduce the background and increase the positive signal, e.g. other methods of lysing the colonies could have been explored. The pMAL expression and screening system is a time consuming one, even using the pinned lid and multiwell system described. The screening process using high temperature and SDS will mean that the protein is denatured and will re-fold when the SDS is removed. This may result in improper conformation.

The bacteriophage expression system offered the advantage that selection of the hCGβ molecule selected the DNA encoding it at the same time. Also the protein was routed to the periplasm thus disulphide bond formation is possible. This system also required optimisation. The failure to find hCGβ fusion protein when gIIIp was expressed was investigated in terms of its toxicity to the host. Recombinant proteins that are toxic to *E. coli* are under a selection pressure to be eliminated. Any *E. coli* which deleted the plasmid would be selected for and outgrow those producing the toxic protein. However no deletion of the plasmid was observed. Though c-myc/hCGβ fusion protein was observed in reasonable amounts in whole cell lysates, an attempt to isolate it from the periplasm failed. The pHEnhCGβ construct includes the hCGβ 20 amino acid signal sequence, used in normal circumstances to direct secretion from mammalian placental cells. In conjunction with the pelB leader sequence in *E. coli* this may disrupt the normal transport pathway to the periplasm. This should not in itself prevent the expression of a fusion protein, which would remain in the cytoplasm as observed in HB2151. It would, however, prevent assembly of recombinant phage. The signal sequence of hCG was therefore removed from the pHEnhCGβ construct but this did not lead to the expression
of gIIIp/hCGβ. Later successful expression of both pFABhCGβ and pFABhCGβΔss suggest that the hCGβ signal sequence does not disrupt expression.

Parmley and Smith (1988) have reported the increased breakdown of recombinant gIIIp compared to native gIIIp when the cloned gene encodes protein of greater than 110 amino acids. hCGβ has 145 amino acids and therefore it is possible that when the gIIIp/hCGβ fusion protein is produced in TG1, it is being taken to the periplasm but is immediately degraded. The c-myc/hCGβ fusion protein may be less susceptible to the proteolytic enzymes due to its smaller size, but may also be degraded when it reaches the periplasm, and therefore is only observed in the cytoplasm. The pFAB3 system has a truncated gene III, where the N-terminus is removed. The N-terminus is important in adsorption to the sex pili of bacteria (Armstrong et al., 1981), this function is not necessary in a recombinant gIIIp. This deletion may make the fusion protein less susceptible to proteolysis, due to its smaller size. It may also make accessibility to the carboxyl terminus of the hCGβ easier (Lowman et al., 1991). This hypothesis was borne out by the faint band of the expected size from pFABhCGΔss in strain NF1829. This was the first proof of gIIIp/hCGβ being made. Subsequent expression in E.coli strains deficient in periplasmic proteases did show a strong band which bound hCG specific antibody. The expression of this protein was shown to be affected by the concentration of IPTG used for induction and also by culture temperature. Growing E.coli cultures at lower temperatures is a common means of controlling proteolysis of recombinant products (Enfors, 1992). Even in the protease deficient strain there is evidence that there is still some proteolysis occurring since a higher production of hCG fusion protein is observed at lower temperatures. It appears that hCGβ may be susceptible to certain E.coli proteases.

Despite expression of the hCG/gIIIp fusion protein observed with the pFAB constructs, no hCGβ was detected on the surface of phage. These constructs had a deletion in the N-terminus of gIIIp, this is the region responsible for superinfection (Crissman et al., 1984). Later work by Orum et al. (1993) with pFAB3 derivatives, pFAB4 and pFAB5 showed that even gIIIp with the N-terminal deletion had a negative effect on superinfection titres. The phage preparation method used in this study for the pFAB3 constructs meant that IPTG was added to the E.coli culture before helper phage since it was presumed that with
the N-terminal deletion, the phage superinfection would not be inhibited. However, from the later work of Orum et al. (1993), it is now clear that the induction of the gIIIp would have lowered the subsequent superinfection rate and hence the production of recombinant phage. Their work also demonstrated the importance of strong repression of the gIIIp through the lac I^r repressor. When this repressor is present, more phage are yielded in the supernatant when compared to the wild type lac I. If gIIIp is completely repressed by the stronger lacI^r then helper phage are more readily able to infect E.coli and addition of IPTG after superinfection allows recombinant phage to be produced. In both the pFAB and pHEN systems used here the lacI^r was present in the E.coli strains used.

Expression of hCGβ on the surface of phages was not achieved until a commercial helper phage was used in their preparation. The lab stocks may have been mutated from repeated amplification and storage at 4°C, leading to inefficiency in packing of the recombinant gIIIp. Therefore phage are produced but no recombinant gIIIp is incorporated into the assembling phage coat. It is interesting that hCGβ was observed on the surface of pHENhCGβ because a gIIIp/hCGβ fusion protein was not detectable in western blots of cell lysates. Yet the fusion protein must have been present in small amounts as it was expressed on the phage surface. This suggests that if phage were prepared in the same way from pFABhCGβΔss expressed in the protease deficient strain 801-H, then a larger proportion of the phage would have the fusion protein on their surface. The fusion protein appeared to be folded at least partially in the same conformation as the native protein since the panel of mAbs recognized the surface expressed hCGβ to varying degrees. However it may be that there was only a low number of recombinant phage. This is supported by the fact that the gIIIp/hCGβ fusion protein, which must have been present for hCGβ to be detected on the surface of the phage, was not detectable on western blot. Perhaps if the pHENhCGβ had been grown in the protease deficient strain then gIIIp/hCGβ fusion protein would be detectable and a higher number of recombinant phage would be produced.

Thus the expression of hCGβ in different prokaryotic systems has been examined. It appears that hCGβ is susceptible to proteolytic enzymes in the periplasm of E.coli and care must be taken when choosing an expression plasmid and the host strain used. Laboratory
stocks of helper phage must also be carefully stored and monitored to avoid detrimental mutations. The results illustrate clearly how important it is to optimise each expression system for individual cloned genes. It has been shown that it is possible to express hCGβ on the surface of phages, but this was at a low level. More work is necessary to optimise this system.

Though the panel of mAbs did bind the surface expressed hCGβ there are doubts about whether it would be folded correctly due to low reactivity with the β1 mAbs. Towards the end of this part of the work, studies by Huth et al. (1994) suggested that hCGβ produced in E.coli does not form disulphide bonds properly, even when directed to the periplasm. Large amounts of aggregates were observed in inclusion bodies, and has to be denatured and re-folded in vitro. Also there is increasing evidence that the carbohydrates are important for the correct folding of hCG (Feng et al., 1995a; Feng et al., 1995b), E.coli does not form complex carbohydrates. As it is essential for analysis of epitopes that the correct conformation is possible in the expression system used, it may be more prudent to use an eukaryotic expression system.
Chapter 4

Antigenicity of hCGβ mutants
4.1 Introduction

4.1.1 Choice of expression system

hCGβ is a complex molecule including 12 cysteines that form six conserved disulphide bridges and is heavily glycosylated. Because of the limitations of the expression of hCGβ in prokaryotic systems (chapter 3) and in order to ensure correct folding of the recombinant molecules, a mammalian expression system was investigated. Factors shown to be important for hCG assembly and folding are disulphide bonds and glycosylation (Suganuma et al., 1989a; Huth et al., 1992a; Bedows et al., 1993a; Furuhashi et al., 1994a; Feng et al., 1995a; Feng et al., 1995b). These are more likely to be formed correctly in a mammalian system than in a prokaryotic system.

If random mutagenesis was to be used to identify the B-cell epitope residues, the screening would be facilitated if the mutant molecules were expressed on the surface of the host cell, in a manner similar to the bacteriophage system. This would allow isolation of the DNA encoding the mutant protein by selection of the surface expressed mutant molecule. A panel of conformationally-dependant mAbs comprising both hCG-specific and hLH cross-reactive specificities was used for the analysis of mutant proteins. In its natural state hCGβ is secreted and not surface bound. An expression system was designed where the hCGβ is fused at the COOH terminus with the H2-D² transmembrane region. This enables analysis of the surface expressed hCG mutants by flow cytometry, this is a useful approach for site-directed mutants also, since unlike in a secreted system there is no need to purify the protein from the tissue culture medium.

Transient expression in COS7 cells (African Green Monkey Kidney cells, derived from CV-1 cells) was investigated. This cell line was developed by Gluzman (1981) for growing early-region mutants of SV40 virus. The COS7 lines have an origin-defective SV40 virus integrated in the chromosomal DNA, this produces large T antigen but no virus particles. If a plasmid with an SV40 origin is introduced into these cells, then the T antigen causes the plasmids to replicate to a high copy number (10,000 to 100,000 /cell).
Any introduced cDNA which is under the control of an appropriate promoter will be highly expressed, until the cell either dies or expels the plasmid due to the over-expression. Two different transfection methods were examined here. The calcium chloride technique where the DNA is mixed with CaCl₂ and phosphate buffer to form a precipitate which is added to the cells (Graham et al., 1973), and the DEAE dextran transfection (McCutchan et al., 1968). Both of these are thought to work by facilitating DNA binding to cells and subsequent uptake by endocytosis. Various other treatments are often combined which may modify membrane structure and enhance DNA uptake, e.g. glycerol shock, DMSO. Chloroquine is also used with DEAE dextran and has been shown to bind DNA (Cohen et al., 1965) and reduce DNA degradation (Luthman et al., 1983).

4.1.2 Mutant analysis of hCGβ

To produce an epitope-specific vaccine it is essential to have knowledge about the residues comprising the B-cell epitopes, so they can either be eliminated or maintained. In the case of hCGβ it is difficult to ascertain the importance of the residues since the B-cell epitopes are all discontinuous except for those on the C-terminus. Earlier work using peptides spanning the whole length of the beta subunit had revealed only partial binding of one mAb (INN-hCG-58) to a linear epitope (Dirnhofer et al., 1994b). An approach using LH/CG chimaeras (Moyle et al., 1990) had revealed some residues important in the hCG-specific epitopes, but very little information about those for the shared epitopes.

Glycine/serine replacements

Prior to the solving of the crystal structure of hCG (which occurred in the middle of this project) it was very difficult to predict where the important residues for the shared epitopes were. hCGβ and hLHβ share 85% homology in the first 110 amino acids, thereafter the C-terminus adopts a poorly ordered structure (Lapthorn et al., 1994). Therefore our initial approach using site directed mutagenesis was designed to change large stretches of linear amino acids in between cysteine residues. Single mutations in the cysteine residues have been shown to drastically affect the folding and subunit formation (Suganuma et al., 1989; Bedows et al., 1993), this is due to the cystine knot motif at the core of hCG. Hence the cysteines on hCG were not mutated. It was hoped that the
correct folding of the whole molecule would still occur despite the large number of consecutive changes, by maintaining the hydrophobicity and hydrophilicity of the side chains. In this way it was expected that the reaction of one or more mAb would be diminished or lost. This would then identify areas of the molecule which could be further analysed by specific mutations or by a directed random mutagenesis using degenerate PCR primers (Peterson and Seed, 1988).

Crystal structure based approach

Targeting shared epitopes

The publication of the crystal structure of hCG allowed more precise mutagenesis (Lapthorn et al., 1994; Wu et al., 1994). The beta hairpin regions of the β subunit, residues 10-33 and 58-87, referred to as the loops 1 and 3 respectively, form the body of the subunit and are held together by the disulphide bond 23-72. Sequences which are relatively conserved in the glycoprotein hormone β subunits are located in the tips of these loops. This region is therefore a good candidate for the cross-reactive epitope sites. Those residues with protruding side-chains which are present in both hLHβ and hCGβ were targeted.

Individual amino acid residues from this region which are surface accessible and not considered to play a critical structural role were selected. To minimise the possibility of introducing large conformational changes, residues were replaced with those occurring at the same position in other glycoprotein hormones from differing species. The changes were designed to introduce dissimilar and opposing sidechain properties (e.g. size, charge, polarity) from the native residues, to disrupt any mAb binding to the given residues. Computer graphic analysis of the potential hCG mutants was performed by Dr Adrian Lapthorn to provide further evidence that the substitutions did not affect the overall protein conformation and that the introduced side-chains could be accommodated.

β1 epitope region analysis

It is obviously crucial in designing an epitope-specific hCG based contraceptive vaccine that the hCG-specific epitopes are maintained. To determine which residues may be involved in the formation of the β1 epitope region a number of mutants were made using
information from the previous mutants (see 4.2.5). The hCGβ specific residues which are on the hCGβ core fragment (as are β1 and β7 epitope regions) are Arg 8, Arg10, Thr 15, Val 55, Asn 58, Asn 77, Tyr 82, Gln 89, Ala 91 and Leu 92. Of these residues Val 55 and Asn 58 are not on the surface and Tyr 82 is not well exposed. As the changes made in loop 3 did not affect the binding of the mAbs available to us from β1 and β7 epitope regions, it was presumed that Asn 77, also on loop 3, does not contribute to these epitope regions. However Arg 8 and 10, Gln 89, Ala 91 and Leu 92 are grouped together and are also well exposed on the surface, further, these residues have been previously implicated in both hCG-specific epitopes and hCGβ-specific epitopes (Moyle et al., 1990). These residues were therefore changed.

It is essential that any changes introduced into hCGβ do not change the overall conformation of the molecule so that the hCG-specific epitopes are retained and the possibility of creation of neo-epitopes is minimised. The surface expression system and the high transfection rate described in chapter 4 allowed for a very effective analysis of small changes brought about by the substitutions in the mutants. The mutants were all analysed by flow cytometry which is ideal for measuring such differences.

4.2 Results

4.2.1 hCGβ surface expression construct

The hCGβ cDNA was cloned into pCDM8 (Seed et al., 1987) into which a DNA fragment from H2-D^β containing the first 17 membrane-proximal amino acid residues, the transmembrane region and cytoplasmic tail had been inserted. This fragment was obtained by PCR amplification of cDNA made from a mRNA liver preparation of a C57BL/10. The primers were based on the published sequence (Reyes et al., 1982). The H-2 D^β sequence differed from the published in that there were 3 additional membrane-proximal amino
acids. The construct was designed so that the C-terminus of hCGβ was next to the N-terminus of the MHC fragment (Figure 4.1).

4.2.2 Transient transfection in COS7 cells

Two transfection methods were assessed, Calcium chloride and DEAE dextran (see section 2.2.5 for optimisation). DEAE dextran gave consistently higher numbers of transfected cells and was used for the Mutants 1 through 22 and the calcium chloride technique was used for the glycine/serine mutants.

