The Effect of Cytokines on Chorionic Gonadotrophin Expression in the Marmoset Monkey Embryo

Louise J. Gameau B.Sc. (Hons)

A thesis submitted in total fulfilment of the requirements for the degree of Doctor of Philisophy

Department of Obstetrics and Gynaecology,
The Faculty of Medicine.
University of Adelaide,
Adelaide, South Australia

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Chapter Four: Quantitative RT-PCR

Chapter Five: ELISA

Chapter Six: Culture of Marmoset Trophoblastic Vesicles and Embryos with Cytokines

EXAMINER: Dr. Leeanda Wilton

SPECIFIC COMMENTS:

1. i) *Repeat lines:* the bottom of pages iii, iv, viii, 50, 168, 243. Appropriate corrections have been made.

ii) *Spelling Errors or too many words:*
page 179, line 3 summrised, should be summarised.
page 201, line 11 iflushingî, should be "flushing".
page 214, 4th line from the bottom, delete "in a".

2. *Chapter 3 describes the expression of mCG and mGAPDH in marmoset embryos and tissues. On page 112 it is concluded that there is expression of mCG-a but not mCG-b or mGAPDH from the 4-cell stage. Although it is qualified that there was a low recovery of RNA, the other possibility is that the embryo was dead. Flushing marmoset embryos at day 13-14 post Estrumate should yield embryos that are at least 8-cell. It is unlikely that a 4-cell embryo would be viable although it may have intact, relatively healthy looking cells. What is the half-life of the mCG RNA in these cells? I would also like to see the source of the oocytes documented. Were these freshly collected from ovaries or flushed from the oviducts/uterus? In my experience, marmoset oocytes retain a normal appearance for several days in vivo or in vitro. The viability of the oocytes needs to be considered before concluding that there was no mCG expression.*

The lack of detection of mCG-β or mGAPDH mRNA at the 4-cell stage may be due to the small amount of RNA recovered from the cells. Pooling of a number of 4-cell embryos may be required to obtain a more accurate indication of gene expression. Although the embryo was recovered by Associate Professor Alex Lopata at day 9/10 after estrumate treatment and examined immediately to confirm viable appearance, an alternative may be that the 4-cell embryo was not viable. Obviously more embryos at these stages would be important to further investigate the expression of mCG-α, -β and mGAPDH. The half-life of mCG RNA in embryonic cells is yet to be determined. The marmoset oocytes were freshly recovered directly from the ovaries (Gilchrist *et al. Biol. Reprod.* 52: 1234-1243, 1995) and while the only assessment of viability was of a visual nature under light microscopy, it can not be discounted that they were not viable, giving rise to a negative result for mCG.

3. *page 114. "In the mouse, cultured 1-cell embryos fail to progress beyond the 2-cell stage,..." This is not always the case and occurs in certain strains.*

page 114, line 16 *please change the above phrase to:* In some strains of mice, cultured 1-cell embryos fail to progress beyond the 2-cell stage,.....

4. *page 168. The suggestion that mCG could be used as a predictor of viability is speculative. Could the lower secretion from the late morula-early blastocyst simply reflect cell number? If it were possible to calculate cell number, what would the results look like if CG expression was expressed per cell?*

As stated on page 168, sentence 3: The differences in mCG levels secreted by individual embryos in culture may be an indicator of viability. This is only speculative and the results may simply reflect a smaller number of cells at this stage of development. If it were possible to calculate cell number, it would be useful to determine the amount of CG expressed per cell.

5. *page 168. I would like to see more discussion of the idea that female embryos have a reduced capacity to differentiate cytotrophoblast, particularly as there is no peer reviewed reference provided. It seems a remarkable suggestion to me and the ramifications to embryonic development of female and male embryos could be discussed. Of course, it is well documented that paternal alleles of some imprinted genes are preferentially expressed in the extra-embryonic tissues but this is independent of gender.*

page 169, first paragraph: This data was presented to me whilst I was in Melbourne learning about trophoblastic vesicle culture (March 1997). However, more investigation into this finding needs to be performed. It has not been published as yet, so at the end of the paragraph: "(personal communication, Alex Lopata, 1997)" should be included. This unpublished data was provided to explain why some embryos might not have formed trophoblastic vesicles in culture.

9. *page 194. It appears to me that none of the control embryos formed trophoblastic outgrowths. If not, was there a problem with the culture system?*

All the control embryos attached to the plastic. If they had been allowed to continue culturing to form trophoblastic vesicles (*as described in Section 2.3.1*), the trophoblastic outgrowths would have been visible, however as they were sacrificed just after attachment the outgrowths are not visible. The fact that these control embryos behaved exactly as they did (i.e. forming primary vesicles) suggests that there is not a problem with the culture system.

10. *Again, I think that the suggestion that the mCG-β expression is indicative of embryo viability is speculative and the text should indicate this. Its absence from degenerate embryos is not surprising. An indicator of viability should predict the potential of apparently normal embryos, not ones that are retarded or degenerate. This has not been established.*

As stated above: on page 168, sentence 3: "The differences in mCG levels secreted by individual embryos in culture may be an indicator of viability" and again on page 209, line 15: "Hence, suggesting that CG-β may be an indicator of viability of the embryo". The idea that mCG-β is an indicator of viability is only speculative and the results may simply reflect a smaller number.

EXAMINER: Associate Professor A.M. Dharmarajan

SPECIFIC COMMENTS:

1. *Repeated lines:* page iii. Appropriate correction has been made.

2. *Discussion of alternate splicing of the LH/CG receptor.*

The LH/CG receptor gene consists of 11 exons, separated by 10 introns (Tsai-Morris *et al. J Biol Chem* 266: 11355-11359, 1991). These 10 introns are all located within the putative extracellular domain, prior to the first transmembrane region. Exon 11 encodes the transmembrane domains, the connecting loops, cytoplasmic tail, the 3' flanking region and a 47-residue segment of the extracellular domain adjacent to the plasma membrane. Truncated forms of the receptor result from splicing at various alternative acceptor splice sites in exon 11 with or without the elimination of exon 9. This has been identified in pig (Loosfelt *et al. Science* 245: 525-528, 1989) and rat testes (Tsai-Morris *et al. J Biol Chem* 265: 19385-19388, 1990; Tsai-Morris *et al.* 1991) and in human ovarian libraries (Bernard *et al. Mol Cell Endocrinol* 71: R19-R23, 1990; Minegishi *et al. Biochem Biophys Res Commun* 172: 1049-1054, 1990). The 3' non-coding region contains 2 functional polyadenylation domains, which are responsible for the formation of 2 major sets of mRNAs, including the alternatively spliced variants, thus leading to the diversity of the LH/CG receptor mRNA transcripts.

3. *EPF in rabbit serum.*

Studies by Sueoka *et al.* (*J Reprod Fertil* 84: 325-331, 1988) have found that the rabbit also produces EPF. Similar to the other species mentioned on page 25, two phases of EPF production was observed during pregnancy. Rabbit ovaries were perfused in this study, however, EPF production did not occur when the oviduct and embryos were not present. Thus, it suggests, as in the other species, that the embryo acts as a stimulus to produce EPF from some other source.

4. *Marmoset embryo culture. Embryos were cultured for several days. Were they examined for viability? When embryos are cultured in serum-free medium they undergo spontaneous apoptosis. It is important to make sure that these embryos are not dying either by necrosis and/or apoptosis. There are several techniques one could use to test these events. I would recommend some discussion on this issue.*

Marmoset embryos were examined each day for viability. If embryonic viability was not clear, Dr. Lou Warnes, an embryologist, examined the embryo. Dr. Lou Warnes also verified the stage at which the embryos were collected.

Only 3 embryos were cultured in serum-free medium, from the time of collection, however, they did not develop further than the stage at what they were collected *(as described in Section 6.5.2.1)*. All the other embryos were cultured in medium with serum, overnight to recover, before the medium was aspirated, collected and replaced with medium with cytokine (LIF or GM-CSF) or medium with serum (control).

The medium with serum, contained fetal calf serum, insulin and transferrin, as well as L-glutamine, penicillin and streptomycin. The medium with cytokine did not contain serum, because serum contains many factors including growth factors; nor did it contain insulin and transferrin. These factors have been demonstrated to affect embryonic development previously (Lopata and Oliva, *Hum Reprod* 8: 932-8, 1993).

Techniques for detecting apoptosis and/or necrosis are usually performed on cells that are not required to be further cultured. It would be ideal if a substance was secreted into the surrounding medium to indicate that either apoptosis or necrosis is occurring, however, such a substance has not been identified to my knowledge, as yet. Annexin V antibody staining analyses the externalisation of phosphatidylserine on the membrane, but this staining is usually in combination with propidium iodide, which is toxic to cells. A study just looking at the occurrence of apoptosis and/or necrosis in the blastomeres of embryos would be of value, however a large number of embryos would be required.

5. *Culture of Marmoset Trophoblastic Vesicles and Embryos with Cytokines.*

The importance of investigating the effect of various cytokines and growth factors in CG expression and secretion from embryonic material will enable us to better understand the factors regulating CG expression and secretion. This information may be used to develop serum-free fully defined medium for the *in vitro* culture of human embryos prior to transfer, thus potentially benefiting *in vitro* fertilisation technology.

Summary

Chorionic gonadotrophin (CG) is one of the first factors produced by the embryo to signal its presence to the mother and thus prevent atrophy of the corpus luteum, thereby ensuring the continued production of progesterone to favour embryonic development. CG, a primate specific glycoprotein hormone, is produced by the trophoblast and is first detected in the human peripheral blood between Days 8-11 after ovulation. However, *in vitro* studies have shown that CG is secreted by the embryo before it can be detected in the peripheral blood.

Cytokines and growth factors are a group of proteins and polypeptides, released from the cell, with a variety of functions, including intracellular communication and alteration and regulation of cell growth and differentiation. Various cytokines and growth factors are involved in the ovulatory process, in the immune response between mother and embryo, as well as in the implantation process. A variety of cytokines have been shown to modify CG expression in human tumour cell lines, modify differentiation of the embryonic cells and to be involved in communication between the mother and the embryo. Various cytokine ligands and receptors for these factors have been found to be expressed by the embryo.

The characterisation of the role of cytokines and growth factors has been conducted predominantly on rodents, with few results attained using primate tissues or embryos. Hence, to

determine the involvement of these factors in the regulation of CG expression, we have used the Common Marmoset (*Callithrix jacchus*) as a model primate.

The purpose of this project was to examine the cellular and molecular processes involved in expression of embryonic signals and the initial interactions that occur between the embryo and the maternal endometrium of the primate. This knowledge will assist in developing culture conditions for improved viability of human IVF embryos, as well as enhancing our understanding of primate reproductive physiology.