4.2.3 Expression of hCGβ on the cell surface

Surface expressed wild type hCGβ was analysed by indirect staining with a panel of mAbs. The protein appeared to be expressed to a high level on the cell surface, with cells very bright under a fluorescence microscope (Figure 4.2) but there was a wide variation in expression from cell to cell depending on the multiplicity of transfection (Figure 4.3). The mAbs were all titrated on hCGβ transfected COS7 cells to give approximately the same staining intensity. The fact that all the mAbs bound well to the hCGβ expressed on the COS7 cells indicated that it was folded correctly, as all these mAbs except OT3A are conformationally dependent (Dimhofer et al., 1994b).

4.2.4 Glycine/serine replacement mutagenesis

The lack of structural information made it impossible to predict the location of the shared epitopes, large stretches of amino acid sequence were changed in hCGβ in an attempt to knock out the binding of one or more mAbs. Since cysteines and prolines are important structurally these residues were left intact (Darby et al., 1993).
a) The cDNA for hCGB was subcloned into pCDM8 so that the C-terminus was in translational reading frame with the transmembrane fragment of H-2 D'. Hind III and Sal I sites allow the easy subcloning of mutant hCGB molecules into the construct.

b) A restriction digest analysis of plasmid DNA of pCDM8 (lane 1) and pCDM8hCGB (lane 2) is illustrated. 1 μg DNA was digested with Hind III and Sal I and run on a 1% agarose gel. Lambda HindIII / EcoR I was run as a size marker (M).
Figure 4.2 Fluorescent microscopy of COS7 cells transfected with hCGβ.

Cells were transfected with wild-type pCDM8hCGβ and stained with INN-hCG-58 and a FITC conjugated anti-mouse. They are shown under both light (a) and fluorescence (b).
Figure 4.3 Example of hCGβ surface expression on COS7 cells.

Cells were harvested and stained 72hrs post-transfection with pCDM8hCGβ. The negative isotype control (CD34) is shown by the black line, cells staining with mAb OT3A are shown by the red fill, and below with mAb INN-hCG-2 (B1) by cyan fill. The differences in amount of hCGβ expressed on the surface of the cells is reflected by the wide range of fluorescence observed.
Four mutants were made replacing stretches between cysteines 9-23, 23-34, 57-72 and 72-88. The primers used are listed in table 2.3 and the substitutions are shown in figure 4.4. The mutants were made using overlapping PCR (Figure 2.1), a representative example is shown in figure 4.5. All mutants were subcloned into pCDM8 containing the transmembrane fragment.

All the mutants were analysed by fluorescent microscopy and all except Mutant C9 (9-23) were additionally analysed by flow cytometry.

**Fluorescence microscopy analysis**

COS7 cells transfected with pCDM8hCGβ or mutant plasmids were stained with various concentrations of a mouse polyclonal antiserum raised against hCG (MS). Qualitative results are presented in table 4.1

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>C9</th>
<th>C23</th>
<th>C57</th>
<th>C72</th>
</tr>
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<tbody>
<tr>
<td>MS 1/500</td>
<td>+++++</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>MS 1/250</td>
<td>+++++</td>
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Table 4.1 Fluorescent microscopy analysis of COS7 cells transfected with glycine/serine mutants.

Staining of COS7 cells transfected with either hCGβ (WT) or Mutants C9, C23, C57 or C72, when mouse polyclonal anti-hCG is used (MS). Normal mouse serum (NMS) is used as a negative control. WT = wild-type. +++++ very bright fluorescence; ++ dim fluorescence; + faint fluorescence; - no fluorescence.

As shown in table 4.1, COS7 cells transfected with wild-type hCGβ stained very strongly with all dilutions of the antiserum, indicating hCG protein was on the surface in appreciable concentrations. In contrast the mutants were only detected when the mouse polyclonal was used at very high concentrations. This indicated that the mutants were
Figure 4.4  Amino acid sequence of hCGβ showing the position of the glyceine/serine site directed mutants and substitutions made.
Figure 4.5 Construction of Mutant 8 by overlap mutagenesis.

a) The two halves of the hCGβ cDNA are PCR amplified using primers which incorporate the residue substitutions and which overlap. b) The two templates are used in a PCR reaction which creates the full length mutated cDNA, this is purified from an agarose gel. c) The DNA is digested overnight with Hind III and Sal I to release the fragment which is ligated into pCDM8 containing H2-Dk transmembrane region. d) Plasmid preparations were digested with Hind III and Sal I ready to be sequenced. All DNA are run on 1% TAE low melting point agarose gels.
either not expressed on the cell surface at the same level as the native hCGβ, or that they were not folded properly and thus many of the epitopes recognised by the components of the antiserum were absent in the mutants.

To further extend the analysis, Mutant C57 was examined with a panel of polyclonal sera. As shown in table 4.2 there is a clear difference in the ability of each polyclonal antiserum to recognise Mutant C57.

<table>
<thead>
<tr>
<th>Ab</th>
<th>WT</th>
<th>C57</th>
<th>UNT</th>
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<tbody>
<tr>
<td>SigCG 1/500</td>
<td>++++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>SigCG 1/250</td>
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<td>-</td>
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<tr>
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<td>-</td>
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<tr>
<td>CD34</td>
<td>-</td>
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Table 4.2  Fluorescent microscopy analysis of COS7 cells transfected with Mutant C57.

Staining of COS7 cells transfected with either hCGβ (WT) or Mutant C57, when different polyclonal anti-hCG are used. Un transfected cells (UNT) were included to see if there was any non-specific binding to COS7 cells. mAb INN-bLH-1 was also tested with mAb CD34 as a negative control.

The polyclonal antiserum P3 bound Mutant C57 better than the other polyclonals used. It is possible that none of the mutants were folding properly and that only the components of the polyclonal antisera which were against linear epitopes were being recognized. P3 appeared to have a larger proportion of such antibodies as it bound to Mutant C57 better than the other antisera. It was therefore used in double staining of mutant C72 in flow cytometry.
Flow cytometry analysis

A more quantitative analysis of COS7 cells expressing the mutants was performed with flow cytometry and either staining with a single mAb (Mutants C23 and C57) or double staining with the P3 polyclonal antisera and the individual mAbs (Mutant C72).

The results are shown in figures 4.6 and 4.7. None of the mAbs previously shown to be conformationally dependent bound to the mutants except INN-hCG-58 and INN-hCG-32 which bound slightly to Mutant C23. mAb OT3A detected all mutants indicating that all were being expressed and some of the expressed mutant was transported to the cell surface. The polyclonal antibody P3 again reacted less with the mutants than with wild-type hCGB.

The double staining on Mutant C72 shows some interesting data which may be explained in terms of the epitope regions of hCGB. In this experiment the polyclonal P3 was used at a concentration of 1/500, and the mAbs were used at 1/200. There was no non-specific binding to the COS7 cells by either the polyclonal or the mAbs as shown on the untransfected cells, except for OT3A. The COS7 cells transfected with wild-type hCGB were 52% positively transfected when single stained with polyclonal P3 (Figure 4.6). When the negative isotype control CD34 or mAb OT3A are incubated with the transfected cells before P3, then a similar result is obtained as a single stain with P3, i.e. approximately 50% positive cells. When the β1 mAb INN-hCG-2 is incubated first then the P3 result drops to 40%, and when any of the other mAbs from epitope regions β2 through β5 are incubated first, the P3 result drops even further to about 25% (Figure 4.6). The single stain on the wild-type hCGB with one mAb INN-bLH-1 gave about 20% cells positive (data not shown), this is similar to the result obtained with all the mAbs tested in the double stains. These results can be explained if the work of Berger et al (1990) is considered. They showed how some mAbs which were defined into epitope regions could bind simultaneously to hCG. If the polyclonal antiserum is considered as a mixture of mAbs then it may be envisaged that the component mAbs which belong to for example the β1 and the β5 region may bind simultaneously to the surface expressed hCGB. However when a mAb preparation is used it may only bind to one site on each hCGB molecule. Thus the cells which express a low amount of hCGB will show a higher fluorescence with
Figure 4.6 Flow cytometry analysis of Mutant C72.

COS7 cells transfected with either wild-type hCGB (top), Mutant C72 (middle) or untransfected (bottom) were indirectly stained with one of a series of mAbs and then with a polyclonal antiserum (P3) to detect the presence of hCGB on the surface of the cell, and to analyse the epitopes changed in Mutant C72. The result of a single stain on wild-type transfected cells are also shown (ssP3).

A marker was set on the negative isotype control CD34, and the number of cells to the right of this marker counted and represented as a black bar for mAb and a grey for polyclonal antiserum. The background staining of 13% in OT3A is not subtracted in above graphs. This experiment was only done once. Results shown are of duplicates and standard errors are indicated.
Figure 4.7 Reaction of Mutants C57 and C23 with mAbs.

COS7 cells were transfected with wild-type hCGβ and Mutant C57 (A), and also in another experiment with Mutant C23 and wild-type hCGβ (B). The transfected cells and untransfected cells were single stained with some of the mAb panel, and analysed by flow cytometry. The results in each case are from a single transfection, with stains done in duplicates and standard errors indicated.
the polyclonal antisera than with the mAb due to simultaneous binding or more than one antibody. When the negative control or OT3A are allowed to bind hCGβ before the polyclonal sera then the binding of the polyclonal antibodies are unaffected (since CD34 will not bind hCGβ and the OT3A binding to the C-terminus is far removed from the binding sites of the epitope regions β1 through β7). Less of the polyclonal antibodies are able to bind when the β1 mAb is incubated first and less still when any of the other mAbs are bound first. This may be because the part of hCGβ to which make up the epitope regions β2 through β5 is immunodominant, so the majority of the antibodies in the polyclonal antisera are against these regions. Another explanation may be that the affinity of the mAb preparation for the hCGβ is higher than the antibodies in the polyclonal serum which compete for the same site and that the β1 mAbs have a lower affinity compared to the β2 - β5 mAbs thus more β1 mAbs are displaced by the P3 Abs giving a slightly lower result for the β1 mAb and slightly higher result for the P3 polyclonal as observed.

The mutants are expressed on the surface of the COS7 cells as shown by reactivity with OT3A and P3, but they do not fold properly. Each mutant lost the reactivity of almost all the mAbs (Figure 4.6 and 4.7). That INN-hCG-58 bound slightly to Mutant C23 is significant as it is the only mAb previously shown to bind linear peptides (Dirnhofer et al., 1994b). Of course it could be argued that there is a critical residue for each mAb in each of the replaced stretches. But this is unlikely and the reduction seen of P3 binding in the double staining of Mutant C72 when OT3A is allowed to bind first (Figure 4.6), implies strongly that almost all reactivity of P3 with the mutants is through the C-terminus.

4.2.5 Site directed mutation based on the crystal structure

After the crystal structure was published (Lapthorn et al., 1994; Wu et al., 1994), mutants were designed which specifically targeted the β3/β5 epitopes. The substituted residues chosen should not disrupt the overall folding of the molecule. The mutants were made in stages, the first being multiple mutants on loops 1 or on loop 3. These were later analysed as single mutants. Then all the mutated residues were combined on one mutant (14) and
further changes were made on this as well as on wild-type hCGβ. Changes were also made on hCGβ to identify residues involved in the hCG-specific epitope region β1. The mutants were all made by overlap PCR mutagenesis except Mutant 14 (made by ligating parts of Mutants 3 and 4). Every mutant hCGβ molecule was fully sequenced to check for PCR induced mutations. The changes introduced in each mutant are listed in tables 4.3 - 4.6 and sequences of representative mutations are shown in figure 4.8. The position of the Mutants 1 - 10 on the hCG molecule is shown in figure 4.9.

Indirect single staining with a panel of mAbs defined the number of positively transfected cells and the result of each mAb was expressed relative to that of mAb OT3A. Each mutant/mAb combination was tested in duplicate and at least three times.

The mAbs to the β1 and the β7 epitope regions and the mAb OT3A bound to wild type and all the mutant hCGβ with about the same relative binding (except for Mutant 19-3). This demonstrates the mutant molecules, as desired, fold to recreate the hCG specific epitope β1 and the hCGβ specific epitope β7 (which becomes masked upon formation of the heterodimer).

**Loop 1 multiple mutants**

The mutations in the loop 1 (Mutant 4; Lys 20, Glu 21, Gly 22, Pro 24 and Val 25) completely abrogated binding of the mAbs specific for the β3 and β6 epitope region and reduced to 24.3% the binding of mAb 3E2. Different mutants were made to pinpoint the amino acids that contribute to the binding of the different mAbs. Mutations of residues Lys 20, Glu 21 and Gly 22 (Mutant 1) totally destroyed all reactivity with the mAb INN-hCG-64 recognizing the hCGβ-specific epitope region β6 without affecting the binding affinities for the β3 mAbs. In contrast Mutant 2 (Pro 24 and Val 25) failed to bind both β3-specific mAbs (INN-bLH-1 and INN-hCG-111). The mAb 3E2 showed a reduction in binding to both Mutant 1 and 2 (56% and 54.3%, respectively). These results are summarised in table 4.7.
Figure 4.8 DNA sequences of wild-type hCGβ and Mutants 3 (a) and 4 (b).
Figure 4.9 The position of the substituted amino acids in Mutants 1-10.

a) The loops 1 and 3 of hCGβ are circled in the area where the mutations were made. The alpha subunit is in blue, the beta subunit is in green and the position of the carbohydrates illustrated in a stick representation. The position on the C-terminus where mAb OT3A binds is shown by a box. b) and c) are approximately orthogonal views of loops 1 and 3. Mutant 1 is highlighted in magenta, Mutant 2 in yellow and Mutant 3 in cyan.

This figure was made by Dr A Lapthorn.
<table>
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<th>Mutant</th>
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Table 4.3 Changes in Loop 1.

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Table 4.4 Changes in loop 3
Table 4.5 Substitutions in further mutants.

Further mutational strategy. Mutants 3 and 4 were combined to give Mutant 14. This has the following changes: Lys 20 → Asn, Glu 21 → Arg, Gly 22 → Glu, Pro 24 → His, Val 25 → Tyr, Arg 68 → Glu, Arg 74 → Ser, Gly 75 → His, Val 79 → His, as well as Val 44 → Ala. Mutants 16 and 22 were made on Mutant 14 and Mutants 19-3 and 19-6 were made on WT hCGβ.