Expression of marmoset CG genes (alpha and beta subunits) was confirmed in pre-implantation marmoset embryos from the eight-cell stage through to the hatching blastocyst stage of development. A method for quantitating marmoset CG-β mRNA expression in marmoset tissue, using competitive reverse transcriptase polymerase chain reaction (RT-PCR) was also developed. Marmoset glyceraldehyde-phosphate-dehydrogenase (mGAPDH) was cloned and partially sequenced to use as an endogenous internal standard to compare tissue types and to confirm the success of the reverse transcription reaction.

An enzyme-linked immunosorbent assay (ELISA) was developed, using an inhouse polyclonal antibody raised to recombinant mCG and purified in our laboratory, to measure the amount of mCG present in plasma of cycling and pregnant

marmoset monkeys and the amount of mCG secreted by marmoset trophoblastic vesicles and embryos.

In vitro culture of marmoset trophoblastic vesicles was used to study the influence of two cytokines, LIF & GM-CSF, known to be present in the uterine milieu at peri-implantation. LIF is known to regulate differentiation of cytotrophoblasts to anchoring trophoblasts and thus presumably decrease CG expression, whereas GM-CSF is known to enhance CG expression. This culture system was then applied to whole marmoset embryos, in an attempt to gain an insight into primate pre-implantation embryo development and the role these two factors play at implantation. Preliminary results suggest that both cytokines promote blastocyst hatching and attachment. However, more embryos are needed to further quantitate mCG-β expression using the competitive PCR assay and to fully understand the full impact of these two factors at implantation.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by any other person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

SIGNED ..... DATE: 01/08/98

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My mother, Julie, my sister, Nicky, our two cats, Crumpet and Leeroyd and my turtle, Thomas.

Finally, I dedicate this thesis to my grand-mother Joy, who passed away in 1996. Thank-you for being my inspiration and keeping me motivated.

Publications

Publications arising from the work in this thesis:

Gameau, L.J., Lopata, A., Norman, R.J. and Simula, A.P. (1998) Temporal expression of chorionic gonadotrophin messenger ribonucleic acids in marmoset pre-implantation embryos. (In preparation, to be submitted to Biology of Reproduction).

Amato, F., Simula, A.P., **Gameau, L.J.** and Norman, R.J. (1998) Expression, characterisation and immunoassay of recombinant marmoset chorionic gonadotrophin dimer and β-subunit. *Journal of Endocrinology* 159: in press.

Conference Presentations

National Meetings:

Gameau, L.J., Simula, A.P. and Norman, R.J. (1995). Quantitation of marmoset CG/LH beta mRNA species in pituitary and placental tissue. *Proceedings of the 26th Annual Conference of the Australian Society for Reproductive Biology.* Melbourne, Australia; September 1995: Abstract 57.

Gameau, L.J., Lopata, A., Norman, R.J. and Simula, A.P. (1996). Chorionic gonadotrophin expression in early primate embryos. *Proceedings of the 39th Annual Scientific Meeting of the Endocrine Society of Australia.* Sydney, Australia; September 1996: Abstract 3.

Simula, A.P., **Gameau, L.J.** and Norman, R.J. (1996). Messenger RNA splice variants of the beta-subunit of marmoset chorionic gonadotrophin. *Proceedings of the 39th Annual Scientific Meeting of the Endocrine Society of Australia.* Sydney, Australia; September 1996: Abstract 55.

Gameau, L.J., Lopata, A., Norman, R.J. and Simula, A.P. (1997). Quantitation of chorionic gonadotrophin expression in pre-implantation embryos of the marmoset monkey (*Callithrix jacchus*). *Proceedings of the 28th Annual Conference of the Australian Society for Reproductive Biology.* Canberra, Australia; September 1997: Abstract 55.

Amato, F., Simula, A.P., **Gameau, L.J.** and Norman, R.J. (1997). Development of a specific and sensitive ELISA for marmoset chorionic gonadotropin. *Proceedings of the 16th Annual Conference of the Fertility Society of Australia.* Adelaide, Australia; December 1997: Miniposter 38.

International Meetings:

Gameau, L.J., Simula, A.P., Amato, F., Lopata, A. and Norman, R.J. (1998). The effect og leukaemia inhibitory facotr (LIF) and granulocyte-macrophage colony stimulating factor (GM-CSF) on chorionic gonadotrophin expression in an *in vitro* model of early primate implantation. *Proceedings of the 31th Annual Meeting of the Society for the Study of Reproduction. College Station, Texas,* USA; August 1998: Abstract 156.

Awards

1994-1997 Australian Research Council Postgraduate Scholarship.

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1995 Finalist: Australian Society for Reproductive Biology Junior Scientist Award.

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1997-1998 Repromed Postgraduate Scholarship.

List of Abbreviations used in this thesis

aa	amino acid
bp	base pair(s)
cDNA	Complementary DeoxyriboNucleic Acid
cpn10	Chaperonin 10
CSP	Conceptus Secretory Protein
CG	Chorionic Gonadotrophin
CG-α	Chorionic Gonadotrophin alpha
CG-β	Chorionic Gonadotrophin beta
CL	Corpora Lutea or Corpus Luteum
CSF	Colony Stimulating Factor
dNTP	DeoxyNucleoside TriPhosphate
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPF	Early Pregnancy Factor
FGF	Fibroblast Growth Factor
FSH	Follicle Stimulating Hormone
GnRH	Gonadotrophin Releasing Hormone
hCG	Human Chorionic Gonadotrophin
hPL	Human Placental Lactogen
ICM	Inner Cell Mass
IFN	Interferon
IGF	Insulin-like Growth Factor
IL	Interleukin
IU	International Unit
Kda	Kilodalton
LIF	Leukaemia Inhibitory Factor
LH	Luteinising Hormone
LH/CG R	Luteinising Hormone/Chorionic Gonadotrophin Receptor
µg	Microgram
µl	Microlitre
mCG	Marmoset Chorionic Gonadotrophin
MQ water	MilliQ Water
mIU	Milli International Unit
mRNA	Messenger RiboNucleic Acid
NGF	Nerve Growth Factor
OD	Optical Density
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PGF_{2_α}	Prostaglandin F_{2_α}
pI	Isoelectric Point
rcDNA	Recombinant DeoxyriboNucleic Acid
rcRNA	Recombinant RiboNucleic Acid
RER	Rough Endoplasmic Reticulum
RT	Reverse Transcription
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
TP	Trophoblastic Protein
TQEH	The Queen Elizabeth Hospital
TSH	Thyroid Stimulating Hormone
UV	Ultra-violet

Chapter One

Review of the Literature

1.1 Introduction

Chorionic gonadotrophin (CG) is secreted by the primate embryo as the first known signal to prevent the demise of the corpus luteum (CL). The maintenance of the CL results in the continued production of progesterone, thereby, maintaining the pregnancy. Many other factors are known to be secreted by the embryo and the maternal endometrium that affect the development of the embryo and its subsequent implantation into the endometrium. Similar factors are also known to influence the expression of CG in human tumour cell lines and cultured placental tissue. There is limited knowledge, however, of the endogenous factors which regulate CG expression in the early primate embryo.

It is the intention of this thesis to investigate the effect of cytokines on chorionic gonadotrophin expression and secretion, using the marmoset monkey as an animal model. Four areas of research will be discussed in this introductory chapter. Firstly, the dynamics of early embryonic development and the process of implantation; secondly, the different signalling mechanisms used by mammals for maternal recognition of pregnancy. Thirdly, a brief review of cytokines and growth factors known to be involved in embryo-maternal interactions and finally, the factors involved in culturing embryos *in vitro*.

1.2 Early Embryonic Development

1.2.1 *Formation of the zygote*

Embryonic development, or embryogenesis, begins at fertilisation. In mammals, fertilisation takes place in the ampulla, the longest and widest part, of the oviduct of the female. The mammalian spermatozoan penetrates the zona pellucida and enters the perivitelline space. Sperm entry triggers the rotation of the metaphase spindle in most species, except humans and sheep and completion of the second meiotic division.

The maternal pronucleus is formed following the completion of the second meiotic division. The sperm head decondenses and the paternal pronucleus also forms. The newly fertilised diploid egg is now called a zygote: DNA replication occurs, the pronuclear membranes break down, the first mitotic division begins and cleavage to a two-cell embryo occurs. The first mitotic or cleavage division is regulated by information inherited from the maternal cytoplasm. As the conceptus begins to express its own genes, the importance of this maternally inherited information decreases and the process of early embryogenesis becomes dependent on the embryonic genome.

1.2.2 *Embryonic cleavage*

1.2.2.1 Cleavage in the oviduct:

The embryo remains in the oviduct for a few days after fertilisation. Cilia of the oviductal epithelial cells gradually sweep the embryo towards the uterus, and cleavage continues whilst in the oviduct. G1 phase, the resting phase characteristic in adult cells, is absent in early blastomeres, *that is* there is no pause between mitosis and DNA replication (or S phase). Thus, in cleavage embryos, DNA replication, mitosis and cytokinesis are continuous (McLaren 1982).

The total size of the embryo does not change during early development, but the number of cells or blastomeres increases, resulting in the daughter cells being half the size of the parent cells at each division.

1.2.2.2 Morula:

Approximately three days after fertilisation, the sixteen-cell stage embryo, or morula, enters the uterus and compaction occurs. The blastomeres flatten against each other, maximising intercellular contact. Gap and tight junctions are formed between the wedge-shaped blastomeres and the cellular organelles become polarised. Microvilli disappear from the lateral and basal cell membranes but remain on the apical surface of each blastomere adjacent to the perivitelline space (Johnson and Everitt 1988; McLaren 1982).

Changes also occur in the composition of the internal constituents, or biochemistry of the blastomeres (Ducibella 1977) and a marked increase in the synthetic capacity of the blastomeres takes place. Changes occur in the synthetic patterns of phospholipids and cholesterol, the net synthesis of protein and RNA increases and the transport of nucleotides and amino acids into the cells rises. At this stage, the morula only measures 70-100 μm in diameter and contains between 20 and 1,000 fmols of free amino acids, 23 ng of protein and only 0·5 ng of RNA (Johnson 1981).

There is substantial evidence to suggest that the developmental fate of early mouse blastomeres is related to their position in the morula (*discussed in Section 1.2.3*). Johnson and Ziomek (1981) found that a relationship exists between the blastomeres, resulting in the differentiation of the inner cell mass (ICM) and the trophectoderm of the eight-cell mouse morula.

At compaction, prior to division, most of the organelles of the blastomeres of the late eight-cell morula are polarised; thus cleavage to sixteen cells, results in some cells being central and others not. The peripheral, polarised cells give rise to the trophectoderm and the central, unpolarised cells to the inner cell mass, or embryoblast, of the blastocyst. Thus, it has been suggested that the process of differential inheritance of organelles leads to the differentiation of the trophectoderm and ICM (Handyside and Johnson 1978; Johnson *et al.* 1981; Johnson and Ziomek 1981).