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<td>Arg 63 → Ser, Gly 65 → Ala</td>
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Table 4.6 Amino acid changes in mutants made to identify residues involved in β1 epitope region.

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Table 4.7  Binding of mAbs to wildtype (WT) and mutant hCGβ molecules, with substitutions in loop 1, expressed on COS7 cells.

- mAb binding equivalent to that of OT3A (cells positive >70% relative to OT3A).
- mAb binding reduced compared to that of OT3A (cells positive 50-70% relative to OT3A).
- mAb binding reduced compared to that of OT3A (cells positive 20-30% relative to OT3A).
- mAb abolished (<10% relative to OT3A).
**Loop 1 single mutants**

For further analysis, mutants were constructed in which only a single amino acid was changed in loop 1. The two β3 mAbs have separate but overlapping binding sites on hCGβ, because a single point mutation Pro 24 \(\rightarrow\) His (Mutant 5) removed all binding of mAb INN-bLH-1 but allowed reduced binding of INN-hCG-111 (59-63%), whereas the mutation Val 25 \(\rightarrow\) Tyr (Mutant 6) prevented binding of INN-hCG-111 and lowered the binding of INN-bLH-1 to 63% (Table 4.7).

**Loop 3 multiple mutants**

In contrast to the above mutations the four mutations at residues 68, 74, 75 and 79 introduced in the Loop 3 (Mutant 3) removed all the reactivity to the mutated molecule of all cross-reactive antibodies yet retained the binding of the hCG-specific mAbs directed to the β1 and β7 epitope regions and to the linear epitope in the C-terminus (Table 4.8). A representative Facs profile is shown in figure 4.10.

**Loop 3 single mutants**

The analysis of the single changes on the Loop 3 gave surprising results. Mutant 7 with a single change of Arg 68 \(\rightarrow\) Glu lost the binding of all the cross-reactive mAbs and, unlike Mutant 3, retained all the specific mAbs including the β6 representative INN-hCG-64. Mutant 8 (Arg 74 \(\rightarrow\) Ser, Gly 71 \(\rightarrow\) Arg) lost only the binding of β3 mAbs and the mAb 3E2. The change of Gly 75 \(\rightarrow\) His, Mutant 9, prevented mAb INN-hCG-64 (β6) from binding. Whilst in Mutant 10, Val 79 \(\rightarrow\) His appeared to have no affect on any of the mAbs except 3E2 which was reduced to 57% (Table 4.8).

**Mutant 14 and further mutants**

The residues changed in loops 1 and 3 were almost all shown to participate in epitopes of the cross-reactive mAbs tested. Those that did not, may still evoke a cross-reactive response if left as the wild type sequence in a vaccine. Therefore the changes in both loops were combined in one mutant (Mutant 14).
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<th>Reactivity</th>
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Table 4.8 Binding of mAbs to wild-type (WT) and mutant hCGβ molecules, with substitutions in loop 3, expressed on COS7 cells.

- mAb binding equivalent to that of OT3A (cells positive >70% relative to OT3A).
- mAb binding reduced compared to that of OT3A (cells positive 50-70% relative to OT3A).
- mAb abolished (<10% relative to OT3A)
Figure 4.10 Representative flow cytometric analysis of COS7 cells expressing hCG Mutant 3.

Events (5000) are shown of cells stained with mAbs OT3A, INN-hCG-2 (hCG-specific) and INN-hCG-58 (cross-reactive with hLH). The negative isotype-matched control (anti-CD34) is shown in each with a dotted line. The number of cells positive in each case is indicated.
Mutant 14 was constructed by making use of the unique Pst I site in hCGβ (Figure 4.11). When sequenced it was revealed to have an additional change introduced during its construction at residue 44 of Val to Ala. Since this is a fairly conservative change it was decided not to correct it. Mutant 14 retained the binding of the hCG-specific and hCGβ-specific mAbs INN-hCG-2, INN-hCG-32 and INN-hCG-68, and as expected all the cross-reactive mAbs failed to react as did hCG-INN-hCG-64 (Table 4.9).

Mutant 14 was the base for further site directed mutations, some of which were also done on wild type hCGβ in order to determine the contribution of these individual residues to epitopes. The mutations made are listed in table 4.5. Mutant 22 which had 2 extra changes in addition to those in Mutant 14 (Arg 63 → Ser and Gly 65 → Ala) also retained the β1 and β7 epitope regions (Table 4.9). Mutant 16 is identical to Mutant 22 except for residue 68 which is changed to Leu instead of Glu (due to error in PCR primer). The staining with INN-hCG-24 on Mutant 16 shifted slightly to the right each time, giving 13% compared to less than 5% with Mutant 22. Mutants 19-3 and 19-6 were constructed to examine the individual influence on epitope formation of the latest residues changed. Mutant 19-6, with only Arg 63 → Ser and Gly 65 → Ala, bound all mAbs equally as well as wild-type hCGβ. Mutant 19-3, the result of a fortuitous PCR error, with an additional change at Arg 60 → His, retained all mAb binding except that of the β1 mAbs (Table 4.9).

**β1 epitope analysis**

It is important to know which residues are involved in the hCG-specific epitope region β1 so that they are not changed in the final vaccine. Therefore changes were made in residues proposed to be part of this epitope region. The changes introduced are shown in table 4.6. Of the mutants made, only Mutant 12 showed any difference to wild-type hCGβ, where a reduction to 60% was seen with the β1 mAbs (Table 4.10).
Figure 4.11 Construction of pCDM8 Mutant 14.

pCDM8 Mutant 4 and pCDM8 Mutant 3 are digested with Pst I. The halves containing the mutations are ligated together to give pCDM8M14. The red lines indicate the mutated area, the blue area is hCG beta subunit, and the orange area is the transmembrane tail.
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Table 4.9 Binding of mAbs to wild-type (WT) and mutant hCGβ molecules, expressed on COS7 cells, Mutants 14 and above. * Mutant 14 has all the changes introduced into Mutant 3 and Mutant 4 as well as V44A. Mutants 19-3 and 19-6 are based on wild-type hCGβ and Mutants 16 and 22 are based on Mutant 14 with the extra mutations indicated.

- **N** mAb binding equivalent to that of OT3A (cells positive >70% relative to OT3A).
- **13%** mAb binds with 13% relative to OT3A
- **< 10%** mAb abolished (< 10% relative to OT3A)
Table 4.10 Binding of mAbs to wild-type (WT) and mutant hCGβ molecules expressed on COS7 cells with substitutions made to analyse the hCGβ-specific β1 epitope region.

<table>
<thead>
<tr>
<th>Mab</th>
<th>Epitope cluster</th>
<th>Reactivity</th>
<th>W.T.</th>
<th>Mut 11</th>
<th>Mut 12</th>
<th>Mut 13</th>
</tr>
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<tr>
<td>INN-hCG-2</td>
<td>B1</td>
<td>CG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INN-hCG-32</td>
<td>B1</td>
<td>CG</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>INN-hCG-22</td>
<td>B2</td>
<td>CG/LH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INN-b1.H-1</td>
<td>B3</td>
<td>CG/LH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INN-hCG-111</td>
<td>B3</td>
<td>CG/LH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INN-hCG-24</td>
<td>B4</td>
<td>CG/LH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INN-hCG-58</td>
<td>B5</td>
<td>CG/LH</td>
<td></td>
<td></td>
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<tr>
<td>INN-hCG-51</td>
<td>B3/5</td>
<td>CG/LH</td>
<td></td>
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<tr>
<td>INN-hCG-20</td>
<td>B3/5</td>
<td>CG/LH</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>3E2</td>
<td>B3/5</td>
<td>CG/LH</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>INN-hCG-64</td>
<td>B6</td>
<td>CGB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INN-hCG-68</td>
<td>B7</td>
<td>CGB</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>OT3A</td>
<td>C term.</td>
<td>CG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- mAb binding equivalent to that of OT3A (cells positive >70% relative to OT3A).
- mAb binding reduced compared to that of OT3A (cells positive 50-70% relative to OT3A).
4.3 Discussion

In order to produce an epitope-specific vaccine, a detailed knowledge of the epitopes and their composite amino acids is required. As previously shown for hCGβ (Dimhofer et al., 1994b), the most difficult epitopes to elucidate are discontinuous B-cell epitopes which are by their nature highly dependent upon the correct folding and conformation of the molecule. hCGβ (residues 1-110) has 85% amino acid homology with hLHβ, and four (β2, β3, β4, β5) of the seven epitope regions present on this fragment elicit antibody responses that cross-react with hLH. This chapter investigated two different approaches in using site-directed mutagenesis on hCGβ to identify residues involved in some of the shared epitopes and to create epitope-loss mutants which retain the desired hCG-specific antigenic areas.

4.3.1 Glycine/serine replacements

The first approach, started before the crystal structure was known, was to replace large stretches between the cysteine residues. The amino acids with hydrophobic side chains were replaced with glycine and those with hydrophilic side chains with serine. The hydrophobic side-chains of some residues are important in forming the hydrophobic core of proteins, and thus maintenance of the hydrophobicity may be important in the folding of the molecule. This drastic measure was hoped to identify areas of the molecule important for reaction with mAbs whilst retaining the overall conformation.

In this investigation changing the cysteine residues was deliberately avoided. This was due to the complex structure of the hCG molecule with its cystine knot. There is much evidence that interruption of any disulphide bond results in structural forms different from the native (Suganuma et al., 1989; Huth et al., 1992; Bedows et al., 1993; Furuhashi et al., 1994). Proline residues in the glycine/serine replacements were also unchanged. The cyclic side chain of proline limits the flexibility of the polypeptide backbone at this residue and as such is often found at the beginning of alpha helix turns and in beta turns. The rigidity of the proline is important in maintenance of the turns in these structures (Darby et
It was anticipated that the cysteine residues would keep the structure intact and that the glycines and serines would also allow a similar positioning to the original residue, whilst being poorly antigenic due to their decreased size.

The results show that the basic premise of maintaining the overall structure has not been met with these substitutions. Only mAb OT3A bound to the mutants and also only faint staining was possible with most polyclonal antiserum tested on the mutants compared to the very bright staining on the wild-type hCGβ. When a double stain was done on cells transfected with Mutant C57 it was observed that after OT3A had bound, less staining was seen with the polyclonal P3, compared to that observed when a single stain with P3 was performed. This suggests that the C-terminal sequence was involved in the binding of the polyclonal P3. Indeed P3 may have been more reactive against the mutants than the other polyclonal antisera tested because it had a higher proportion of antibodies specific for the C-terminus.

The complete failure of the correct folding of the mutant hCGβs can be attributed to the choice of glycine as a replacement residue and also to a lesser degree the decrease in glycosylation possible on the mutants. Glycine actually has no side chain, thus is not considered hydrophobic, which may have affected the folding of the molecule. This also makes the polypeptide backbone very flexible where it is present and along with proline is known as a helix breaking residue which it makes unstable (though proline is considered such for the opposite reason - it's great rigidity). Alanine would have been a better choice, it is considered hydrophobic and also has a small side chain and thus would not be particularly antigenic. Alanine has been used as a replacement amino acid in stretches of hFSHβ with no apparent affect on the intracellular folding (Roth et al., 1993). Mutant C9 and Mutant C23 have destroyed the N-linked recognition sequences of Asn 13 and Asn 30 respectively. It has been shown that the N-linked oligosaccharides help with the folding of the beta subunit (Feng et al., 1995a; Feng et al., 1995b). This may also have hindered the folding of the mutants. However since both sites need to be missing to see a large decrease in the folding and secretion it is unlikely that this played a major role. It is not known whether COS7 cells are able to glycosylate hCG in the same way as when it is produced in vivo. If some glycosyltransferase enzymes essential for the processing of the
carbohydrates are not expressed in COS7 cells then the final sugar moieties may well be different, this could affect the folding of the molecule. However, based on the binding observed with the panel of mAbs on the wild-type hCG expressed on the transfected COS7 cells, it is presumed that the hCGβ is folding correctly.

In conclusion the use of glycine has made the β subunit unstable and prevents proper folding. The mutants do reach the surface of the COS7 cells where they can be identified with polyclonal antisera and a mAb specific for a linear epitope on the C-terminus. Also it is probably advisable to maintain the N-X-S/T recognition site in any mutants to allow glycosylation.

4.3.2 Crystal structure based mutations

The analyses presented here of the second group of site-directed mutants have permitted identification of amino acid residues important in mAb binding to hCGβ. They also maintain the criteria of losing shared epitopes whilst maintaining sufficient conformation to allow undiminished binding of other mAbs against desired epitopes. Substitution of the amino acids at positions 68, 74, 75 and 79 creates a mutant hCGβ (Mutant 3) which has completely lost the binding of all the cross-reacting murine mAbs used in this study yet retains the epitopes specific for the hCG holo-hormone. Remarkably, substitution of just Arg 68 with Glu (Mutant 7) also eliminates these cross-reacting epitopes but the mutant is still folded such that the hCG-specific epitope regions β1, β6 and β7 are maintained since the relevant mAbs bound Mutant 7 as well as they did the wild type hCGβ.

Analysis of the mutants provides important information with respect to both hCG-specific and cross-reacting epitopes. The changes caused by mutation are most probably due to differences in the structure of the side chains of the substituted amino acids which could modify antibody binding probably by alteration of a contact residue or by stereochemical inhibition. The constraints on the fold caused by the cystine knot and the disulphide at 23-72 means that the mutants are unlikely to cause local changes in the tertiary folding (Robinson et al., 1995).
4.3.3 Epitope mapping

Loop 1 mutations

Residues Lys 20, Glu 21 and Gln 22 are involved in the hCGβ-specific cluster β6 which is masked in the holo-hormone. Examination of the crystal structure shows that these amino acids are close to the α subunit; Glu 21 is partially buried and probably forms a salt bridge with Lys α45, so that binding of a mAb to this region would be blocked stereochemically in the heterodimer. The only hCG-specific residue in this area is Asn 77; this is an Asp in hLHβ and the negative charge of Asp may account for the failure of mAbs raised to this region of hCGβ to bind hLHβ. Moyle et al (1990) showed that the hCG-specific mAb B112 also binds hLH which has been mutated at position 77 from Asp to Asn. The substitutions in the C-terminal hairpin loop (Mutant 3) also abolished binding of the mAb to β6 (INN-hCG-64) suggesting that the structural changes also disrupt binding around the Asn at position 77. Further analysis by the single point Mutants 7 - 10 showed that only Gly 75 -> His was responsible for this effect. Therefore it is presumed that INN-hCG-64 and probably other mAbs which have mapped to epitope region β6, are binding to the area shown in figure 4.9b, comprising of Lys 20, Glu 21, Gly 22 and Gly 75.

Residues Pro 24, Val 25 and Arg 68 contribute unequivocally to epitope region β3 as demonstrated by the complete loss of binding of β3 mAbs when these residues are mutated. Results from Mutant 8 (Arg 74 → Ser, Gly 71 → Arg) would imply that Gly 71 may also be involved since INN-bLH-1 was raised against bovine LH which has Pro rather than Arg at residue 74. This is also borne out by the absence of an effect on β3 mAbs by Mutant 20 (Arg 74 → Ser). Clearly the two β3 monoclonals INN-hCG-111 and INN-bLH-1 have slightly different binding profiles as evidenced by the effects of substitutions in Pro 24 and Val 25. As INN-hCG-111 binding is affected by the substitution in Mutant 1, it could be argued that residues Lys 20, Val 24, Pro 25, Arg 68 and Gly 71 all contribute to this epitope. These proposed sites would allow the β3 and β6 mAbs to bind hCGβ simultaneously as previously shown (Berger et al., 1990).
**Loop 3 mutations**

Arginine dominates the functional epitopes of human growth hormone (Jin et al., 1992) and in the core of hCGβ the arginines at positions 68 and 74 project from the surface (Figure 4.9), and are likely to be highly immunogenic. Arg 68 showed the unexpected result that substitution of this residue on Mutant 7 reproduces the dramatic elimination of the cross-reacting β2, β3, β4, β5 and β3/5 epitopes achieved by the changes made in the C-terminal hairpin loop in Mutant 3. Arg 74 (substituted in Mutant 20) appears to have no part in the reaction of all these mAbs, except 3E2, with hCGβ. This is not surprising since all β3 and β5 specific mAbs show low cross-reactivity with porcine LHβ which differs from hCGβ at residue 74 by having Pro (Berger et al., 1990). Cosowsky et al. (1995) have shown that a mutation of Arg 74 → Pro largely eliminates the binding of B105, another mAb cross-reactive with hLH. The mAb 3E2 appears to be sensitive to all the mutations made here (with the exception of Gly 75 → His). If it is presumed that changes of greater than tenfold in relative antibody binding are necessary to identify an antibody binding residue (Prasad et al., 1993) then only Arg 68 and Arg 74 are implicated in mAb 3E2. If 3E2 binds to the tip of the hCGβ subunit, then the other mutations may be causing slight structural alterations to which this mAb may be particularly sensitive. The changes observed in the N-terminal loop can then be seen to have an additive effect, where the more changes introduced, the less 3E2 binds. This mAb appears to have a different binding region to β3 or β5 mAbs and this would explain why this mAb proved difficult to assign to the previously established epitope regions (Berger 1994, unpublished data). Possibly, the other cross-reactive mAbs used in our study are directed towards epitopes further along the beta hairpin towards Pro 70. We have not been able to discriminate the epitope regions β2, β3, β4 and β5 with the changes made here. Further mutagenesis studies would be required to identify the residues constituting each of the epitope regions on hCGβ unambiguously.

**Further mutations**

After analysing the immunogenicity of Mutant 3 (presented in chapter 5) the continuing mutation strategy was to join Mutants 3 and 4 and to place further mutations on the resulting Mutant 14. Two such mutants were made, Mutant 22 (with additional Arg 63 → Ser, Gly 65 → Ala) and Mutant 16, identical to Mutant 22 except for residue 68 which is
Leu. Mutant 14 and 22 as expected failed to bind the cross-reactive mAbs and the β6 mAb whilst retaining the epitopes for the hCG-specific and hCGβ-specific mAbs. It was interesting that Mutant 16 showed a slight difference to Mutant 22 where the β4 mAb INN-hCG-24 facs profile of fluorescence was shifted slightly to the right. The only difference between this and Mutant 22 is the substitution at position 68. The leucine does not have as complete an inhibitory effect as does a glutamic acid at this position. This is presumably due to some interaction of the positive charge of the arginine with the binding site of the mAb, glutamic acid changes the charge so this may actually repel a similarly charged area in the binding site. Leucine does not have a charge and so may still allow adjacent residues to partake in binding interactions.

With the panel of mAbs employed no further information on epitopes can be inferred from additional mutations on Mutant 14. However it is hoped that by testing the immunogenicity of such mutants that a decrease in the LH cross-reactivity will be observed.

**hCG-specific mutants**

Three mutants were made to pinpoint the residues involved in the hCG-specific epitope region β1. These showed that Arg 8 and 91 were not involved, but the replacement of Arg 10 led to a 40 % reduction in binding suggesting Arg 10 may play a minor role in antibody binding. Mutants 19-3 and 19-6, constructed in order to see the effect of changing Arg 63 and Gly 65 on the wild-type hCGβ, revealed interesting data related to the β1 epitope region. Substitutions at positions 63 and 65 alone had no effect on the binding of any of the mAbs, however, a fortuitous PCR error also changed Arg 60 → His in one clone (19-3). This mutant lost the binding of the β1 mAbs, and thus implicated Arg 60. This residue (Arg 60) is presumably a critical binding residue for the hCG-specific mAbs. Arg 60 is a shared residue with LH, therefore the adjacent residues have to be examined to determine where the hCG-specificity comes from. The hCG-specific residue 89 is contiguous with Arg 60 on the surface (Figure 4.12). Table 4.11 illustrates the reactivity of hCG, hLH and Mut 19-3 with β1 mAbs.
It appears that the specificity of the β1 mAbs is dictated by the presence of the Gln at position 89. In hLHβ the slightly larger side chain of arginine may prevent binding due to steric hindrance or to the increased electropositive charge. It is predicted that a mutant of hCGβ with Gln 89 → Arg would also prevent the reaction of the β1 mAbs, thus proving the source of the hCG-specificity. Arg 10 may also contribute slightly to the hCG-specificity as seen by the slight decrease in mAb binding to Mutant 13. It is difficult to explain why a histidine at position 60 prevents binding of the β1 mAbs. Perhaps the electrostatic interactions between the charged residues are of importance for this area of the antibody/antigen contact site (histidine is either uncharged or positively charged, depending on the local environment, it may be uncharged in this position). It may be that charge neutralisation is more important towards the centre of the contact area than on the edge where water can dissipate the charge effects (Davies et al., 1988). This would imply that residue 60 is towards the centre of the contact area. A model of the proposed binding area of the β1 mAbs is proposed in figure 4.12

<table>
<thead>
<tr>
<th></th>
<th>89</th>
<th>60</th>
<th>61</th>
<th>β1 mAb binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>hLHβ</td>
<td>Arg</td>
<td>Arg</td>
<td>Asp</td>
<td>-</td>
</tr>
<tr>
<td>hCGβ</td>
<td>Gln</td>
<td>Arg</td>
<td>Asp</td>
<td>+</td>
</tr>
<tr>
<td>Mut 19-3</td>
<td>Gln</td>
<td>His</td>
<td>Asp</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.11. Amino acids present at positions 89, 60 and 61 in Mut 19-3, hLHβ and hCGβ and their reactivity with β1 mAbs.

It has been demonstrated that it is possible to remove several shared epitopes on hCGβ whilst retaining the hCG-specific epitopes of the β1 region. This approach has identified several of the residues involved in the B-cell epitopes, both those shared with LH and specific to hCGβ. The importance of the substituted side-chain is also demonstrated. The most effective substitutions made in this investigation were those where a change of charge was introduced, e.g Arg for Glu. This indicates that for these particular mAbs the electrostatic interactions with the antigen are important for effective binding.
Figure 4.12 Proposed β1 hCG-specific epitope region.

Space filling diagram of hCG. The alpha subunit is in blue and the beta subunit in green. The proposed area for the hCG-specific epitope region β1 is indicated by a black oval. Residues shown by mutation to be important in the formation of the epitope region are highlighted: Arg 10 and Arg 60 are in red (with only Arg 10 labelled for ease of identification) and Gln 89 is in orange. (made in Rasmol using coordinates from file pdb1hcn.ent).
Chapter 5

Immunogenicity of hCGβ mutants
5.1 Introduction

This work has produced mutant hCGβ molecules which have lost varying numbers of hCG-specific and hLH shared epitopes. Those which have lost shared epitopes are potential fore-runners for the production of a genetically-engineered contraceptive vaccine which may be safer and more effective than the formulation already used in certain clinical trials (Jones et al., 1988; Talwar et al., 1994). This stage of the investigation therefore sought to examine whether one of these mutant hCGβ molecules was capable of eliciting an anti-hCG B-cell response and whether this response showed less cross-reactivity with hLH than that raised against wild-type hCGβ.

The expression system used in this work, whilst ideal for analysis of the mutants, does not produce secreted mutant protein suitable for purification and subsequent immunisation. Recent technological advances in the ability to deliver nucleic acid plasmids to tissues in vivo (Wolff et al., 1990; Lin et al., 1990; Williams et al., 1991) and also to express the proteins encoded therein have opened up a new field in vaccination studies. Initial studies by Wolff et al (1990) showed that naked DNA and RNA injected into the skeletal muscle of mice expressed levels of reporter enzymes comparable to those observed in transfected cell lines in vitro. The plasmid DNA remained unintegrated in the muscle cells and the expression was long lasting (2 months after initial injection). Later work showed that biolistic devices could also be used to deliver smaller amounts of DNA to other tissues and achieve expression (Williams et al., 1991). These biolistic devices use DNA coated onto 1 - 5 μm gold particles which are propelled into the tissue by pressurised helium gas.

The immunological breakthrough came when Tang et al (1992) demonstrated the presence of antibodies specific for human growth hormone (hGH) after immunisation with a plasmid encoding hGH into skin cells of mice via a hand held biolistic device. The induction of antibodies following genetic immunisation has since been shown in a variety of species and against diverse antigens including, haemagglutinin (HA) matrix protein and nucleoprotein (NP) from influenza A virus (Ulmer et al., 1993), gp 120 and gp160 from human immunodeficiency virus (Wang et al., 1993; Rhodes et al., 1994), surface glycoprotein from rabies (Xiang et al., 1995). In addition to antibody production the HA
and NP genetic immunisation resulted in protection to challenge with a lethal dose of influenza A virus.

Genetic immunisation has been shown to induce both antibody and cytotoxic T-cell responses (Coney et al., 1994; Hoffman et al., 1994). Genetic immunisation, since it does not involve lengthy purification procedures, has the potential of yielding immunogenicity results in a relatively short time. It has been shown that an immune response is evoked equally well whether the encoded protein is secreted, retained in the cell or membrane bound (Ulmer et al., 1993; Davis et al., 1994; Xiang et al., 1995). The type of immune response desired may be evoked by directing the encoded protein to the appropriate site. For example, the HIV response is similar to that seen with live whole virus since the synthesized protein is in the cell and thus can be presented on MHC class I molecules to produce a T-cell mediated immunity. Also addition of genes for immunological adjuvants in the expression vector can further direct the appropriate immune response (Raz et al., 1993). By using different promoters and regulatory elements in the vector construct, the expression level of the endogenously synthesized protein may be controlled (Vogel et al., 1995). Therefore in the absence of available purified protein, genetic immunisation was used to examine the immunogenicity of hCGβ Mutant 3.
5.2 Results

5.2.1 Wild-type hCGβ DNA injected mice

To determine the time course of antibody production against hCG in response to DNA immunisation, sera were taken from BALB/c mice two weeks after each injection of wild-type DNA (wild-type sera) and analysed for their reactivity with hCG protein. All ten mice produced antibodies which recognised hCG protein. In the strongest responders there was a measurable antibody response two weeks after the second DNA immunisation, though most of the mice produced a measurable response only after the 3rd injection (Figure 5.1). Surprisingly seven out of 10 mice show a decrease in response after the fourth DNA immunisation. This may be due to storage artifacts; the third bleed having been stored at 4°C for longer than the fourth bleed was more likely to have partially evaporated, thus making it more concentrated. There is also a variability amongst individuals within the group of mice. Five out of 10 mice show a high response with end point titres above 100,000 (data not shown). Two have a low response, but this is still higher than that of the pre-immune sera. The mice vaccinated with plasmid alone showed no response (data not shown).

This ELISA was performed by Daniel Cooper (BSc student) under my supervision.

5.2.2 Mutant 3 DNA injected mice

The sera from the BALB/c mice taken two weeks after the fourth immunisation with Mutant 3 DNA (mutant sera) showed a marked difference in hCG reactivity to that seen with the wild-type sera. After development of the ELISA substrate for one hour there was little signal (compare the wild-type sera with the mutant sera in figure 5.2 a). If the substrate was allowed to develop for a further 17 hours (i.e. overnight), then an appreciable signal was seen for all mice (Figure 5.2b). This was considered valid since the pre-immune sera controls remained negative and the duplicates remained tight. After an
equivalent time the signal from all the wild-type sera reached saturation (data not shown). The mutant sera were also heterogeneous in their antibody response as can be seen clearly in the overnight signal. Five mice have levels approaching saturation, three have O.D. readings between 0.5 and 1.5 and two are less than 0.5. Overall the response is significantly lower than with the wild-type hCG DNA (Figure 5.2).

5.2.3 Reactivity of wild-type and mutant sera with hLH

The four best responders from the wild-type and mutant sera were tested for their ability to recognise hLH protein. One of the four wild-type sera tested recognised hCG far better than it did hLH (mouse B2). The other three wild-type sera recognised hCG and hLH to a similar extent, with perhaps two slightly biased to hCG (B5 and C1) and one slightly biased to hLH (C4) (Figure 5.3). This indicates that despite five hCG-specific epitope regions mapped on the beta-subunit the antibodies raised against wild-type hCGβ are primarily directed to the cross-reactive epitopes. The mutant sera showed surprising results, three recognised hLH better than they did hCG (E1, E3 and E4) and this was quite marked in two of the sera (E1, E4). The fourth mutant serum gave a higher signal with hCG (E2) (Figure 5.4).
Figure 5.1 Time course of immune response to hCGβ DNA vaccination.

Serum samples were taken the day before each injection, which were given at two week intervals. Sera were assayed for binding to 1μg/ml hCG coated directly on ELISA plate. Detection was with an alkaline phosphatase conjugated anti-mouse IgG. 1 hour after addition of substrate, O.D. was measured at A₄₁₀. All samples were tested in duplicate and standard errors are shown.
Figure 5.2 Comparison of response of mutant sera and wild-type sera.