1.2.2.3 Blastocyst:

Cleavage of the sixteen-cell embryo yields a thirty-two-cell stage, in which fluid is secreted internally to form a small blastocoele (cavity). Due to the tight junctions between adjacent blastomeres, fluid accumulation is possible (the process of cavitation). The embryo is now termed a blastocyst.

Division to sixty-four cells results in an enlarged blastocoele, with the blastocyst consisting of two distinctive cell types: the trophectoderm cells surrounding the blastocoele cavity and the ICM. The ICM, within the blastocoele, lies against part of the trophectoderm. The trophectoderm will give rise to the trophoblast and results in the formation of the placenta and fetal membranes, whereas the ICM differentiates to form all the fetal tissues and organ systems (Johnson and Everitt 1988).

1.2.2.4 Hatching:

"Hatching" occurs approximately two days after the formation of the blastocyst and is characterised by the extrusion of the blastocyst from the surrounding zona pellucida. There are at least two schools of thought on the process of hatching: 1) the blastocyst pulsates and wears a hole in the zona pellucida and 2) enzymes, which are probably maternally derived, attack the zona, causing it to degenerate. Hatching from the zona pellucida allows the blastocyst to expand rapidly. Nutrients are derived from the secretions of the uterine glands (Moore and Persaud 1993).

1.2.3 *Embryonic Genome Activation*

During cleavage in the oviduct, there is a 'switch' between the maternal and embryonic genomes, dependent on the species. In the mouse, the most actively studied species, the 'switch' occurs during the 2-cell stage. At this time, associated with the maternal-embryonic genome switch, is the so-called 2-cell block, which occurs when embryos are cultured *in vitro* in simple medium (Goddard and Pratt 1983; Muggleton-Harris *et al.* 1982; Pratt 1987; Pratt and Muggleton-Harris 1988; Schultz 1986; Telford *et al.* 1990). The block can be circumvented by improved culture media (Chatot *et al.* 1989), however, the block does occur when there is a transition from maternal to embryonic control and obviously murine embryos are very sensitive to culture conditions at this time.

In domestic species: cow, sheep and pig, the transition from maternal to embryonic control varies: between the 4-8-cell stage in the pig and between the 8-16-cell stage in the cow and sheep. Developmental blocks *in vitro* also occur in these domestic animals (for a review see Telford *et al.* 1990).

Studies with human embryos suggest that activation of the embryonic genome occurs at the four to eight-cell stage (Braude *et al.* 1988). Autoradiographic studies using [^{3}H]uridine incorporation, have demonstrated pre-mRNA synthesis in 4-cell embryos and rRNA synthesis at the 6-8-cell stage (Tesarik *et al.* 1986; Tesarik *et al.* 1987; Tesarik *et al.* 1986b). Human embryos cultured *in vitro* arrest at the 4-8-cell stage (Braude *et al.* 1988) and these embryos have an increased sensitivity to culture conditions at this stage.

Recent research has shown the presence of *de novo* transcripts for the paternal allele of the myotonin protein kinase (MPK) gene in human 1-cell embryos (Daniels *et al.* 1995), which is a predominantly muscle-specific protein kinase (Salvatori *et al.* 1994; Whiting *et al.* 1995). The transcripts of ZFY (a region essential for spermatogenesis; Koopman *et al.* 1991) and SRY (gene that encodes the Y-linked testis-determining factor; Clépet *et al.* 1993), both Y-linked and therefore sperm-derived genes have also been detected in human 1-cell zygotes by RT-PCR (Ao *et al.* 1994; Fiddler *et al.* 1995).

Daniels and collegues (1997) also using RT-PCR, developed an assay to detect two house-keeping genes: HPRT and APRT and two tissue-specific genes: α-globin and β-globin in single red blood cells and fibroblasts and subsequently in human oocytes and embryos. The two house-keeping genes and α-globin were expressed constitutively and from the 1-cell stage, respectively, whilst β-globin was only rarely expressed (in 1 out of 11 embryos). The difference in expression between α- and β-globin may be explained by the fact that α-globin contains a CpG-island. Active genes are characterised by under-methylation (Cross and Bird 1995) and paternally-inherited genes that contain CpG-islands may become active, following fertilisation, due to their hypomethylated state (Daniels *et al.* 1997).

All cells in the morula stage of development express the Na+-K+ adenosine triphosphatase (ATPase) and the cell adhesion molecule E-cadherin. These two molecules, however, become

redistributed to the basolateral plasma membrane of the mural trophoectoderm (Cross *et al.* 1994). Wiley *et al.* (1990) suggest that the Na+-K+ ATPase establishes a transcellular Na+ gradient which drives the process of cavitation, however, other transport mechanisms may also be used (van Winkle and Campione 1991).

Arceci and associates (1992a) have found that the receptor for Colony Stimulating Factor 1 (CSF-1), encoded for by the *c-fms* gene is first detected in cleavage stage mouse embryos. After the blastocyst stage, *c-fms* expression becomes restricted to the trophoblast cells, in which a cell-specific promoter drives its expression.

The activity of promoters for Interferon-tau (IFN-τ: Cross *et al.* 1994) and Chorionic Gonadotrophin-α (CG-α: Liu *et al.* 1994), are repressed by the ectopic expression of *Oct-4* in trophoblasts. *Oct-4* is a POU domain-containing transcription factor which is expressed during cleavage by all cells of the embryo. *Oct-4* expression is down-regulated by cells which differentiate (Palmieri *et al.* 1994), thus *Oct-4* is presumed to transactivate the expression of genes which are critical for maintaining the undifferentiated state. *Oct-3*, another POU domain-containing transcription factor, is also expressed in totipotent and pluripotent stem cells of the pre-gastrulation embryo (Rosner *et al.* 1990). This gene maintains totipotency in blastomeres and is inactivated as cellular differentiation begins.

1.2.4 *Implantation*

Attachment of the blastocyst to the endometrial epithelium occurs between 4.5 and 6 days after fertilisation in rodents and primates. An elaborate interaction occurs between the uterine epithelium and the conceptus at this time. Firstly, the conceptus establishes physical and nutritional contact with the maternal endometrium at the site of implantation and secondly, the conceptus emits signals that act upon the maternal pituitary-ovarian axis. Failure of these contacts or signals results in luteal regression, as well as, deprivation of essential nutrients and arrest of growth of the conceptus, leading to loss of the conceptus (Cross *et al.* 1994; Johnson and Everitt 1988).

The blastocyst attaches adjacent to the ICM, the area that represents the embryonic pole. The trophoblast starts to rapidly proliferate, displacing endometrial cells and then differentiates into two layers: 1) the inner cytotrophoblast and 2) the outer syncytio-trophoblast. The cytotrophoblast is a layer of mononucleated cells that are mitotically active. As this layer proliferates, the cells migrate into the syncytiotrophoblast, a mass of multinucleated cells in which no cell boundaries are discernible.

The syncytiotrophoblast gradually erodes or transforms the maternal endometrial stroma (process of decidualisation), allowing the blastocyst to slowly embed itself into the endometrium. Around the implantation site, the maternal stroma, or decidual cells, become laden with glycogen and lipids. Upon degeneration, these cells provide the implanting embryo with nutrients. Other events

occurring at this time include recruitment of inflammatory and endothelial cells, transepithelial invasion of trophoblasts into the endometrium and apoptosis of the uterine endometrium (Weitlauf 1988).

The syncytiotrophoblast is responsible for the majority of the production of chorionic gonadotrophin. This glycoprotein hormone enters the maternal peripheral blood and is the basis for detecting pregnancy in primates (Filly 1988).

1.3 Maternal Recognition of Pregnancy

For pregnancy to be successful, the conceptus must be recognised by the mother. *That is*, there must be "maternal recognition of pregnancy" (Short 1969). This leads essentially to the rescue and maintenance of the functional corpus luteum (CL) and thus the production of progesterone. Quite a few factors are involved in these processes, the presence of hormones and cytokines included, plus the presence of a viable blastocyst.

Basu (1985) defines maternal recognition of pregnancy as a functional relationship between the uterus, the corpus luteum and the embryo itself. The uterus recognises the presence of the embryo and the embryo prevents luteal regression, thus the corpus luteum produces a sufficient amount of progesterone to maintain the pregnancy. There must be a signal from the pre-implantation embryo to the uterus or ovaries that either suppresses

prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; a luteolysin) production from the uterus and/or stimulates the corpus luteum by a luteotrophic mechanism. Before the embryo can produce any signal, however, there must be activation of the embryonic genome. The human embryonic genome is thought to be activated between the four- and eight-cell stages of pre-implantation development (Braude *et al.* 1988).

Embryonic signals vary considerably between species. In domestic animals, *for example*, the major embryonal secretory peptide is trophoblastic protein (TP; oTP, bTP), which acts locally on the endometrium to prevent production of $PGF_{2\alpha}$. In pigs, oestrogens are released and play a role in preventing regression of the corpus luteum, whilst in horses, both oestrogens and conceptus secretory proteins are produced during early pregnancy to suppress $PGF_{2\alpha}$ production. In primates, including man, a polypeptide hormone, chorionic gonadotrophin (CG) is produced that acts directly on the ovary to provide luteotrophic support (Hearn 1986).

1.3.1 *Embryonic signals in primates - Chorionic Gonadotrophin*

The mechanism of luteolysis is poorly understood in primates. It is known that it is uterine independent, *that is* intraovarian effects of prostaglandins, oxytocin and other undefined agents cause the luteolysis of the corpus luteum.

The first studies of maternal recognition in primates were conducted by Hearn and associates (Hearn 1979; Hearn 1986; Hearn *et al.* 1988a; Hearn *et al.* 1988b; Hearn *et al.* 1991). One such study involved the immunisation of marmoset monkeys, baboons and rhesus monkeys against the beta subunit of hCG, resulting in the suppression of fertility, whilst the antibody titres remained high. As the antibody levels decreased, recurrent abortions interspersed with occasional live births were experienced by female marmosets. Female rats have also been immunised against hCG-β, resulting in partial or complete infertility, although a threshold titre of antibody was implied to interfere with pregnancy (Hulme *et al.* 1980).

It is widely known that maternal recognition of pregnancy in primates appears to involve independent interactions between the uterus and embryo, as well as between the ovary and embryo. The conceptus produces CG which results in the prolongation of the life of the CL and its progesterone production. The continual secretion of progesterone allows the maintenance of an endometrium that is permissive to embryonic development, implantation and fetal and

placental development to term. Chorionic gonadotrophin secreted by the embryo directly interacts with the ovary, preventing luteolysis of the CL.

There are likely to be other luteotrophic signals produced by the primate embryo. Studies by Webley *et al.* (1989) have shown a changing responsiveness of marmoset luteal cells to progesterone during normal conception cycles. They suggest that there is another agent causing luteotrophism of the CL. Perhaps other hormones, *for example*, inhibin, relaxin and cytokines and growth factors may be involved. The involvement of inhibin has been investigated. The human CL and placenta express mRNA for inhibin (Davis *et al.* 1987; Petraglia *et al.* 1987; Petraglia *et al.* 1989). Studies by Webley and associates (1991) have shown higher concentrations of inhibin in the peripheral circulation of marmosets by Day 9 after ovulation in conception cycles compared with non-pregnant luteal-phase controls.

1.3.1.1 Chorionic Gonadotrophin:

Chorionic Gonadotrophin (CG) is a member of the glycoprotein hormone family, which are of pituitary or placental origin. They include Luteinising Hormone (LH), Follicle Stimulating Hormone (FSH) and Thyroid Stimulating Hormone (TSH), produced by the pituitary, and CG produced by the placenta/embryo. LH and FSH are involved in gametogenesis and steroid hormone synthesis

in both the ovaries and testes, whilst TSH regulates the synthesis and secretion of thyroid hormones.

The glycoprotein hormones, in turn, are members of the cystine-knot growth factor superfamily, along with Nerve Growth Factor (NGF), Transforming Growth Factor (TGF)-β and Platelet Derived Growth Factor (PDGF)-BB (Lapthorn *et al.* 1994). The proteins in this superfamily all have homologous cystine-knot subunits and form homodimers or heterodimers.

Chorionic gonadotrophin has a molecular weight in humans of 38,000 daltons; ~34% of the molecule is carbohydrate. It consists of 2 non-covalently bonded subunits, α and β. Generally, both individual subunits are not biologically active, although there is some evidence to suggest that CG-α does some biological actions of its own (Blithe and Iles 1995). The α subunit is common to all pituitary glycoprotein hormones and is species specific, whilst the β subunit interacts with the receptor and is hormone specific (Pierce *et al.* 1971).

1.3.1.2 Structure and Chemistry of hCG-α & hCG-β:

The α-subunit of human CG (hCG) has a molecular weight of approximately 14,900 daltons: 10,200 of which represents the protein portion and 4,700 daltons is the carbohydrate portion. Human CG-α consists of 92 amino acid residues and has five disulphide bonds. Two asparagine-linked carbohydrate moieties are attached to the α-subunit at positions 52 and 78.

The hormone-specific β-subunit of hCG has a molecular weight of approximately 23,000 daltons: 16,000 represent the protein portion, whilst 7,000 daltons is the carbohydrate part. The amino acid sequence is 145 residues, with six disulphide bridges. At the amino acid level, hCG-β shares 81% sequence homology with hLH-β, up to the serine at position 121, at which point, hCG-β has an additional 24 amino acid residues rich in serine and proline. The extra amino acids on hCG-β are often referred to as the 'C-terminal extension', or 'C-terminal peptide'. Human CG-β has two asparagine-linked carbohydrate units at positions 13 and 30, and four serine-linked carbohydrate complexes at positions 121, 127, 132 and 138.

The 3-D structure has been resolved (Lapthorn *et al.* 1994), showing both the α- and β-subunits to have similar structures, where three of their disulphide bridges form a cystine knot. It was also determined that a segment of the β-subunit wraps around the α-subunit in a 'seat belt' configuration, to stabilise the intact hCG molecule. Both the α- and β-subunits consist of 2 β-hairpin loops on one side of the central cystine knot and a long loop on the other side (Lapthorn *et al.* 1994). The long loop of the α-subunit is longer than the corresponding loop of the β-subunit.

1.3.1.3 Biology and Immunology of CG:

Structural similarities exist between hLH and hCG, thus their biological and immunological properties are very similar. The C-

terminal extension of hCG-β is not necessary for biological activity, due to its absence in hLH-β. Moreover, Louvet and his colleagues (1974) have found that antibodies specific for this tail piece failed to neutralise the biological activity of hCG. Notwithstanding, the highly glycosylated C-terminus is thought to contribute to the much longer biological half-life of hCG compared to hLH, due to the negatively charged sialic acid residues on the carbohydrates (Morell *et al.* 1971; Van Hall *et al.* 1971). Human CG also exhibits structural homologies to the other glycoprotein hormones, hFSH and hTSH, which would explain an intrinsic FSH- and TSH-like activity of hCG.

1.3.1.4 Production and Secretion of CG:

The synthesis of each subunit is independently regulated, as well as unbalanced, so that there is an excess of the α-subunit (Vaitukaitis 1974). Several workers have also observed that there is a greater amount of hCG-α and -β synthesis in the first-trimester compared with that in full-term (Daniels-McQueen *et al.* 1978; Landefeld *et al.* 1976).

It has been suggested that the α and β genes are related (hCG-α and -β show 31% homology) and thus they probably diverged from a common ancestral gene, similar to the globin genes (Fiddes and Goodman 1980). The α-subunits of all four human glycoprotein hormones (CG, LH, FSH and TSH) are encoded by a single gene (Boothby *et al.* 1981; Fiddes and Goodman 1981). The β-subunit of hCG is

encoded by a cluster of six genes on chromosome 19 (Fiddes and Goodman 1981). However, Fiddes and Talmadge (1984) have proved that only two hCG-β genes are functional and encoding the correct sequence. Although Bo and Boime (1992) using RT-PCR have indicated that at least five, and possibly all six CG-β genes are transcribed *in vivo*. Moreover, studies by Strauss *et al.* (1994) using transgenic mice have shown that CG-β genes 1 and 2 are active in the mouse brain, whilst the transcripts found in the mouse placenta are derived from CG-β genes 5, 3 and 8, in a ratio similar to that found in the human placenta. Strauss and associates have also found that CG-β transcripts were only detectable in the mouse placenta late in gestation, from day 14 onwards, in contrast to human placenta, where CG-β peaks early in pregnancy (Strauss *et al.* 1994).

Both subunits are synthesised as precursors, pre-α and pre-β, including a hydrophobic signal peptide extension. The pre-α signal peptide is 24 amino acids long, whilst that of pre-β contains 20 amino acid residues (Birken *et al.* 1981). Translation of the peptide pre-subunits takes place in the ribosomes of the rough endoplasmic reticulum (RER; Sharon 1984) . The signal peptides are cleaved off whilst the peptide is still attached to the ribosomes. They are then transported through the RER channels to the Golgi bodies. Within the golgi body, glycosylation of the hCG-subunits takes place. Two types of glycosylation occur: N-linked and O-linked.

Following glycosylation, the association of the α- and β-subunits to form the hCG dimer occurs. A significant amount of α- and β-subunits do not combine, but are secreted as free subunits, however they are devoid of biological activity (Catt *et al.* 1973). The carbohydrate moieties seem to play an important role in the folding and assembly of the subunits (Bielinska *et al.* 1989). A block in the assembly of the dimer occurs when inhibitors of carbohydrate addition are used (Ruddon *et al.* 1979).

Chorionic gonadotrophin was first isolated from the urine of pregnant women in 1927 by Aschheim and Zondek (Canfield *et al.* 1971). Placental syncytiotrophoblast cells have been established as the main producer of hCG (Pierce & Midgley 1963; Thiede & Choate 1963, both cited in Canfield *et al.* 1971), secreting it into the blood of both the mother and foetus. Renal excretion of hCG occurs, via the mother. Urinary hCG can be used as an indicator of pregnancy as early as 6 to 8 days after ovulation. Plasma levels of hCG rise dramatically, doubling in concentration every 1 - 3 days (Lenton *et al.* 1982); levels range from 160 to 200 IU/ml between 40 and 90 days of pregnancy. The hCG level then drops to about 10 mIU/ml, where it remains until a slight rise during the third trimester. These data agree with others (Reuter *et al.* 1980 and references within). In the early stages of pregnancy, CG is found as a heterodimer, however, both subunits as separate molecules are present in large quantities in the later stages of pregnancy (Vaitukaitis 1974). It has been well established that hCG levels are higher in multiple birth pregnancies

than in singleton pregnancies (Reuter *et al.* 1980 and references within).

Other tissues also secrete CG in the human, including brain, pituitary, testis, liver and colon (Braunstein *et al.* 1979; Braunstein *et al.* 1975; Chen *et al.* 1976; Yoshimoto *et al.* 1979; Yoshimoto *et al.* 1977). A recent article demonstrated the presence of hCG-β in the normal human cyclic endometrium by *in situ* hybridisation (Wolkersdörfer *et al.* 1998). An earlier *in* situ hybridisation study by Bonduelle and associates (1988) has demonstrated the presence of the β-subunit of CG in three of seven 6-8-cell human embyros.

1.3.1.5 CG Receptor

The LH/CG receptor has a common specificity for both LH and CG. Binding of CG is a rapid, reversible, saturable and dependent on a number of variables, including concentration of both the receptor and hormone. To invoke a maximal response, only 10 to 15% of the receptor binding sites may be saturated, thus a very low concentration of CG or LH will induce a response. Receptors for LH/CG have been found in the uterus of several animals, including the rabbit (Jensen and Odell 1988) and pig (Ziecik *et al.* 1986). Reshef *et al.* (1990) detected the presence of LH/CG receptors in non-pregnant human uterus, placenta, fetal membranes and decidua, using light microscope immunohistochemistry. The rabbit blastocyst has also been demonstrated to contain LH/CG receptors (Khan-Dawood and Dawood

1984). Circulating lymphocytes in pregnant women have also been shown to express the LH/CG receptor gene (Lin *et al.* 1995). The LH/CG receptor has also been found in the human fallopian tubes (Lei *et al.* 1993b), ovary (Minegishi *et al.* 1997), umbilical cords (Rao *et al.* 1993) and brain (Lei *et al.* 1993a).

1.3.2 *Embryonic signals in ruminants - Trophoblastic Protein*

Ovine trophoblastic protein (oTP) was identified by Godkin *et al.* (1982b), who demonstrated that the synthesis of oTP occupies the exact window during which maternal recognition of pregnancy occurs in the sheep, *that is*, between days 13 and 21 of pregnancy (Godkin *et al.* 1984b). Ovine TP has also been shown to be the major translational product of a day 16 conceptus, producing up to 500 μg of oTP in a 30 hour culture period *in vitro* (Ashworth and Bazer 1989).

The discovery of bovine TP followed in 1987 by Helmer and associates (1987). In the cow, maternal recognition of pregnancy is initiated at around day 15 and this is the time at which the conceptus begins to secrete large amounts of a protein that is immunologically related to oTP. This protein is known as bTP.