A) 1 hour substrate development.
B) after overnight substrate development.

Data from individual mice are shown. hCG was coated directly onto the ELISA plate and a 1 / 100 dilution of mice sera used. Detection was with an alkaline phosphatase conjugated anti-mouse IgG, and the reaction with the substrate measured at A_{410} both after 1 hour and after overnight incubation. The pre-immune sera are also shown as negative controls. All samples were tested in duplicate and standard errors are shown.
Figure 5.3  Comparison of reaction of wild-type sera with hCG and hLH.

Only the four best responders of the wild-type injected mice were analysed. Sera were used in fourfold serial dilutions from 1/100 to 1/102400, with hCG (1 μg/ml) or hLH (1 μg/ml) coated ELISA plates. Detection was with an alkaline phosphatase conjugated anti-mouse IgG and the O.D. signal was measured at A410 one hour after the addition of the substrate. All samples were done in duplicate, pre-immune sera is shown as a negative control.
Figure 5.4  Comparison of reaction of mutant sera with hCG and hLH.

Only the four best responders of the Mutant 3 injected mice were analysed. Sera were used in fourfold serial dilutions from 1/100 to 1/102400, with hCG (1 μg/ml) or hLH (1 μg/ml) coated ELISA plates). Detection was with an alkaline phosphatase conjugated anti-mouse IgG and the O.D. signal was measured at A415 after overnight incubation with the substrate. All samples were done in duplicate, pre-immune sera is shown as a negative control.
5.3 Discussion

Genetic immunisation has been successfully used to examine the immunogenicity of Mutant 3 and compare it to that of wild-type hCGβ. An immune response was observed in all mice vaccinated with mutant or wild-type DNA and those immunised with plasmid alone showed no response (data not shown). However, as observed in previous DNA immunisation studies (Davis et al., 1993; Hoffman et al., 1994) the IgG response measured was variable amongst the population of mice used. This phenomena is also common with protein immunisation. It could be caused by human error, where there is variability in the treatment of the mice by the handler with the result that some mice are more stressed than others. Stress may affect the immune response (Matthews et al., 1995). Another consequence of different stress levels in the mice are different relaxation states of the muscles to be injected. If a muscle is tense, not all the DNA may enter. Also the mouse may jerk its leg and the needle position within the muscle tissue may vary. Many investigators choose to pool sera for these reasons. The variability may be reduced by using anaesthetized mice when injecting. This will allow the intra-muscular injection to be more easily and reproducibly administered.

The Mutant 3 injected mice gave a significantly lower hCG-specific antibody response than did the wild-type injected mice. Even the best responders of the Mutant 3 DNA injected mice were only as good as the poorest responders of the wild-type hCGβ DNA immunised mice. This could be due to the loss of a major B-cell or T-cell epitope due to the changes introduced in Mutant 3. Wild-type hCGβ has only four residues different from Mutant 3. It has been shown that only one of these changes, Arg 68 to Glu, has a significant effect on the binding of the panel of mAbs tested (the mAbs were produced from a BALB/c immunised mouse). Perhaps the structure recognised by these mAbs, which is destroyed in Mutant 3, is an immunodominant B-cell epitope in Balb-c mice with residue 68 being critical. This would explain why Mutant 3 elicits such a poor antibody response when compared to wild-type hCG. Another possibility is that a T-cell epitope has been eliminated by the changes in Mutant 3. Previous work producing T-cell hybridomas by immunisation of BALB/c mice with hCGβ have not indicated a T-cell epitope in the area
of Mutant 3 (Rouas et al., 1993). There was, however, indications of a T-cell epitope including residues 20, 21 and 22. It is a theoretical possibility that the changes in Mutant 3 may prevent the processing of this T-cell epitope, perhaps by preventing access of proteases to specific sites. However, examining the substitutions made and their proximity to residues 20 - 22, this would seem unlikely.

Of the four mutant sera tested, three contained antibodies which resulted in a higher signal in an ELISA with hLH than with hCG. Three out of four wild-type sera recognised hCG equally as well as hLH. This implies that in vivo most of the immune-response is directed against the shared epitopes. In the fourth mouse given wild-type hCGβ DNA (B2), the hCG-specific epitopes must have been more readily recognised as this serum had a much greater reactivity with hCG than with hLH. The mutant sera data indicates that the region changed in Mutant 3 is immunodominant in BALB/c mice, and therefore when it is removed, the level of antibodies raised is much lower. Amongst this decreased antibody response, there appear to be more antibodies which cross-react onto hLH than are hCG-specific. This may mean that there are still more shared epitopes on Mutant 3 than hCG-specific epitopes. However, antibodies raised against shared epitopes should still react equally well with hCG as with hLH and thus should give similar results on both proteins. It could be argued that the changes made must have made part of the molecule look more like hLHβ, however this is unlikely as the area involved is virtually identical in both hLHβ and in hCGβ. There may be a technical problem in the assay which is the source of this peculiar result. hCG and hLH protein are both known to dissociate quite readily on storage to their subunits, but hLH is more prone to this (Berger, pers comm). Thus, there may be more free hLHβ than hCGβ in the protein preparations coated on the ELISA well. Since the beta subunit is the immunogen, there will be antibodies raised which are specific for free subunit and these will bind more in the hLH assay giving a falsely higher signal.

The recombinant form of hLH and hCG is more stable and less readily dissociates (Lapthorn pers comm), repeating the assay with recombinant proteins will demonstrate if this is the cause. Another possible explanation is that urinary hCG preparations contain a lot of hCG core fragment (hCGcf) which is believed to be a degradation product (de Medeiros et al., 1992). Loop 2 is missing from hCGcf, but is presumably present in most of the pituitary derived hLH. Antibodies raised against B-cell epitopes on loop 2 will
therefore bind to a greater proportion of the hLH molecules in the assay than to the hCG molecules. This could also give a false positive result for the reaction of the mutant sera with hLH. These technical problems should be overcome by using the recombinant proteins.

Despite the uncertainties expressed above, it is clear that removing an apparently immunodominant shared epitope on the hCG beta subunit still leaves areas on the mutant protein which are capable of eliciting cross-reacting antibodies. These areas will have to be identified and eliminated in order to create a totally hCG-specific immunogen. Analysis of the mutants with the panel of mAbs has shown that the residues changed on loops 1 and 3 (in Mutants 3 and 4) are all involved in the formation of shared epitopes except for residues 74, 75 and 79. The panel of mAbs used, whilst adequate for the analysis here, is limiting in that all the cross-reactive specificities used, except the β3 mAbs, seem to cover a very similar area (as indicated by loss of reactivity of all with Arg 68 → Ghu). Therefore though changes at residues 74, 75 and 79 have not affected any of our panel of mAbs, other epitopes to which we did not have mAbs may be affected. For example, a mutation at Arg 74 has been shown to affect the binding of mAb B105 (Cosowsky et al., 1995). The continuing strategy is to combine Mutant 3 and Mutant 4 to make Mutant 14 and all future mutants will use this and not wild-type hCGβ as the template. The panel of mAbs used should be expanded to include more cross-reactive mAbs against hCG and also mAbs raised against hLH, as no further information will be gained with the existing mAbs. Polyclonal antisera raised against hLH may also be used in the COS7 expression system to quickly determine whether areas of hLH cross-reactivity have been removed on future mutants. The mutant sera produced here may be used to identify these areas and it is possible to make mAbs to the mutant protein which would also aid the identification of further shared epitopes. Prior to this a systematic approach of changing all shared residues which are not important structurally and analysing the immune response elicited will provide a steady, although perhaps not rapid means of developing a more refined contraceptive vaccine.
Chapter 6

General Discussion
Human chorionic gonadotropin is currently undergoing clinical trials as a potential immunocontraceptive (Talwar et al., 1994). However, the epitopes it shares with hLH could prohibit its widespread acceptance in this application. This project has sought to create epitope-loss mutants which have lost these shared epitopes by substitution with other residues. In this way the principle of creating epitope-specific vaccines is demonstrated, where deleterious T- and B-cell epitopes are removed, maintaining the desired epitopes which elicit the required immune response by the correct folding of the molecule.

6.1 Expression systems

Three expression systems for the analysis of hCGβ mutants were examined. Two used a prokaryotic host and one used a mammalian cell line. Of the two prokaryotic systems, the pMAL produces a fusion protein with the subcloned hCGβ which would facilitate purification of mutated molecules, whilst the bacteriophage system would allow isolation of mutant protein together with the gene encoding it by the surface expression of hCGβ. The pMAL system gave high levels of expression but only with the vector which restricted expression to the bacterial cytoplasm. Human chorionic gonadotropin β has six disulphide bonds, which have since been shown to be involved in the intricate cystine knot motif (Lapthorn et al., 1994), and is unlikely to fold properly in the reducing environment of the bacterial cytoplasm. Indeed, unpublished data from this study and also work from another group have found cytoplasmic E.coli expression of hCGβ to result in insoluble product forming in inclusion bodies (Huth et al., 1994). This is quite a common occurrence for recombinant proteins expressed in E.coli and indicates that the protein has not folded properly. Molecular chaperones are normally responsible for inhibiting the formation of such aggregates and foldases, such as protein disulphide isomerase increase the rate of folding. The molecular chaperones BiP, Erp72 and Erp94 have been found associated with unfolded, unglycosylated hCGβ expressed in Cho cells (Feng et al., 1995b). Overexpression of the bacterial equivalents of these chaperones (e.g. DNAk for BiP) may lead to higher levels of properly folded protein in a bacterial system. Derman et al. (1993) discovered that mutations that inactivate thioredoxin reductase allow an oxidative state in
the bacterial cytoplasm and permit the formation of disulphide bonds in recombinant proteins expressed cytoplasmically. Work in both these areas of disulphide bond formation and molecular chaperones holds promise for the future expression of mammalian genes in E. coli. The bacteriophage system had the advantage that the expressed protein is transported to the periplasm, where disulphide bonds can assemble. However, the surface expression of hCGβ was low and considerable effort would have been necessary to further optimise the system. A mammalian expression system was designed which allowed surface expression of hCGβ to facilitate analysis of mutant molecules. COS7 cells transiently transfected with hCGβ stain very strongly in fluorescent analysis using a panel of conformationally dependent mAbs. Thus, the COS7 expression system allowed fine analysis of hCGβ mutants using flow cytometry.

6.2 Epitope analysis

At the time the COS7 expression was set up, the crystal structure of hCG was published (Lapthorn et al., 1994; Wu et al., 1994). This provided hitherto unknown information about the relative positioning of the amino acid residues in the subunits and permitted precise mutagenesis, targeting potential shared epitopes. A number of site-directed mutants were therefore constructed. The analysis of these mutants revealed information about the epitopes on hCGβ as discussed in some detail in section 4.3 and summarized in table 6.1. Earlier works (summarized in Bidart et al., 1993) had defined the antigenic areas on hCGβ, with the shared epitope regions (β2 - β5) being confined to a single large area at one end of the molecule. The β3 region had different binding patterns than the other shared regions and was the only one which would allow simultaneous binding to the β6 region. The β2, β4 and β5 regions overlapped with the chain-specific β6 region. The hCG-specific β1 and β7 regions were located at the other end of the molecule (figure 1.6). The results of this present study are all in complete accordance with these earlier studies. The residues involved in the formation of the β3 region included the critical residue, Arg 68, on loop 1 for β2, β4 and β5 but also specific to β3 were residues on loop 3. This explains the molecular differences suggested by simultaneous binding studies. β1 was
shown to be located near the cystine knot, quite distant from the shared epitope regions, but there was no overlap with β7 observed with the residues so far examined. The molecular differences between β2, β4 and β5 have not been discerned with this study and further work is required to define these regions.

<table>
<thead>
<tr>
<th>epitope region</th>
<th>specificity</th>
<th>residues involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1</td>
<td>hGPH, hGPHα</td>
<td></td>
</tr>
<tr>
<td>α2</td>
<td>hGPH, hGPHα</td>
<td>α 13 - 18</td>
</tr>
<tr>
<td>α3</td>
<td>hGPH, hGPHα</td>
<td></td>
</tr>
<tr>
<td>α4</td>
<td>hGPH, hGPHα, oLHα</td>
<td>α 17 - 22</td>
</tr>
<tr>
<td>α5</td>
<td>hGPH, hGPHα, oLHα</td>
<td></td>
</tr>
<tr>
<td>α6</td>
<td>hGPH, oLHα</td>
<td>α 33 - 42</td>
</tr>
<tr>
<td>α7</td>
<td>hGPH</td>
<td></td>
</tr>
<tr>
<td>β1</td>
<td>hCG, hCGβ</td>
<td>β 10, β 60</td>
</tr>
<tr>
<td>β2</td>
<td>hCG, hCGβ, hLH, hLHβ</td>
<td>β 68</td>
</tr>
<tr>
<td>β3</td>
<td>hCG, hCGβ, hLH, hLHβ</td>
<td>β 24, β 25, β 68, β 71</td>
</tr>
<tr>
<td>β4</td>
<td>hCG, hCGβ, hLH, hLHβ</td>
<td>β 68</td>
</tr>
<tr>
<td>β5</td>
<td>hCG, hCGβ, hLH, hLHβ</td>
<td>β 68</td>
</tr>
<tr>
<td>β6</td>
<td>hCGβ</td>
<td>β 20, β 21, β 22, β 75, β 77</td>
</tr>
<tr>
<td>β7</td>
<td>hCGβ</td>
<td></td>
</tr>
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<td>β8</td>
<td>hCG, hCGβ</td>
<td>β 137 - 144</td>
</tr>
<tr>
<td>β9</td>
<td>hCG, hCGβ</td>
<td>β 110 - 116</td>
</tr>
</tbody>
</table>

Table 6.1 Epitope regions defined on hCG, their specificity and any residues demonstrated to be involved in the binding of mAbs (12,191). Those determined by the present study are in bold. hGPH = human glycoprotein hormone, which all share common α-subunit.