Ovine TP, alternatively known as trophoblastin (Martal *et al.* 1979), or oTP-1 (Godkin *et al.* 1984b), has a molecular weight of ~20 Kda. It consists of 3-4 non-glycosylated, polypeptide isoforms with

pIs between 5·3 and 5·8 (Roberts 1989). Bovine TP consists of multiple isoforms with molecular weights of 22 or 24 Kda and pIs between 6·3 and 6·8 (Anthony *et al.* 1988). When both TPs are injected into the uterine lumen of their respective animal, the life of the corpus luteum is extended by influencing the output of $PGF_{2\alpha}$ (Roberts 1991).

Both oTP and bTP have been molecularly cloned by Imakawa and associates, revealing the existence of multiple mRNA species, whose sequences are highly conserved (Imakawa *et al.* 1987; Imakawa *et al.* 1989). The oTP and bTP mRNAs are about 1 kilobase in length and code for a 195 amino acid protein, having a 23-residue signal peptide (Charpigny *et al.* 1988; Roberts *et al.* 1989; Stewart *et al.* 1987).

Both TPs show a structural relationship to type I IFNs (IFN-α & IFN-β). The cDNA clones of oTP and bTP share 30% homology with IFN-β and approximately 55-65% homology with IFN-αs. However, 80% homology is seen between the TPs and a group of 'long' IFNs (Stewart *et al.* 1990). These long IFNs are named either, $IFN\text{-}\alpha_{II}$ (Capon *et al.* 1985), or as now recommended by Hauptmann *et al.* (1985), IFN-ω. In 1994, Roberts reported evidence that IFN-τ, a distinct Type I subtype of interferons, is produced by mononucleate trophectoderm cells of ruminant conceptuses. He suggests that IFN-τ has a primary function of triggering maternal responses to the presence of the embryo and thus, rescuing the corpus luteum of pregnancy (Roberts 1994). Therefore, he infers that IFN-τ is TP, thus TP and IFN-t are now used interchangeably.

Ovine TP and bovine TP have certain structural characteristics in common with all the IFN-α and IFN-ω proteins. Four cysteines forming two intrachain disulphide bridges are conserved, leading to the same general three dimensional form for each of the molecules. There are three other characteristics that confirm the similarity between the TPs and the IFNs: 1) potent antiviral activity (Roberts *et al.* 1989); 2) inhibition of growth of bovine kidney epithelial cells in culture, as well as, inhibition of incorporation of ^{3}H-thymidine into activated lymphocytes (Roberts *et al.* 1989); 3) competition for binding to the IFN-α receptors found in the uterus and other organs of the sheep (Knickerbocker and Niswender 1989; Stewart *et al.* 1987).

The day 15 sheep conceptus can produce up to 500 μg of oTP in a 30 hour culture period *in vitro* (Ashworth and Bazer 1989). This amount far exceeds that required to saturate the type I IFN receptors which are present in the uterine epithelium, thus the action of oTP may also be endocrine, as well as autocrine/paracrine. However, Godkin *et al.* (1984a) have attempted to detect the presence of TP within the peripheral blood, without success.

Culture studies have observed the production of oTP by sheep conceptuses as early as days 8 and 10 of pregnancy (Ashworth and Bazer 1989; Farin *et al.* 1990). *In situ* hybridisation studies have detected oTP mRNA in low concentrations as early as day 10 of pregnancy (Farin *et al.* 1990; Farin *et al.* 1989b). On day 13, the amount of oTP mRNA increases several-fold, and is more abundant than actin

mRNA (Farin *et al.* 1989a; Hansen *et al.* 1988). This mRNA is localised to the trophectoderm of the conceptus. In the cow, similar results are seen, however, the events are delayed two or three days (Farin *et al.* 1990). Trophoblastic protein has also been implicated in influencing the pattern of secretion of endometrial proteins (Godkin *et al.* 1984a; Salamonsen *et al.* 1988; Sharif *et al.* 1989; Vallet *et al.* 1987).

1.3.3 *Embryonic signals in other animals*

1.3.3.1 pig:

Bazer *et al.* (1986) have extensively reviewed the theory of maternal recognition of pregnancy in pigs. They believe that in cycling animals, $PGF_{2\alpha}$ is secreted by the uterine endometrium into the uterine vasculature and transported to the corpus luteum to exert its luteolytic effect. However, the pig conceptus secretes oestrogens that redirect the secretion of $PGF_{2\alpha}$ into the uterine lumen, where it can be metabolised or deactivated, thus preventing luteolysis of the corpus luteum (Bazer *et al.* 1989b; Fischer *et al.* 1985).

Conceptus secretory proteins (CSP) are also secreted by the swine embryo. There are two major classes of protein, with differing molecular weights. Both groups of proteins are secreted between Days 10·5 and 18 of pregnancy (Godkin *et al.* 1982a). One or more of the CSPs has antiviral activity (Mirando *et al.* 1990), however, Harney and Bazer (1989) have found that porcine CSPs are not antiluteolytic. A number of other factors make up porcine CSP,

including retinoid binding proteins, plasminogen activator and plasmin/trypsin inhibitor, and various enzymes. The pig conceptus appears to be dependent upon the protein factors that the endometrium secretes throughout the whole of pregnancy (Roberts and Bazer 1988).

1.3.3.2 mare:

Pregnancy establishment in the mare appears to be a fairly fragile process. There is a high incidence (20-25%) of early embryonic loss at around the time of maternal recognition of pregnancy, as well as a high incidence of pseudopregnancy (15-20%) (Sharp *et al.* 1989b). $PGF_{2\alpha}$ is the uterine-produced luteolysin in cyclic mares. However, it appears that the inhibitory effect of the conceptus is relatively transient (Sharp *et al.* 1984).

The equine pre-implantation conceptus actually migrates through the entire uterus 12-14 times per day (Ginther 1984; as cited by Bazer 1992), thus the conceptus secretions affect the entire endometrium, inhibiting the production of $PGF_{2\alpha}$. Equine embryos secrete proteins between Days 12 and 14 of gestation, however, the role of these CSPs are not known (Sharp *et al.* 1984). Oestrogen is also secreted by the embryo between Days 8 and 20 of pregnancy, but still the role of this hormone is unknown in the horse at this time.

Bazer (1992) suggests that the production of CSP and/or oestrogens by equine conceptuses may provide a signal for the

initiation of maternal recognition of pregnancy by inhibiting the endometrial production of $PGF_{2\alpha}$ either directly or indirectly.

The equine trophoblast also produces equine CG, however, the role of this form of CG in the horse is not completely understood (Murphy and Martinuk 1991).

1.3.3.3 mouse:

In mice, the first signal from the conceptus appears to be a pregnancy-specific immunosuppressive substance called Early Pregnancy Factor (EPF). This factor was first detected by Morton and associates in pregnant mice by a rosette inhibition test, within the first 24 hours after fertilisation (Morton 1985; Morton *et al.* 1974; Morton *et al.* 1980; Nahhas and Barnea 1990). EPF has been found in the serum of mice, humans and sheep, however it appears unlikely that the embryo would actually produce this factor. It has been suggested that the embryo acts as a stimulus to produce EPF from some other source. This hypothesis has been tested on the mouse (Morton *et al.* 1980) and it has been found that EPF is secreted as two components, A and B. Component A appears to be oestrus-dependent and oviductally-derived, whereas, component B is pregnancy-dependent and produced by the ovary. EPF has also been characterised and purified in the form of a polypeptide from serum of pregnant women (Mehta *et al.* 1989).

In 1994, EPF was identified as an extracellular form of chaperonin 10 (cpn10), one of the highly conserved heat shock

family of proteins (Cavanagh and Morton 1994). Cavanagh and Morton's group have demonstrated the requirement of EPF/cpn10 for optimising embryonic development in the mouse (Athanasas-Platsis *et al.* 1994; Cavanagh *et al.* 1994; Somodevilla Torres *et al.* 1994).

1.4 Cytokines and Growth Factors

1.4.1 *What are cytokines?*

Cytokines are a heterogeneous group of proteins and polypeptides released from a cell with a variety of functions, including intercellular communication and altering of cell proliferation, differentiation, secretion or rate of migration. Originally identified as soluble molecules, released from activated immune cells and responsible for signalling between the cells of the immune and haematopoietic systems, cytokines have in recent years been found to have new functions and targets in both normal and pathophysiological conditions.

Currently, there are at least 60 different cytokines, divided into four distinct families. These families are:

1. Interleukins (ILs)
2. Colony Stimulating Factors (CSFs)
3. Interferons (IFNs) and
4. Growth Factors (GFs).

A list of the common cytokines and growth factors and their acronyms is shown in *Table 1.1*.

Table 1.1 Common Cytokines and Growth Factors and their acronyms.

Cytokine	**Acronym**
Interleukins	*ILs*
Colony Stimulating Factors	*CSFs*
CSF-1 or Macrophage CSF	M-CSF or CSF-1
Granulocyte-Macrophage CSF	GM-CSF
Granulocyte CSF	G-CSF
Interferons	*IFNs*
Growth Factors	*GFs*
Epidermal GF	EGF
Fibroblast GFs	FGFs
Insulin-like GFs	IGFs
Leukaemia Inhibitory Factor	LIF
Platelet Activating Factor	PAF
Platelet-derived GF	PDGF
Transforming GFs	TGFs
Tumour Necrosis Factors	TNFs

Cytokines, generally have a number of properties in common. Most cytokines are glycosylated, thus there is a range of molecular weights for some factors, due to the extent of glycosylation. However, glycosylation is not essential for the expression of biological activity of most cytokines. They have a low molecular weight, ranging from 6-60 kilodaltons, and consist of between 100 and 200 amino acid residues, including a signal sequence for secretion. The exception for a signal peptide is Interleukin-1 (IL-1)

(Mizel 1989). Most cytokines are monomers or dimers, except Tumour Necrosis Factor (TNF), which is a trimer (Arakawa and Yphantis 1987)

They are synthesised and secreted by a number of cell types, including: activated immune and mesenchymal cells. Cytokines are extremely potent and usually found only in the picomolar range. Production of cytokines is often transient and is regulated at the transcriptional or translational level. Consequently, cytokines exert their biological effects by binding to a high affinity receptor on the plasma membrane of the target cell. Binding to the receptor causes many intracellular activities, *for example* RNA and protein synthesis, and altered cellular behaviour. Receptors of cytokines are classified into receptor families based on common structural features. These families, including those containing tyrosine kinase domains in their cytoplasmic region and others which generate their cytoplasmic signals only in conjunction with additional molecular components (see review of Taga and Kishimoto 1995).

Cytokines function as intercellular communicators between various cells of the immune system. Thus, communication can be at close range or within a small area of tissue (autocrine or paracrine), or perhaps systematically throughout the entire organism (endocrine). All cytokines are pleiotrophic, *that is* they generally exert multiple biological effects on a variety of cell types, including haematopoietic, epithelial and mesenchymal cells. Individual cytokines have multiple overlapping cell regulating

actions, but they do not act in isolation. They appear to interact in a network by: *first* inducing each other; *second* transmodulating cytokine cell surface receptors and *third* by synergistic, additive or antagonistic interactions on cell function. Furthermore, the resultant activity of a cytokine may be modified by the presence or absence of binding proteins, *for example* Insulin-like Growth Factor Binding Proteins (IGFBPs), or soluble receptors, *for example* soluble IL-2 receptor (sIL-2R) which may alter the binding of the cytokine to its receptor.

Many of the known cytokines seem to be involved in the different phases of reproduction in both the female and the male. A substantial amount of work has been conducted in rodents on embryo-maternal interactions. Cytokines are known to be produced by the murine maternal reproductive tract, affecting embryonic development and the implantation process. A small amount of research has also been done on 'spare' human embryos.

There are many recent reviews on cytokines and growth factors and the effect they have on early embryonic development and implantation. The reviews are quite extensive and as cytokines and growth factors have very many different functions I will not discuss every cytokine and growth factor. Instead, I will highlight the various cytokines and growth factors and their receptors that are important: 1) in the oviduct, 2) in the uterus, 3) at implantation and 4) in the regulation of CG expression and secretion, paying particular attention to the four cytokines used in this thesis (LIF, GM-CSF, TGF-β & IL-6).

1.4.2 *Cytokines play a role in the oviduct*

Development of the embryo starts in the oviduct, after fertilisation. The embryo is under the dominion of factors secreted by the oviductal epithelial cells, as well as those factors secreted by the embryonic cells. Differences occur in the origin (maternal or embryonic) and stage-specific timing of appearance of growth factor transcripts (Rappolee *et al.* 1988; Rappolee *et al.* 1990; Schultz and Heyner 1992; Watson *et al.* 1992; Watson *et al.* 1994b). The onset of embryonic transcriptional activity has been compared between several species of mammal (Telford *et al.* 1990, *also mentioned in Section 1.2.3*). Thus, a particular transcript found at the two-cell stage, *for example*: the insulin receptor, will be of embryonic origin in the mouse embryo, but of maternal and embryonic origin in the sheep embryo.

The various cytokines and growth factors and their receptors found in the oviduct or that are known to influence early embryonic development whilst in the oviduct are listed in *Table 1.2.*

*Superscript numbers indicate the following references: [1]Srivastava *et al.* 1996; [2]Barmat *et al.* 1997; [3]Zhao and Chegini 1994; [4]Kurachi *et al.* 1994; [5]El-Danasouri *et al.* 1993; [6]Morishige *et al.* 1993; [7]Smotrich *et al.* 1996; [8]Pfeifer and Chegini 1994; [9]Keltz *et al.* 1996; [10]Gordon *et al.* 1996; [11]Schell *et al.* 1994; [12]Dalton *et al.* 1994; [13]Arceci *et al.* 1992a, 1992b; [14]Arceci *et al.* 1989, Bartocci *et al.* 1986; [15]Pampfer *et al.* 1991; [16]Murphy and Barron 1993; [17]Hunt 1993, Hunt *et al.* 1993; [18]Suzuki *et al.* 1996; [19]Carlsson *et al.* 1993, Zhang *et al.* 1994; [20]Watson *et al.* 1992; [21]Xia *et al.* 1996, Gabler *et al.* 1997; [22]Viuff *et al.* 1995; [23]Schmidt *et al.* 1994; [24]Gandolfi *et al.* 1995; [25]Rosselli *et al.* 1994; [26]Watson *et al.* 1994; [27]Stevenson and Wathes 1996; [28]Doré *et al.* 1996; [29]Tuo *et al.* 1995; [30]Swanchara *et al.* 1995; [31]Wiseman *et al.* 1992.

Table 1.2 Cytokines and growth factors and their receptors found in the oviduct or known to influence embryonic development in the oviduct*.

Factor	human	baboon	mouse	rat	cow	sheep	pig
IL-1α			+[12]				
IL-1β	+[1]		+[12]				
IL-1RA	+[1]						
IL-2	+[1]						
sIL-2R	+[1]						
IL-6	+[1,2]						
IL-8	+[1]						
IL-10	+[1]						
IL-11	+[1]						
CSF-1	+[2]		+[13,14,15]				+[29]
c-fms			+[13,14]				
GM-CSF	+[3]						
IFN-γ	+[1]						
EGF	+[2,4]	+[11]	-[12]		-[20]	-[26]	+[30]
EGF-R	+[5,6]	+[11]					+[30]
TGF-α	+[7]	+[11]	+[12]		+[20]		
HB-EGF			+[12]				
aFGF					+[21]		
bFGF					+[20,21,22]	+[26]	
FGF-R					+[21]		
IGF-I	+[8]		+[12,16]	+[19]	+[23]	+[26,27]	+[31]
IGF-II			+[16]		+[20]	+[26,27]	+[31]
IGFBPs	+[8]					+[27]	
IGF-IR	+[8]			+[19]	+[20]	+[26]	
insulin R					+[20]	+[26]	
LIF	+[2,9]						
NGF					-[20]		
PDGF-A					+[20]		
PDGF-B					+[22]		
PDGF-AR					+[20]		
SCF	+[1]						
SF			+[13]				
c-kit			+[13]				
TGF-β			+[12]		+[20]	+[27,28]	
TNF-α	+[1]		+[17]			+[26]	
VEGF	+[10]						
activin					+[24]		
endothelin					+[25]		
lactoferrin			+[12]				
MIF[*]			+[18]				
MIP-1α[§]	+[1]						

[*]MIF = macrophage migration inhibitory factor; [§]MIP-1α = macrophage inflammatory protein 1 alpha.
+ = presence of ligand or receptor; - = failure to detect ligand or receptor; a blank space = no information available as yet.

1.4.2.1 Leukaemia Inhibitory Factor:

Two recent reports have localised LIF to the human oviduct. Keltz and co-workers (1996) found that LIF mRNA is expressed in the oviduct, with expression varying slightly during the menstrual cycle. The greatest expression of LIF was seen associated with ectopic pregnancy, with levels of mRNA expression higher in the distal portions of the oviduct. They also investigated LIF protein biosynthesis in tubal epithelial and stromal cell cultures, demonstrating that estradiol and progesterone did not modulate LIF expression. The other study by Barmat and associates (1997) characterised growth factor gene expression by passaged co-culture cell lines, including cultures from segments of fresh human oviduct and buffalo rat liver. They found by Northern hybridisation that LIF is expressed in the human oviduct. Another report by Srivastava *et al.* (1996) determined the concentrations, presence or absence of various cytokines and growth factors in human oviductal fluid, follicular fluid, amniotic fluid and seminal fluid. LIF was found in moderate concentrations in human oviductal fluid and in small amounts in amniotic fluid.

1.4.2.2 Granulocyte-Macrophage Colony Stimulating Factor:

The study by Srivastava and co-workers (1996) mentions GM-CSF protein in the oviduct, however levels are negliglible in both human oviductal fluid and amniotic fluid. Zhao and Chegini (1994), using RT-PCR, *in situ* hybridisation and immunohistochemistry

found that human oviduct expresses GM-CSF mRNA and protein and GM-CSF α and β recepetor mRNA. Changes in expression were seen during the menstrual cycle, thus regulation is probably modulated by ovarian steroids.

1.4.2.3 Transforming Growth Factor-β:

Dalton *et al.* (1994), using Northern blot and immuno-histochemistry, localised TGF-β_1 and TGF-β_2 to the secretory epithelial cells of the murine oviduct. They also detected immunostaining in the serosa and smooth muscle, with less immunostaining for TGF-β_1 and intense immunostaining for TGF-β_2 and TGF-β_3. The abundance of mRNAs for TGF-β was not affected by ovariectomy and was relatively constant during the pre-implantation period. TGF-β transcripts have also been found in the oviduct of the cow (Watson *et al.* 1992) and sheep (Watson *et al.* 1994b).

1.4.2.4 Interleukin-6:

Srivastava and associates (1996) detected IL-6 in human oviductal fluid and in amniotic fluid. Negligible levels were found in follicular fluid and in seminal fluid. The authors suggest that IL-6 and other proinflammatory cytokines may play a role in defending the oviduct against infection. Barmat and co-workers (1997) also found IL-6 mRNA was expressed by human oviductal cell cultures.

1.4.3 *Cytokines play a role in the uterus*

Embryonic development continues in the uterus. The zygote reaches the sixteen-cell, or morula stage and passes through the isthmus of the oviduct into the uterus. As mentioned before, the morula undergoes compaction and differentiation to two cell lines: a) the outer trophectodermal layer and b) the inner cell mass. Cleavage of the sixteen-cell morula results in thirty-two cells and blastocyst formation. Two days after the formation of the blastocyst, hatching occurs, followed by implantation into the maternal endometrial epithelium, which will be mentioned later.

The embryo/fetus is now under the influence of factors produced by the uterine epithelium, as well as those factors secreted by the differentiating embryonic cells. The cytokines and growth factors and their receptors that are found in the uterus or that are known to influence embryonic development whilst in the uterus are listed in *Table 1.3*.