6.3 Immunogenicity of Mutant 3

The DNA immunisations provided limited information on the immunogenicity of Mutant 3 in BALB/c mice. IgG antibodies which recognised hCG were raised in all of the
immunised mice. However, there was a large reduction in the reactivity of the sera from the Mutant 3 DNA immunised mice tested for binding to hCG protein compared to that of sera from wild-type hCGβ DNA immunised mice. Mutant 3 has four residues different from wild-type hCGβ. The difference in antibody response measured on the mutant molecule could indicate that there has been a loss of conformation. However, this appears not to be the case in vitro, since when Mutant 3 is expressed on the surface of COS7 there is no loss of reaction with the conformationally-dependent mAbs which do not map to the β2 - β6 area. It is unlikely that the situation is different in vivo. The low antibody response against the Mutant 3 could mean that a major B- or T-cell epitope has been lost. Very recent data (not shown) on the hCG-specific antibody response raised to Mutant 7 (Arg 68 → Glu) in DNA immunised BALB/c mice show a higher response than that of mice immunised with Mutant 3, incorporating all four changes in loop 3. It is possible that all or some of the changes at positions 74, 75 and 79 disrupt a T-cell epitope in the β chain not previously identified in a study on T-cell epitopes of the β chain due to limitations of the screening system used in that study, where the APC used was unable to present some of the peptides of hCGβ (Rouas et al., 1993). Alternatively it could be that the region changed on loop 3, especially the arginines 68 and 74, is amongst the most immunogenic on hCGβ. Jin et al. (1992) analysed the importance of individual residues to the B-cell epitopes on human growth hormone and discovered that arginine residues played a major role in 19 out of 21 functional epitopes defined. Of the mutants analysed in this present study, the arginine residues at 68, 60 and to a lesser extent 10, have been shown to be pivotal in different epitopes. In Mutant 3, two of the four residues changed are arginines. These residues protrude from the surface of hCGβ and are probably highly immunogenic. Though residue 74 has not been implicated with our mAbs, other studies have certainly demonstrated its importance in shared epitopes (Cosowsky et al., 1995). With the removal of this hCGβ immunogenic area, there has been a fall in antibodies made in the mouse which recognise hCG. Antibodies may be made against the new structure but these may not recognise the native hCG. What is left is the response elicited by the rest of the molecule.
6.4 Solving future immunogenicity problems

If the strategy is to gradually add more mutations onto the hCGβ subunit, then this early result with Mutant 3 raises important issues about how immunogenic an 'LH-free' hCG structure would be. In the present study, many of the replacement amino acids were selected from some of the other human glycoprotein hormones. This was to ensure the correct folding of the mutant molecule. In the context of developing a vaccine to be given to healthy women, to have these residues in the final product may be undesirable in case cross-reactivity is produced, however, it is unlikely that the introduction of one amino acid will re-create the epitope existing on the other glycoprotein hormone. Future work will probably introduce poorly immunogenic residues, whilst checking that the overall conformation is unchanged. Thus it is easily envisaged that what will be left after all the LH-cross reactivity is removed will be a very poor immunogen, raising few hCG-specific antibodies, as already observed with Mutant 3. In fact the wild-type hCGβ has been found to be poorly immunogenic in humans (Shahani et al., 1982) and was coupled with an ovine alpha subunit to increase its immunogenicity. The implication from the Mutant 3 immunogenicity data is that there is no increased immune response to the unchanged areas on hCGβ if another area is changed, each epitope region appears to have a finite immunogenicity when presented in the same way.

The concept of antigenic competition has been studied with respect to the surface glycoproteins of influenza virus, neuraminidase (NA) and HA. Both HA and NA are highly immunogenic but when presented together on an intact viral particle HA is dominant over NA in immunogenicity. This is due to intravirionic antigenic competition, where the majority of the T and B-cell priming which occurs is specific for HA (Johansson et al., 1987b), presumably since HA appears on the viral surface in greater numbers than NA, therefore there is a skewed immune response at the individual epitope level. If the HA and NA are presented as separate purified proteins then they each give a similar immune response (Johansson et al., 1987a). The implications for these results could be that removal of a dominant antigen will allow an alternative antigen to elicit a good immune response. It is prevented from doing so normally because of increased numbers of the dominant antigen (as in HA and NA) or perhaps because of a higher number of
precursor T and B-cells which recognize the structures on the dominant antigen more readily and so a situation of clonal dominance occurs. However the biased repertoire theory has been shown to be not valid in at least one situation, that of an inbred mouse strain mice immunised with tobacco mosaic virus protein where it was proposed that a random selection of the available antigen-specific B-cell clones occurs.

In the case of hCGβ there is some evidence that the shared epitopes may be immunodominant in humans. Analysis of sera from 18 subjects immunised with the HSD showed that mAb 206, which cross-reacts with hLH, inhibited binding of the sera to hCG from 40 to 90% in 39 out of 40 random samples tested, compared to 20% inhibition in only two out of 40 samples by an hCG-specific mAb (Deshmukh et al., 1993). The epitope recognized by mAb 206 was dominant throughout the immunisation course, and the polyclonal antibodies against this region were shown to neutralise the binding of hCG to its receptor. The preponderance of anti-hCG antibodies against the epitope region of mAb 206 may be due to a higher immunogenicity of this region. In this present study all the cross-reactive mAbs examined mapped to the same area on the tertiary structure of the hCGβ. In this study when mice were immunised with a mutant hCGβ which is changed in an area where several cross-reactive mAbs map, the antibody response against hCG declined dramatically. Taken together these observations could imply a situation where the area of hCG which determines the majority of the cross-reactivity with hLH also elicits the majority of the anti-hCG immune response. However, it has to be borne in mind that this is drawing conclusions from immunogenicity results from two different species with different immunisation schedules and in different studies. Nevertheless, both the work in humans and the present work in mice suggest the comparatively poor immunogenicity of the hCG-specific areas in both species. The work presented here also suggests that there is no increased immune response against the hCG-specific areas when the competition of the cross-reactive area is removed. If this is the case, then in the future it will be necessary to find means of dramatically increasing the immunogenicity of a solely hCG-specific immunogen. If on the other hand the results seen with the Mutant 3 immunisations are due to a previously undiscovered T-cell epitope in the ‘cross-reactive’ area, then additional T-cell help will have to be introduced. This could be examined in the
future by attaching a universal T-cell epitope to the mutant hCGβ molecule and observing if there is any increase in immunogenicity to the hCG-specific regions.

These immunogenicity results are from an inbred mouse strain. In order for a vaccine to be effective it would have to work in an outbred human population with diverse MHC haplotypes. The relative potency of individual epitopes may be different not only between the different species, but also between individuals within the species. It is important therefore, to attempt to ascertain how well the human immune system might recognise the mutant hCGβ. A number of approaches can be taken before a full scale clinical trial.

There already exists human sera raised to wild-type hCGβ during the course of clinical trials (Deshmukh et al., 1993; Deshmukh et al., 1994). This sera could be analysed for its reactivity with the mutant proteins in comparison to wild-type hCGβ. Alternatively human monoclonal antibodies could be raised against hCGβ and their binding specificities mapped. This would give some indication of which areas of hCGβ are immunogenic in humans. Also a phage library of human heavy and light chains could be screened with the mutant protein (Winter et al., 1994). However there are limitations with the phage library system as the heavy and light chain pairings selected are not necessarily those that existed in vivo.

The addition of powerful, universal T-cell epitopes could be one approach to increasing the overall immunogenicity of a final hCG-specific contraceptive vaccine. Such universal T-cell epitopes are found in the malaria circumsporozoite protein (Sinigaglia et al., 1988) and also influenza HA (Roche et al., 1990). Also the use of suitable adjuvants could help to increase the immunogenicity of the vaccine. Another method suitable for use with a contraceptive vaccine is the use of prolonged-release vaccine delivery systems. Encapsulating antigen in microspheres has been shown to give a prolonged antibody response removing the need for booster injections (Stevens et al., 1990; Singh et al., 1992). Alternatively the complement system may be exploited to increase the immune response against the immunogen. Dempsey et al. (1996) demonstrated how fusing the murine C3d to hen egg lysozyme (HEL) increased the immunogenicity of the HEL by 10,000 fold in immunised mice. The C3d molecule appears to signal the immune response to target antigens to which it is attached.
6.5 Accommodation of side-chains

An interesting concept which can be observed in this work is that of accommodation of side chains in substituted amino acids. Mutant 7 which has a substitution at position 68 of glutamic acid for arginine results in the complete loss of binding of mAb INN-hCG-24; however, when a leucine is substituted instead (Mutant 16), some binding of the mAb is maintained (13%). It is presumed that the charge change associated with the substitution of Arg $\rightarrow$ Glu repels a negatively charged side chain in the antibody binding site. When the arginine is replaced by a leucine, there is a loss of charge, but no charge change. This must allow the antibody binding site to approach the epitope, but the reaction is still much weakened. This indicates that the electrostatic interaction between the epitope and this particular mAb is important for the strength of the reaction, and also that the Arg 68 is a critical residue. Where possible, large changes have deliberately been introduced in the present mutants in order to disrupt mAb binding as much as possible. Now that the important residues for binding of many mAbs has been demonstrated it would be interesting to substitute different residues in these positions. This would provide information on the relative importance of different types of interaction between antibody and antigen. A similar approach could be used to analyse the structural contributions of different side chains in different positions on hCGβ.

6.6 Future use of hCG as an immunocoontraceptive

There are drawbacks with all methods of hCG contraception currently being tested. The HSD approach using the ovine alpha chain has the problems already alluded to with possible cross-reactivity not only with hLHβ but also with the other human glycoprotein hormones through shared epitopes on the alpha chain. Berger et al. (1990) have defined two epitope regions shared between ovine alpha and human alpha chain. Also only 80% of women tested produced sufficiently high titres of antibody necessary for a contraceptive effect, even when using a tetanus toxoid or diptheria toxoid carrier to provide T-cell help (Talwar et al., 1994). The use of tetanus toxoid as a carrier had previously been observed to lead to carrier-induced epitope suppression. Schutze et al. (1989) have shown that
administering an immunogenic carrier prior to hapten/carrier immunisation suppresses the anti-hapten response. This is due to the prior expansion of carrier-specific B-cells. Thus on a second encounter with carrier plus hapten there is a competition between the greater number of the existing memory B-cells and priming of naive B-cells which recognize the hapten (Schutze et al., 1989). A similar phenomenon may be occurring with the tetanus toxoid linked hCGβ. This was overcome to some extent in the HSD trials by alternating diptheria toxoid with tetanus toxoid (Gaur et al., 1990). Alternatively increasing the ratio of hCG:carrier may also overcome the suppression (Schutze et al., 1989). The antibody levels of the women in the trial needed constant monitoring to determine when to give booster injections, and contraceptive levels of antibody were not reached until three months after the initial injection. These immunogenicity problems may be overcome by using one of the universal T-cell epitopes, and also trying one of the methods mentioned above for increasing immunogenicity, especially encapsulating the HSD in microspheres. One problem that would not be overcome by these measures is the failure rate observed with contraceptive levels of antibody. One pregnancy was recorded over 1224 cycles. This is the equivalent of one unwanted pregnancy per year in a group of 100 women, compared to a failure rate of 0.1 - 0.4 per year in women taking the contraceptive pill (Johnson et al., 1995).

The WHO approach is more acceptable in terms of the lack of cross-reactivity with other hormones. However doubts have been raised about its efficacy and safety since antibodies against the β8 epitope region may still bind the C-terminus when hCG is in complex with its receptor (Dimhofer et al., 1994c). This could result in an autoimmune reaction against the ovary. The neutralizing ability of anti-CTP antibodies has also been questioned (Dimhofer et al., 1993), though the hCGβ-CTP has been shown to prevent pregnancy in baboons when used with Freund’s complete adjuvant (Stevens et al., 1981). The efficacy of the C-terminus peptide in humans will be determined in a current phase II clinical trial. The poor immunogenicity of the peptide may be overcome by using the strategies discussed. Perhaps using a MAP type system with more than one peptide would help combining a peptide with a universal T-cell epitope.
Although the hCG which is produced in relatively high levels during pregnancy is actually of fetal origin, the mother does show immune-tolerance to the protein. This tolerance has been broken successfully in clinical trials by presenting the protein conjugated to tetanus toxoid and diptheria toxoid T-cell epitopes. The autoreactive CD4^ sup T helper cells presumably are tolerized by the trace amounts of hCG that exist in normal humans (Birken et al., 1996). This tolerance may also explain the reversibility of hCG-vaccines which is observed after contraceptive levels of antibodies have fallen sufficiently (Talwar et al., 1994). In the absence of the foreign helper T-cell epitope, the native hCG is unable to provoke antibody formation.

Another factor that ought to be considered when discussing hCG as a contraceptive vaccine are the new roles for hCG that are being uncovered as the result of recent research on the hormone and its receptor. The receptor has been found on non-gonadal reproductive tissue in non-pregnant females and also on circulating lymphocytes during pregnancy (Reshef et al., 1990; Lin et al., 1995). The function of the receptors in these tissues is as yet unclear, but needs to be considered if hCG does have a role other than maintenance of progesterone during pregnancy. There have also been reports of hCG in the urine of healthy non-pregnant women (Chen et al., 1976; Armstrong et al., 1984) and recently it has been isolated from normal human pituitary (Birken et al., 1996). There is as yet no known function of pituitary hCG. The potential pathogenesis which could result at the pituitary during a long-term use of an anti-hCG contraceptive vaccine will have to be evaluated. It does appear likely that hCG is used in vivo to stimulate proliferation of some cells (Rivera et al., 1989; Gillot et al., 1996). This growth factor activity is normally confined to early stages of fetal development, but it increasingly seems that many tumours secrete hCG and/or its subunits for this purpose. This could point towards another use of an hCG vaccine, in preventing tumour growth. Tumours in nude mice, derived from a transplanted human lung cancer cell line, were shown to undergo necrosis and growth inhibition on treatment with anti-hCGα antiserum raised in goat (Kumar et al., 1992). hCG vaccines could be developed to treat hCG-secreting cancers. It is interesting to note here that there has been a report of hCG activity that apparently contradicts previous notions on hCG growth factor activity. Lunardi-Iskander et al. (1995a) reported hCG and hCGβ preparations inhibiting the growth of a Kaposi sarcoma cell line in vitro. They
propose this could be due to competition between hCGβ and a growth factor needed by these cells (Lunardi-Iskander et al., 1995b). However, they were also proposing that the hCG is binding the hCG receptor and not an alternative growth factor receptor. It has been suggested that the use of a crude hCG preparation in their assays could have affected the observations (Berger et al., 1995)

6.7 Epitope-specific vaccines

Thus it has been demonstrated that even with a molecule like hCGβ which has complex non-contiguous B-cell epitopes, it is possible to make radical changes in side-chain properties which remove unwanted shared epitopes whilst maintaining sufficient conformation to allow undiminished binding of other mAbs against desired epitopes. This shows that the concept of creating epitope-specific vaccines is sound. It is feasible to alter residues in one area of the molecule without disrupting other regions. The early immunogenicity data presented here highlights what may be one problem area with this strategy. In removing B-cell epitopes from a molecule, especially those that are immunodominant, the overall immunogenicity of the molecule decreases. However, as already discussed in detail there are various strategies for overcoming this limitation.
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Identification and selective destruction of shared epitopes in human chorionic

In Press

Berger P, Bidart JM, Delves PJ, Dirnhofer S, Hoermann R, Isaacs N, Jackson AM,
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Immunochemical mapping of gonadotropins. (1996) Ares Serona Sympoisia Series In
Press.
Identification and selective destruction of shared epitopes in human chorionic gonadotropin beta subunit

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Received 27 March 1996; revised 7 May 1996; accepted 14 May 1996

Abstract

The feasibility of producing epitope-specific antigens by mutation of the gene is demonstrated, the aim being to eliminate unwanted surface epitopes yet allowing the natural folding of the protein to maintain the desired epitope(s). The model protein is the β subunit of human chorionic gonadotropin (hCGβ) which previously has been used as an immunological contraceptive vaccine but has extensive cross-reaction with human luteinizing hormone. Of a series of mutants made, the mutant with substitutions of Glu for Arg 68, Ser for Arg 74, His for Gly 75 and His for Val 79, lost the ability to react with a panel of cross-reacting monoclonal antibodies while retaining the discontinuous and linear epitopes specific to the holo-hormone. In addition, allocation of amino acid residues to established epitope clusters could be made: residues 24, 25, 68 and 71 probably contribute to the cluster termed β3, residues 20, 21, 22, 75 and 77 to cluster β6 and residue 68 to clusters β2, β4 and β5.

Keywords: hCG; hLH; Epitope; Protein engineering; Mutagenesis; Epitope-specific vaccine
1. Introduction

Human chorionic gonadotropin (hCG) is one of a family of four heterodimeric glycoprotein hormones which comprise human leutinizng hormone (hLH), follicle stimulating hormone and thyroid stimulating hormone. The hormones share a common alpha subunit transcribed from a single gene and differ with respect to hormonal properties by virtue of their different β subunits. Human CG plays a unique role in the maintenance of pregnancy and can be detected in the maternal circulation 7 days post-fertilization. It has long been considered a target for a vaccine-based contraceptive, using the human β subunit (Talwar, 1994). Talwar et al. (1994) have used a heterospecies dimer (HSD) involving the ovine alpha chain and hCGβ. In phase 2 clinical trials it was shown that neutralizing antibodies were produced in response to the HSD. However, because hCGβ shares an 85% amino acid homology with hLHβ (in the first 110 amino acids), the antibodies produced also cross-react with hLH. To avoid this cross-reactivity the unique hCGβ-C- terminal peptide (hCGβ-CTP) has been proposed as a vaccine (Jones et al., 1988). A drawback with this latter approach is that the hCGβ-CTP is poorly immunogenic (Ramakrishnan et al., 1979) and those antibodies that are elicited may not effectively neutralise native hCG (Dirnhofer et al., 1993).

Our approach has been to produce a genetically engineered hCGβ which has lost those epitopes giving cross-reactions with hLHβ but still retains the correctly folded hCG-specific discontinuous epitope(s). In this way we hope to produce an epitope-specific molecule (Roitt, 1989). This strategy is equally applicable to other molecules, not just for removing discontinuous epitopes, but for removing one function of a bi- or multifunctional molecule.

Whilst progress has been made in identifying the amino acids involved in the binding of hCG-specific mAbs (Bidart et al., 1985; Moyle et al., 1990) less is known about the cross-reactive epitopes, apart from limited competition mapping (Schwarz et al., 1986; Berger et al., 1990). In contrast to linear epitopes, which may be identified with overlapping synthetic peptides, discontinuous epitopes are difficult, if not impossible, to identify without structural knowledge. A procedure involving random mutagenesis on the gene for the candidate protein, followed by expression and selection with monoclonal antibodies (mAbs) is a possible method for identifying key residues contributing to an epitope, which can then be removed (Peterson and Seed, 1988). In the present study, the crystal structure of hCG
(Lapthorn et al., 1994; Wu et al., 1994) is used in conjunction with sequence data of related hormones to select rationally residues that are involved in discontinuous cross-reacting epitopes. Mutations of these residues have been engineered and certain mutants have lost the ability to bind a panel of hCGβ/hLHβ cross-reactive mAbs whilst retaining epitopes specific for hCGβ.

2. Materials and methods

2.1. Antibodies

All mAbs except OT3A and 3E2 have been described previously (Berger et al., 1990). OT3A was a gift from Dr Wim Stevens (Organon Technika, Netherlands) and recognises hCGβ 133–139. 3E2 was a gift from Professor Roger Ekins (UCL, UK) and binds both hCGβ and hLHβ. The FITC-conjugated F(ab′)2 rabbit anti-mouse Ig is from Dako (Cat No. F313). All mAbs are of the subclass IgG1 (except INN-hCG-64, INN-hCG-68 and 3E2 which are only known to be IgG). An attempt was made to map mAb 3E2 into the previously defined epitope clusters (unpublished data), however it did not fit into any one particular epitope cluster, but had characteristics of both β3 and β5. The isotype control for flow cytometry analysis is the QB-END/10 anti-CD34 (Quantum Biosystems, Cambridge).

2.2. Expression vector construct and production of mutants

Full length hCGβ cDNA was cloned from human placental third trimester RNA (a gift from Dr Sally Pemble, CRC Molecular Toxicology Group, UCL, UK) using RT-PCR and the sense cloning primer 5'ACCGGAATTCCAGGGGCTCCTGCTGGTGG3' (corresponding to nucleotide (nt) −51→−33) and the antisense cloning primer 5'TTGGTGACTTGCGGAGGATCGGGGTGGTCC3' (nt 414→435). The hCG cDNA was cloned into pCDM8 (Seed and Aruffo, 1987) in which a DNA fragment from H2-Db containing the 17 membrane-proximal amino acid residues, the transmembrane region and cytoplasmic tail had been inserted. This fragment was obtained using RT-PCR amplification of RNA from a spleen of a C57BL/10 mouse with the sense primer 5'GCGTTGGTCGACCATGAGGGCTGCCTGAGCC3' (nt 547→566) and an antisense primer 5'CACAGGAGAGACCTGAACACATCG3' (nt
809→832). The sequence of hCGβ was identical to that previously published (Talmadge et al., 1984).

The mutants were produced by an overlap PCR mutagenesis method (Horton and Pease, 1991). The primer sequences were: Mutant 1 sense 5'GAGAACCACGGAGTGGCCCCGTGATCACCCGTC3', antisense 5'GGCCTGCACGGTTTCTCCACACAGGGAGGTGGC3'; Mutant 2 sense 5'CCACTACTGCATCACTACCGTCAACACCACCATCTGTGC3', antisense 5'CGGTGATGAGTATGATGGCACGCCCTCTTCTTCTC3'; Mutant 3 sense 5'GGCTGCCCCTCCACCTGAAACCACCACGTCTCTCTACGCCGTG3', antisense 5'CGTGGAGGGGCGACGCGAGGGAGCTGCATGTGGACTG-4AG3'; Mutant 4 sense 5'GGAGGCCGAGGGGCGTGAACCCCGTG3', antisense 5'GACGTTGATACACGCCTGGAGCCCTCCTTCCTC3'; Mutant 5 sense 5'GAAGGGGCTGCCCCTACTGCATCACCGTC3', antisense 5'GGGTGACGGTGATGCAGTAGGGCCAGCCCTCCTTC3'; Mutant 6 sense 5'GAAGGGGCTGCCCCTACTGCATCACCGTC3', antisense 5'GGGTGACGGTGATGCAGTAGGGCCAGCCCTCCTTC3'; Mutant 7 sense 5'CCATCGAGCTCCTGGCTGCCCCTGC3', antisense 5'CAGGGGAGCTGCATGTGGACTG-4AG3'; Mutant 8 and Mutant 20 sense 5'CTGCCCGTGGCGTGACCACCGT3', antisense 5'CCAGGGAGCTGCATGTGGACTG-4AG3'; Mutant 9 sense 5'CTGCCCGTGGCGTGACCACCGT3', antisense 5'CCAGGGAGCTGCATGTGGACTG-4AG3'; Mutant 10 sense 5'GAAGGCCGACGTCTCCTTCACGCAGGCGG3', antisense 5'GAGACGGTGGGCTGATGCAGTAGGGCCAGCCCTCCTTC3'. As the template, hCGβ cDNA was used to generate all the mutants apart from Mutant 4 which used Mutant 1 as its template.

The sequence of all the mutations were verified using double stranded DNA sequencing (Sequenase, United States Biochemicals) and a range of hCGβ internal and CDM8 primers.

2.3. Transfections, surface expression, staining and FACS analysis

COS7 cells were transfected using a modified DEAE dextran-chloroquine method (based on Seed and Aruffo, 1987). Briefly, 1.5 x 10⁶ cells were seeded into an 80 cm² flask on the day before transfection. Transfection mixture (6 ml) (10% NuSerum [Becton Dickinson, Bedford, MA], 1–2 µg/ml supercoiled DNA [CsCl or PEG prepared] and 250 µg/ml DEAE dextran) was added to the washed monolayer and left in a 37°C incubator for 60 min. Chloroquine was then added to a final concentration of 200 µM and the cells incubated for a further 120 min. The transfection mixture was then removed, the monolayer washed with PBS and 3 ml of 10% DMSO (in PBS) added for 2 min. The cells were washed again and complete medium
added (Dulbecco's Modified Eagle Medium, 10% FCS). The cells were split 1:1 24 h later and harvested 65–72 h after transfection. A transfection efficiency of 20–50% was routinely obtained.

Cells were stained in duplicates of $2 \times 10^5$ cells for each mAb tested. Following washing of the harvested cells with 10% FCS, 0.02% NaN₃ in PBS, they were incubated with 100 µl of mAb for 30 min on ice, washed twice in PBS; 0.02% NaN₃ and then incubated with 100 µl of rabbit anti-mouse FITC. Following washing, the cells were fixed in 1% formaldehyde in PBS, and FACS analysis performed using a Becton-Dickinson Facscan. Markers were set on the negative control which was routinely an anti-CD34 IgG1. All cells to the right of this marker were deemed to be positively transfected. All mAbs were titrated in order to give roughly the same staining intensity. Each mutant/mAb combination was tested at least three times.

3. Results

hCGβ consists of 145 amino acid residues which include 12 cysteines that form 6 conserved disulphide bridges (Lapthorn et al., 1994; Wu et al., 1994). It is heavily glycosylated with N-linked carbohydrates at positions Asn 13 and Asn 30 in addition to four O-linked carbohydrates in the C-terminus at Ser 121, 127, 132 and 138. To ensure correct folding of the recombinant molecules we opted for expression in mammalian cells. A construct consisting of hCGβ with a C-terminal extension of the H2-Dβ transmembrane region, cytoplasmic region and the first 17 amino acids proximal to the membrane was made, so that wildtype and mutant hCGβ is expressed on the surface of transfected cells. The mAb OT3A, which recognizes a linear amino acid sequence on the C-terminus of hCGβ (residues 133–139), can be used to quantitate the expression level of the wildtype and mutant recombinant proteins following transient transfection in the COS7 cells as shown on Fig. 1 for Mutant 3.

Using our panel of mAbs, nine distinct epitope clusters on hCGβ designated β1–β9 have previously been defined (Berger et al., 1990; Dirnhofer et al., 1994). These can be related to each other graphically using a cylindrical Mercator’s projection (Schwarz et al., 1986; Berger et al., 1990). The β1–β5, β8 and β9 clusters are accessible on the free and assembled hCGβ-subunits, whereas β6 and β7 are located on the free hCGβ-subunit only. β8 and β9 are on the unique C-terminus of hCGβ. Whilst the β1, β6, β7, β8 and β9 clusters are specific to hCG and/or hCGβ, the mAbs to the other epitope clusters cross-react with hLH and hLHβ. All the mAbs used
Fig. 1. Representative flow cytometric analysis of COS7 cells expressing hCGβ Mutant 3. Events (5000) are shown of cells stained with mAbs OT3A (C-terminus), INN-hCG-2 (β1, hCG specific) and INN-hCG-58 (β5, cross-relative with LH). The negative isotype-matched control (anti-CD34) is shown in each with a dotted line. The number of cells positive in each case is indicated.
(Table 1) apart from OT3A recognize discontinuous sequences on hCGβ, because reduction and alkylation of hCGβ abolishes their binding (Dirnhofer et al., 1994), (N.B. 3E2 not included in this study). Each of the mAbs used in this study bind to the wild type hCGβ transiently expressed on the surface of COS7 cells, as summarized in Table 1. This suggests that the folding of the recombinant wild type hCGβ is similar to that seen in the native hCGβ and not compromised in the construct.

The region of hCGβ on which the cross-reactive epitopes are located has not been determined before. The three dimensional structure of hCG (Lapthorn et al., 1994) determined by X-ray crystallography has revealed that each subunit of hCG has a cysteine-knot growth factor fold. This fold is characterised by a knot of three disulphides with two twisted β hairpins on one side and a variable length loop on opposite sides of the knot. The β subunit structure is elongated and flat in appearance and wraps around the α subunit making a large dimer interface. Residues β90-110 are intimately involved in dimerisation and form part of the presumed receptor binding site, whilst the hCG-specific residues of the C terminus are not seen in the structure and are presumed to adopt a poorly ordered, flexible structure.

The beta hairpin regions of the β subunit, residues 10–33 (loop 1) and 58–87 (loop 3), which we refer to as the N and C-terminal hairpins, respectively, form the body of the subunit and are held together by the cystine 23–72. Sequences which are relatively conserved in the β hormones are located in the tips of these loops. This region is therefore a good candidate for the cross-reactive epitope site. Individual amino acid residues from this region which are surface accessible and not considered to play a critical structural role were selected. To minimise the possibility of introducing large conformational changes, residues were replaced with those occurring at the same position in other glycoprotein hormones from differing species. The changes were designed to introduce dissimilar and opposing side-chain properties (e.g. size, charge, polarity) from the native residues, in order to disrupt any mAb binding to the given residues. Computer modelling of the potential hCG mutants was performed to confirm that substitutions did not affect overall protein conformation. Eleven mutant hCGβs were constructed using overlapping PCR mutagenesis (Horton and Pease, 1991), targeting residues on either the N or C-terminal hairpins (Fig. 2).