*Superscript numbers indicate the following references: [1]Simón *et al.* 1996; [2]Kauma *et al.* 1990; [3]Simón *et al.* 1993a; [4]Simón *et al.* 1993b; [5]Simón *et al.* 1995b; [6]Boehm *et al.* 1989, Yagel *et al.* 1989; [7]Haynes *et al.* 1993; [8]Tabibzadeh *et al.* 1989; [9]Noble *et al.* 1996; [10]Daiter *et al.* 1992, Kanzaki *et al.* 1992; [11]Azuma *et al.* 1991; [12]Jokhi *et al.*1994; [13]Pampfer *et al.* 1992; [14]Sharpe-Timms *et al.* 1994, Giacomini *et al.* 1995; [15]Jokhi *et al.* 1994; [16]Russell *et al.* 1993; [17]Haining *et al.* 1991a, 1991b; [18]Imai *et al.* 1995; [19]Watson *et al.* 1994; [20]Horowitz *et al.* 1993; [21]Cordon-Cardo *et al.* 1990, Rusnati *et al.* 1990; [22]Gao *et al.* 1995; [23]Charnock-Jones *et al.* 1994b, Cullinan *et al.* 1996; [24]Kojima *et al.* 1994, Arici *et al.* 1995, Chen *et al.* 1995, Delage *et al.* 1995, Tabibzadeh and Babaknia 1995, Vogiagis *et al.* 1996, Laird *et al.* 1997; [25]Kanzaki *et al.* 1994; [26]Kojima *et al.* 1995; [27]Arici *et al.* 1996; [28]Tabibzadeh *et al.* 1991, Hunt *et al.* 1992, Terranova *et al.* 1995; [29]Charnock-Jones *et al.* 1993; [30]Osuga *et al.* 1995; [31]Ace and Okulicz 1995; [32]Fazleabas *et al.* 1994; [33]Takacs *et al.* 1988, Takacs and Kauma 1996; [34]Wegmann 1990c, Tangri and Raghupathy 1993; [35]Lin *et al.* 1993; [36]Jacobs *et al.* 1992, Robertson *et al.* 1992; [37]Robb *et al.* 1998; [38]Bartocci *et al.* 1986, Arceci *et al.* 1992a, 1992b; [39]Arceci *et al.* 1989; [40]Robertson and Seamark 1992, Sanford *et al.* 1992, Robertson *et al.* 1996; [41]Robertson *et al.* 1994; [42]Huet-Hudson *et al.* 1990; [43]Brown *et al.* 1989, Tong *et al.* 1996; [44]Tamada *et al.* 1991, Paria *et al.* 1994a; [45]Das *et al.* 1994; [46]Wordinger *et al.* 1992, 1994, Taniguchi *et al.* 1998; [47]Kapur *et al.* 1992; [48]Bhatt *et al.* 1991, Shen and Leder 1992; [49]Yang *et al.* 1996; [50]Yang *et al.* 1995a; [51]Das *et al.* 1992; [52]Roelen *et al.* 1994; [53]Doré *et al.* 1996; [54]Hunt 1993, Hunt *et al.* 1993, Roby and Hunt 1995; [55]Roby *et al.* 1996; [56]Geisert *et al.* 1991, Kirby *et al.* 1996; [57]Vogiagis *et al.* 1994; [58]Riley *et al.* 1994; [59]Tuo *et al.* 1995; [60]Zhang *et al.* 1992b; [61]Kim *et al.* 1995; [62]Katsahambas and Hearn 1996, Gupta *et al.* 1997; [63]Letcher *et al.* 1989, Wiseman *et al.* 1992; [64]Ko *et al.* 1991, 1994; [65]Chastant *et al.* 1994; [66]Hofig *et al.* 1991; [67]Anegon *et al.* 1994; [68]Gupta *et al.* 1996; [69]Grünig and Antczak 1995; [70]Hofmann and Anderson 1990; [71]Paria *et al.* 1994b; [72]Hrabé de Angelis *et al.* 1995; [73]Carlone and Rider 1993; [74]Carlsson and Billig 1991; [75]Lennard *et al.* 1995; [76]Zhang *et al.* 1994; [77]Yang *et al.* 1995; [78]Yang *et al.* 1994; [79]Lea *et al.* 1995; [80]Yelavarthi *et al.* 1991; [81]Das *et al.* 1997; [82]Yamanouchi *et al.* 1997.

Table 1.3 Cytokines and growth factors and their receptors found in the uterus or known to influence embryonic development in the uterus before the time of implantation*.

Factor	human	primate†	mouse	cow	sheep	pig	others§
IL-1α	+[1]		+[33]				
IL-1β	+[1,2,3]		+[33]				
IL-1RtI	+[3,4]		+[33]				
IL-1RA	+[1,5]						
IL-2	+[6]		+[34]				+ [70h]
IL-4	+[7]		+[35]				+ [70h]
IL-5			+[35]				
IL-6	+[8]		+[36,37]				
IL-10			+[35]				
IL-11	+[9]		+[38]				
CSF-1	+[10-12]		+[39,40]			+[60]	
c-fms	+[11,13]		+[40]				
GM-CSF	+[12,14]		+[37,41]				
GM-CSFRα	+[15]		+[42]				
GM-CSFRβ			+[42]				
IFN-γ	+[7,12]		+[35]				+ [70h]
IFN-R	+[16]				+[16]		
EGF	+[17,18]	+[31M]	+[43]				
EGF-R	+[18,19]	+[31M/32B]	+[43,44]			+[61]	+[71b/72s]
TGF-α	+[18,20]		+[45]				
HB-EGF			+[46]			+[62]	
bFGF	+[21]		+[47]			+[63]	+[73b/74r]
IGF-I	+[22]	+[31M]	+[48]	+[56]		+[64,65]	+[75r]
IGF-II	+[22]			+[56]		+[65]	-[76h]
							+[77r]
IGF-IR		+[31M/32B]	+[48]			+[66,67]	
IGF-IIR						+[66]	
LIF	+[12,23-25]	+[32M]	+[49,50]		+[57]	+[68]	+[50,78b]
LIFR	+[23,26]		+[50,51]				+[79b]
SCF	+[25]						
TGF-β	+[2,27]	+[31M]	+[52]		+[58]	+[69]	+[80h]
TGF-β R		+[31M]	+[53]			+[69]	
TNF-α	+[28]		+[54]				+[70h/81r]
TNF-α R			+[55]				
VEGF	+[29]						+ [82b]
PD-ECGF	+[30]						
activin							+[83h]
endothelin					+[59]		

†primates = rhesus monkey (M) or baboon (B).
§others = rat (r), rabbit (b), skunk (s), or horse (h).
+ = presence of ligand or receptor; - = failure to detect ligand or receptor; a blank space = no information available as yet.

1.4.3.1 Leukaemia Inhibitory Factor:

Many studies have detected LIF in the endometrium of different species (*as listed above*). Expression in the luminal and glandular epithelium of the human endometrium has marked cyclical changes during the menstrual cycle, suggesting a paracrine/autocrine role for LIF in endometrial function (Vogiagis *et al.* 1996). Laird *et al.* (1997) demonstrated the presence of LIF in uterine flushings obtained from normal fertile women from Day 7 after the LH surge reaching maximal levels at Day 12 after the LH surge. Women of unexplained infertility had significantly lower levels than fertile women. A few groups have looked at the secretion of LIF by culturing cells from endometrial biopsies, showing that expression is dependent on cell type and time of collection (stage of menstrual cycle; Chen *et al.* 1995; Delage *et al.* 1995; Laird *et al.* 1997 1347). Delage and co-workers (1995) found that LIF production of endometrial cultures obtained from women with repeated failures of embryonic implantation and unexplained primary infertility is significantly lower than that obtained from normal fertile women.

Ace and Okulicz (1995) investigated by semi-quantitative RT-PCR the expression of LIF mRNA in populations of cDNA isolated from oestrogen and progesterone dominated rhesus monkey endometrial tissue samples. They found that LIF is progesterone-dependent and expressed during the secretory phase of the menstrual cycle of the rhesus monkey.

Uterine expression of LIF has been demonstrated in the mouse and shown to be most abundant in the uterine endometrial glands specifically on Day 4 of pregnancy, which coincides with implantation in the mouse (Bhatt *et al.* 1991). Shen and Leder (1992) showed that LIF expression varies during the estrous cycle, suggesting that perhaps LIF is regulated by ovarian steroid hormones. Recently, Yang *et al.* (1995a) localised LIF protein and receptor and gp130 in the mouse uterus during early pregnancy. The localisation of the protein was very similar to that of LIF mRNA (Bhatt *et al.* 1991; Shen and Leder 1992). Yang and co-workers also investigated expression of LIF (Yang *et al.* 1994) and LIFR and gp130 in the rabbit (Yang *et al.* 1995b).

1.4.3.2 Granulocyte-Macrophage Colony Stimulating Factor:

In the mouse, synthesis of GM-CSF by uterine luminal and glandular epithelial cells of the uterus is regulated by ovarian steroids, in particular oestrogen (Robertson *et al.* 1992; Robertson *et al.* 1996b). Seminal components at mating are also known to modulate GM-CSF production (Robertson *et al.* 1996a; Robertson and Seamark 1990a). Sharpe-Timms *et al.* (1994) localised GM-CSF to the human uterine epithelial cells by immunohistochemistry and Giacomini and co-workers (1995) found that human uterine epithelial cells are the major source of GM-CSF, using an endometrial explant culture system. GM-CSF has also been proposed to be produced in the uterus by T lymphocytes (Athanassakis *et al.* 1987; Wegmann *et al.* 1989)

and leucocytes, fibroblasts and endothelial cells (Le *et al.* 1990). The actual implications of GM-CSF within the endometrium remains to be determined, however, in the murine model, GM-CSF supports attachment and outgrowth of murine blastocysts *in vitro* (Armstrong and Chaouat 1989) and promotes placental growth and fetal survival and prevents spontaneous abortions in abortion-prone mice (Wegmann *et al.* 1989).

1.4.3.3 Transforming Growth Factor-β:

Kauma *et al.* (1990) demonstrated the presence of TGF-β bioactivity within all tissues of the maternal-fetal interface, including decidua, placenta and placental membranes. They also found that all of these tissues expressed mRNA for TGF-β and thus conclude that TGF-β may regulate the local maternal immune response and prevent rejection of the foetus. Ace and Okulicz (1995) investigated the expression of TGF-β_2 and its receptor, finding that TGF-β_2 was induced in the secretory phase. This is consistent with a study by Chegini *et al.* (1994), who showed similar increases in human endometrium during the early and mid-secretory phase.

Expression of TGF-β has also been investigated in the mouse uterus. Das and co-workers (Das *et al.* 1992; Tamada *et al.* 1990) using Northern blot and immunocytochemistry, demonstrated that all three TGF-β isoforms (TGF-β_1, TGF-β_2 & TGF-β_3) are expressed in a cell-type specific manner in the peri-implantation mouse uterus. This differential expression suggests that each isoform may have a unique role in embryo-uterine interactions. The embryonic

expression of the receptors for TGF-β have also been examined by immunohisto-chemistry, *in situ* hybridisation and RT-PCR (Roelen *et al.* 1994). Their results are consistent with the view that TGF-β may be involved in embryo implantation.

Both the sheep (Doré *et al.* 1996) and the horse (Lea *et al.* 1995) endometrium express TGF-β during early pregnancy. However, as both these animals undergo restructuring and modifying of the endometrium for a subsequent estrous cycle and/or pregnancy, they suggest that TGF-β plays a role in the remodelling process.

1.4.3.4 Interleukin-6:

Tabibzadeh and co-workers (1989) have investigated the secretion of IL-6 from human endometrial explant cultures and found that this secretion is modulated by oestrogen. One of the sites of IL-6 secretion in the mouse uterus has been localised to the uterine epithelium (Robertson *et al.* 1992). Jacobs *et al.* (1992) also investigated IL-6 secretion in the mouse, using cultured endometrial cells. They propose that the two cell populations they examined: uterine stromal and uterine epithelial cells, communicate with each other via IL-6 and thus play a role in regulating the implantation process.

1.4.4 *Cytokines play a role at implantation*

Attachment of the blastocyst to the endometrial epithelium occurs about six days after fertilisation, in the human. As

mentioned above, an elaborate interaction occurs between the uterine epithelium and the conceptus. Failures in implantation account for the abortions in about one-third of normal human pregnancies (Wilcox *et al.* 1988). In farm animals, failures in implantation account for almost 80% of embryonic loss (Roberts *et al.* 1992a; Roberts *et al.* 1990a). Thus the implantation process is very important for proper placental development and to sustain the foetus for the full term of gestation.