The first series of mutants (1–4) were made with a number of point mutations in each. These were then further refined by single mutations (Mutants 5, 6, 7, 9, 10 and 20) and Mutant 8 which contains an additional PCR induced mutation of residue 71. The results of staining wild type and mutant hCGβ expressed on the surface of COS7 cells with the panel of
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- mAb binding equivalent to that of OT3A (cells positive >70% relative to OT3A).
- mAb binding reduced compared to that of OT3A (cells positive 50-70% relative to OT3A).
- mAb binding reduced compared to that of OT3A (cells positive 20-30% relative to OT3A).
- mAb abolished (< 10% relative to OT3A).

Table 1
Binding of mAbs to wild type (WT) and mutant hCGβ molecules expressed on COS7 cells. All mAbs recognise conformationally-dependent epitopes except OT3A which is against a linear epitope (a.a. 133-139) on the C terminus.
Mutant | amino acids changed  
--- | ---  
1 | 20 Lys → Asn  
21 Glu → Arg  
22 Gly → Glu  
2 | 24 Pro → His  
25 Val → Tyr  
3 | 68 Arg → Glu  
74 Arg → Ser  
75 Gly → His  
79 Val → His  
4 | 20 Lys → Asn  
21 Glu → Arg  
22 Gly → Glu  
24 Pro → His  
25 Val → Tyr  
5 | 24 Pro → His  
6 | 25 Val → Tyr  
7 | 68 Arg → Glu  
8 | 74 Arg → Ser  
71 Gly → Arg  
9 | 75 Gly → His  
10 | 79 Val → His  
20 | 74 Arg → Ser

Fig. 2. Amino acid substitutions and their position on hCGβ. (a) Table of the mutant constructs with their amino acid substitutions. (b) The N-terminal and C-terminal hairpins which have been mutated are circled on a ribbon diagram of the hCG heterodimer produced using SETOR (Evans, 1993). The hCGα subunit is in blue, the hCGβ subunit in green and the extent of the carbohydrate is illustrated in a stick representation, coloured by atom type (C, white; O, red; N, blue). The hCGβ C-terminus, for which the structure is unknown, is illustrated by a series of points with a box indicating the residues implicated in the OT3A binding site. The spacefilling representations (c) and (d) are approximately orthogonal views of the N- and C-terminal hairpins, respectively, with the subunits and carbohydrates coloured as in (b). Mutant 1 is highlighted in magenta, Mutant 2 in yellow and Mutant 3 in cyan.
mAbs are summarized in Table 1. The mAbs to the β1 epitope cluster and the mAb OT3A bound to wild type and all the hCGβ mutants with about the same relative binding strength. This demonstrates that all eleven mutant molecules fold to recreate the hCG specific epitope β1 and the hCGβ specific epitope β7.

The mutations in the N-terminal hairpin loop (Mutant 4; mutations of Lys 20, Glu 21, Gly 22, Pro 24 and Val 25) completely abolished binding of the mAbs specific for the β3 and β6 epitope cluster and reduced to 24.3% the binding of mAb 3E2. Different mutants were made to pinpoint the important amino acids that contribute to the binding of the different mAbs. Mutations of residues Lys 20, Glu 21, and Gly 22 (Mutant 1) totally destroyed all reactivity with the mAb INN-hCG-64 recognizing the hCGβ-specific epitope cluster β6. Mutant 2 (mutations of Pro 24, and Val 25) failed to bind both β3-specific mAbs (INN-bLH-1 and INN-hCG-111). The mAb 3E2 showed a reduction in binding to both Mutant 1 and 2 (56% and 54.3%, respectively).

In contrast to the above mutations, the four mutations at residues 68, 74, 75, and 79 introduced in the C-terminal hairpin loop (Mutant 3) removed all the reactivity to the mutated molecule by all cross-reactive antibodies, yet retained the binding of the hCG-specific mAbs directed to the β1 and β7 epitope clusters and to the linear epitope in the C-terminus.

For further analysis six more mutants were constructed, in each of which only a single amino acid was changed. The two β3 mAbs have separate but overlapping binding sites on hCGβ, because a single point mutation Pro 24→His (Mutant 5) removed all binding of mAb INN-bLH-1 but allowed reduced binding of INN-hCG-111 (59–63%), whereas the mutation Val 25→Tyr (Mutant 6) prevented binding of INN-hCG-111 and lowered the binding of INN-bLH-1 to 63%. The analysis of the single changes on the C-terminal hairpin loop gave surprising results. Mutant 7 with a single change of Arg 68→Glu lost the binding of all the cross-reactive mAbs but, unlike Mutant 3, retained all the specific mAbs including the β6 representative INN-hCG-64. Mutant 8 (Arg 74→Ser, Gly 71→Arg) lost only the β3 mAbs and the mAb 3E2. The change of Gly 75→His, (Mutant 9), prevented mAb INN-hCG-64 (β6) from binding, whilst in Mutant 10, Val 79→His appeared to have no effect on any of the mAbs except 3E2 which was reduced to 57%.

4. Discussion

We have identified amino acid residues important in mAb binding to hCGβ, and shown that it is possible to create mutated molecules which
have lost undesirable shared epitopes whilst maintaining sufficient conformation to allow undiminished binding of other mAbs against desired epitopes. hCG/3 (residues 1-110) has 85% amino acid homology with hLH/3, and four (β2, β3, β4, β5) of the seven epitope clusters present on this fragment elicit antibody responses that cross-react with hLH. Substitution of the amino acids at positions 68, 74, 75 and 79 creates a mutant hCG/3 (Mutant 3) which has completely lost the binding of all the cross-reacting murine mAbs used in this study yet retains the epitopes specific for the hCG holo-hormone. Remarkably, substitution of just Arg 68 with Glu (Mutant 7) also eliminates these cross-reacting epitopes, but the mutant is still folded such that the hCG-specific epitope clusters β1, β6 and β7 are maintained since the relevant mAbs bound Mutant 7 as well as they did the wild type hCG/3.

Analysis of the mutants provides important information with respect to both hCG-specific and cross-reacting epitopes. The changes caused by mutation are most probably due to differences in the structure of the side chains of the substituted amino acids which could modify antibody binding probably by alteration of a contact residue or by stereochemical inhibition. The constraints on the fold caused by the cysteine-knot and the disulphide at 23-72 means that the mutants are unlikely to cause local changes in the tertiary folding (Robinson et al., 1995).

Residues Lys 20, Glu 21 and Gln 22 are involved in the hCG/3-specific cluster β6 which is masked in the holo-hormone. Examination of the crystal structure (Fig. 2) shows that these amino acids are close to the alpha subunit; Glu 21 is partially buried and probably forms a salt bridge with Lys α45, so that binding of a mAb to this region would be blocked stereochemically in the heterodimer. The only hCG-specific residue in this area is Asn 77; this is an Asp in hLH/3 and the negative charge of Asp may account for the failure of mAbs raised to this region of hCG/3 to bind hLH/3. Moyle et al. (1990) showed that the hCG-specific mAb B112 also binds hLH which has been mutated at position 77 from Asp to Asn. The substitutions in the C-terminal hairpin loop (Mutant 3) also abolished binding of the mAb to β6 (INN-hCG-64) suggesting that the structural changes also disrupt binding around the Asn at position 77. Further analysis by the single point mutants 7–10 showed that only Gly 75→His was responsible for this effect. Therefore, we presume that INN-hCG-64, and probably other mAbs which have mapped to epitope region β6, are binding to the area shown in Fig. 2c, comprising of Lys 20, Glu 21, Gln 22 and Gly 75.

Residues Pro 24, Val 25 and Arg 68 contribute unequivocally to epitope cluster β3 as demonstrated by the complete loss of binding of mAbs to β3 when these residues are mutated. Results from Mutant 8 (Arg 74→Ser, Gly
71 → Arg) would imply that Gly 71 may also be involved as INN-bLH-1 was raised against bovine LH which has Pro rather than Arg at residue 74. Clearly the two β3 monoclonals INN-hCG-111 and INN-bLH-1 have slightly different binding profiles as evidenced by the effects of substitutions in Pro24 and Val25. As INN-hCG-111 binding is affected by the substitution in Mutant 1, it could be argued that residues Lys20, Val24, Pro25, Arg68 and Gly71 all contribute to this epitope. These proposed sites would allow the β3 and β6 mAbs to bind hCGβ simultaneously as previously shown (Berger et al., 1990).

Arginine dominates the functional epitopes of human growth hormone (Jin et al., 1992) and in the core of hCGβ the arginines at positions 68 and 74 project from the surface (Fig. 2c and 2d), and are likely to be highly immunogenic. With respect to Arg68, we have already referred to the unexpected finding that substitution of this residue on Mutant 7 reproduces the dramatic elimination of the cross-reacting β2, β3, β4, β5 and β3/5 epitopes achieved by the changes made in the C-terminal hairpin loop in Mutant 3. Arg74 (substituted in Mutant 20) appears to have no part in the reaction of all these mAbs, except 3E2, with hCGβ. This is not surprising since all β3 and β5 specific mAbs show low cross-reactivity with porcine LHβ which differs from hCGβ at residue 74 by having Pro (Berger et al., 1990). Cosowsky et al. (1995) have shown that a mutation of Arg74 → Pro largely eliminates the binding of B105, another mAb cross-reactive with hLH. The mAb 3E2 appears to be sensitive to all the mutations made here (with the exception of Gly75 → His). If it is presumed that changes of greater than 10-fold in relative antibody binding are necessary to identify an antibody binding residue (Prasad et al., 1993) then only Arg68 and Arg74 are implicated in mAb 3E2. If 3E2 binds to the tip of the hCGβ subunit, then the other mutations may be causing slight structural alterations to which this mAb may be particularly sensitive. The changes observed in the N-terminal loop can then be seen to have an additive effect, where the more changes that are introduced the less 3E2 binds. This mAb appears to have a different binding region to β3 or β5 mAbs and this would explain why this mAb proved difficult to assign to the previously established epitope clusters (unpublished data). Possibly, the other cross-reactive mAbs used in our study are directed towards epitopes further along the beta hairpin towards Pro70. We have not been able to discriminate the epitope clusters β2, β3, β4 and β5 with the changes made here. Further mutagenesis studies would be required to identify unambiguously the residues constituting each of the epitope clusters on hCGβ.

In order to produce an epitope-specific vaccine, a detailed knowledge of the epitopes and their composite amino acids is required. As previously shown for hCGβ (Dirnhofer et al., 1994), the most difficult epitopes to
elucidate are discontinuous B-cell epitopes which are by their nature highly dependent upon the correct folding and conformation of the molecule. Protein antigens intended for use as vaccines, with epitopes eliciting protective immune responses, may also present epitopes capable of inducing undesirable effects (Fig. 3). For example, an epitope may be shared with self-antigens of the immunized host, as in the case of *Trypanosoma cruzi* and heart tissue (Van Voorhis and Eisen, 1989), provoking a potentially damaging autoantibody response. Many microbial immunogens possess powerful B-cell epitopes that dominate the immune response but fail to be protective because they are subject to rapid variation in structure (e.g. human immunodeficiency virus, malaria, African trypanosomiasis), competing with and limiting the response to conserved epitopes which could provide an effective vaccine. In T-cell mediated immunity, T-cells that are primed by a foreign antigen with sequence homology to self proteins may cross-react with cryptic epitopes displayed on cells that have processed the self protein (Moudgil and Sercarz, 1993). Candidate vaccines may also possess T-cell epitopes which compromise the response to the immunogen either through biasing the TH1/TH2 response towards a non-protective subset or by induction of a suppressive T-cell population (Moudgil and Sercarz, 1993).

The use of synthetic peptide vaccines incorporating linear T and B-cell epitopes (Arnon and Van-Regenmortel, 1992), is not useful where discontinuous B-cell epitopes are involved. Another approach using monoclonal

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**Fig. 3.** Schematic diagram of the strategy to make epitope-specific candidate vaccines.
anti-idiotype vaccines has been tried (Thanavala et al., 1986), though this is a difficult route. A logical strategy is to allow the natural folding of the protein itself to generate the required epitope while at the same time removing unwanted epitopes by appropriate mutation of the gene. The effects of these mutations on epitope expression can be monitored by reactions with specific mAbs, it clearly being essential to avoid changes in amino acid residues which affect folding of the desired epitope.

Using this approach we have shown that even with a molecule like hCGβ, which has complex non-contiguous B-cell epitopes, it is possible to make radical changes in side-chain properties which remove unwanted epitopes yet maintain other desirable epitopes. These changes have helped to identify several of the residues involved in the B-cell epitopes which are shared with hLH. We are now preparing to test the Mutants 3 and 7 in immunogenicity studies but, in order to develop a more specific contraceptive vaccine, it is important to ensure there is no remaining cross-reactivity with hLH. If these Mutants 3 and 7 still retain epitopes capable of evoking cross-reactive antibodies we will use these new antibodies to monitor removal of the residual unwanted structures by further mutation, i.e. extending our basic strategy. The studies reported herein relate to the abolition of cross-reactive epitopes recognized by murine mAbs. Clearly such studies are constrained by the fact that these antibodies represent those produced by an inbred strain of rodent. In an outbred human population with diverse MHC haplotypes the relative potency of individual epitopes may be different and immunogenicity studies in vivo in higher primates and in vitro using human lymphoid cell cultures will be required to confirm the loss of undesired epitopes. It will also be important to establish that the mutations have not led to the creation of neo-epitopes which could provoke the development of antibodies that are cross-reactive with other self antigens. These studies would open the way for testing hCGβ mutant molecules as improved candidates for an hCG contraceptive vaccine with predictable advantages over the hCGβ C-terminal peptide or the entire unaltered hCGβ currently being assessed for control of fertility.

Acknowledgements

This work was funded with grants from the Medical Research Council (London), the Special Trustees of the Middlesex Hospital, the Cleveland Immunological and General Trust and the Research Council, Austria (P102717-MED). The authors also thank Organon Technika for the mAb OT3A and Quantum Biosystems for the mAb QB-END/10.
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