Once the blastocyst attaches to the endometrium, adjacent to the inner cell mass, the trophoblast starts to rapidly proliferate and differentiate into one of three cell types: 1) villous syncytiotrophoblasts, 2) extravillous anchoring trophoblasts, or 3) invasive intermediate trophoblasts [Nachtigall, 1996 #1424; and references therein]. There is growing evidence that cytokines and growth factors mediate these differentiation pathways (Strickland and Richards 1992) and these are listed in *Table 1.4*.

*Superscript numbers indicate the following references: [1]Laird *et al.* 1994, Masuhiro *et al.* 1991, Tabibzadeh *et al.* 1990a; [2]Kariya *et al.* 1991; [3]Silen *et al.* 1989, Yagel *et al.* 1989b, Kauma *et al.* 1994, Frank *et al.* 1995; [4]Sawai *et al.* 1995; [5]Nishino *et al.* 1990; [6]Garcia-Lloret *et al.* 1989; [7]Lysiak *et al.* 1994; [8]Arici *et al.* 1995; [9]Graham *et al.* 1992, Matsuzaki *et al.* 1992; [10]Tabibzadeh *et al.* 1989, Inoue *et al.* 1994, Tabibzadeh *et al.* 1995; [11]Fazleabas *et al.* 1994; [12]Simón *et al.* 1994a; [13]Abbondanzo *et al.* 1996; [14]Chaouat *et al.* 1990; [15]Armstrong and Chaouat 1989; [16]Jacobs *et al.* 1992; [17]Wegmann *et al.* 1993; [18]Robb *et al.* 1998; [19]Bartocci *et al* 1986, Pollard *et al.* 1987, Simón *et al.* 1994b; [20]Wegmann 1990, Kanzaki *et al.* 1991, Robertson *et al.* 1991; [21]Brown *et al.* 1989; [22]Das *et al.* 1994; [23]Taniguchi *et al.* 1998; [24]Markoff *et al.* 1995; [25]Stewart *et al.* 1992, Stewart 1994, Lavranos *et al.* 1995; [26]Tamada *et al.* 1990; [27]Pampfer *et al.* 1994; [28]Chakraborty *et al.* 1995; [29]Gupta *et al.* 1997; [30]Gupta *et al.* 1996; [31]Carlone and Rider 1993; [32]Jakeman *et al.* 1993.

Table 1.4 Cytokines and growth factors and their receptors found to modulate hatching and implantation*.

Factor	human	baboon	mouse	cow	sheep	pig	others§
IL-1α	+[1]						
	-[2]						
IL-1β	+[3]		+				
IL-1R tI			+[12]				
IL-1RA			NE[13]				
IL-2			-[14]				
IL-3			+[14,15]				
IL-6	+[4]		+[16]				
IL-6R	+[5]						
IL-10			+[17]				
IL-11			+[18]				
IL-11R			+[18]				
CSF-1/*c-fms*	+[6]		+[19]				
GM-CSF			+[14,15,20]				
IFN-γ			-[14]				
EGF		+[11]	+[21]				
TGF-α	+[7]						
EGFR			+[21]				
HB-EGF			+[22]				
bFGF			+[23]			+[29]	+rat[31]
IGFs		+[11]					
IGFBPs			+[24]				
LIF	+[8]		+[25]		+		
gp130	+[4]						
TGF-β	-[9]		+[26]			+[30]	
TNF-α	-[10]		-[14,27]				
VEGF			+[28]				+rat[32]

§others = rat (r), rabbit (b), skunk (s), or horse (h).
+ = cytokine/GF could possibly have a beneficial effect; - = cytokine/GF could possibly have a detrimental effect; NE = no significant effect; a blank space = no information available as yet.

1.4.4.1 Leukaemia Inhibitory Factor:

Gene knockout studies by Stewart *et al.* (1992) have shown that maternal expression of LIF is essential for blastocyst implantation. Messenger RNA for LIF peaks at the time of implantation in human endometrium (Charnock-Jones *et al.* 1994b) and the human blastocyst contains mRNA for the receptor at this time (Charnock-Jones *et al.* 1994b; Sharkey *et al.* 1995). The presence of the

receptor on uterine luminal epithelium indicates a role for LIF in regulating human embryo implantation (Cullinan *et al.* 1996). The expression of LIF receptor is also seen in the human placenta (Kojima *et al.* 1995), which may suggest that LIF is responsible for growth and differentiation of the trophoblasts during pregnancy. The gp130 receptor subunit of LIF has also been localised to the trophoblasts of the human placenta using immunohistochemistry (Sawai *et al.* 1995b).

Arici and co-workers (1995), used endometrial cell culture to show that LIF is regulated by other cytokines, including IL-1, TNF-α, PDGF, EGF and TGF-β, which induced expression and IFN-γ, which inhibited the LIF expression induced by the other cytokines. They also demonstrated that LIF expression is not directly regulated by steroid hormones. This same group have also shown that LIF acts on human cytotrophoblasts to differentiate to anchoring trophoblasts, thereby increasing their production of fibronectin and decreasing hCG production (Nachtigall *et al.* 1996).

Lavranos *et al.* (1995) cultured 8-cell mouse embryos and found that LIF increase the number of embryos hatching and exhibiting trophoblast outgrowth. Endometrial cells were cultured in the presence or absence of oestradiol and/or progesterone, but expression of LIF was not significantly enhanced.

1.4.4.2 Granulocyte-Macrophage Colony Stimulating Factor:

Chaouat *et al.* (1990) examined the effect of various cytokines, including GM-CSF on fetal survival of abortion-prone (CBA/J x DBA/2) mice. Injections of both IL-3 and GM-CSF improved fetal survival and increased fetal and placental weight. GM-CSF has also been shown to stimulate proliferation of murine placental cells *in vitro* (Armstrong and Chaouat 1989). Robertson and associates (1991) cultured murine embryos of different stages with various concentrations of GM-CSF, showing that GM-CSF-treated embryos progress more quickly through to hatching and attachment than controls. Embryonic development was also assessed by ^{3}H-thymidine incorporation into implanted murine blastocysts, demonstrating that embryos cultured in the presence of GM-CSF incorporated more label than controls.

1.4.4.3 Transforming Growth Factor-β:

A study by Graham *et al.* (1992) localised TGF-β at the human fetal-maternal interface, using immunohistochemistry. They established trophoblast cell cultures and noted an antiproliferative effect of TGF-β, thus suggesting a regulatory role for TGF-β in proliferation and differentiation of the trophoblast. As mentioned in *Section 1.4.3.3*, in the mouse differential expression of the TGF-β isoforms is exhibited in the peri-implantation uterus (Das *et al.* 1992; Tamada *et al.* 1990) and the receptor is expressed by embryos (Roelen *et al.* 1994), suggesting a possible role for TGF-β in the implantation process.

1.4.4.4 Interleukin-6:

As mentioned in *Section 1.4.3.4*, IL-6 secretion from two populations of cultured murine endometrial cells and their modulation by oestrogen, suggests communication between uterine stromal and epithelial cells via IL-6 allowing regulation of the implantation process (Jacobs *et al.* 1992). Receptors for IL-6 (IL-6R and gp130) have been localised to the trophoblasts of the human placenta by immunohistochemistry (Nishino *et al.* 1990; Sawai *et al.* 1995b). Thus IL-6 may play a role in the human at the time of implantation.

1.4.5 *Cytokines play a role in CG expression*

Chorionic gonadotrophin's most widely recognised function is sustaining the life of the corpus luteum of pregnancy. However, it seems to also be involved in implantation, as well as trophoblastic differentiation. Although CG is essential for a viable pregnancy in primates, an understanding of the factors regulating expression is lacking. A number of cytokines have been cultured with human cytotrophoblast cell lines, using CG secretion as an end point, as shown in *Table 1.4*. Secretion of trophoblastic protein (TP), the ruminant analogue of CG has also been investigated.

Table 1.4 Cytokines and growth factors and their receptors play a role in expression of CG, or its analogue, trophoblastic protein (TP)*.

Factor	human	primate	cow	sheep	pig
IL-1α	+[1]				
IL-1β	+[2,3]				
IL-2					
IL-3					
IL-6	+[4]				
IL-10					
IL-11					
CSF-1	+[5]				
GM-CSF	+[5]			+[13]	
EGF	+[6]				
TGF-α	+				
FGF	+[7]				
insulin	+[8]				
LIF	+[9]				
	-[10]				
LIFR					
TGF-β	-[11]				
TNF-α	+[12]				
	NE[2]				

+ = cytokine/GF could enhance CG expression or secretion; - = cytokine/GF could suppress CG expression or secretion; NE = no significant effect; a blank space = no information available as yet.
*Superscript numbers indicate the following references: [1]Masuhiro *et al.* 1991; [2]Silen *et al.* 1989, [3]Yagel *et al.* 1989b, Steelee *et al.* 1992; [4]Nishino *et al.* 1990; [5]Garcia-Lloret *et al.* 1989; [6]Beneviste *et al.* 1978, Ritvos *et al.* 1988, Maruo *et al.* 1987, Morrish *et al.* 1987; [7]Oberbauer *et al.* 1988; [8]Ren and Braunstein 1991; [9]Sawai *et al.* 1995a, 1995b; [10]Nachtigall *et al.* 1996; [11]Matsuzaki *et al.* 1992, Morrish *et al.* 1991; [12]Li *et al.* 1992; [13]Imakawa *et al.* 1993.

A group from Ōsaka, Japan, has shown that human chorionic gonadotrophin released from trophoblasts can be induced by trophoblast-derived IL-1 and TNF-α, using IL-6 and the IL-6-receptor system (Li *et al.* 1992; Masuhiro *et al.* 1991). Trophoblast-derived IL-6 stimulates hCG release, acting through the IL-6 receptor on human trophoblasts (Nishino *et al.* 1990). These authors also discuss

another regulatory network, a GnRH and GnRH-R system, which regulates hCG release by trophoblasts.

Bartocci and associates (1986) found that there is a 1000-fold increase in the level of CSF-1 in the uterus which appeared to be regulated by CG. When CSF-1 is added to cultures of human term cytotrophoblast, differentiation to syncytium and an increase in hCG and hPL occurs (Garcia-Lloret *et al.* 1989).

EGF stimulates hCG secretion in human choriocarcinoma cell lines (Benveniste *et al.* 1978; Ritvos *et al.* 1988). Maruo *et al.* (1987) and Morrish *et al.* (1987) both found that EGF stimulates the release of hPL as well as hCG from normal early placenta and thus, suggested that EGF plays a role in the differentiation of the trophoblast and that this resulted in increased secretion of hCG and hPL from the syncytiotrophoblast (Morrish *et al.* 1987).

Ren and Braunstein (1991) have found that insulin stimulates the production and secretion of hCG from the choriocarcinoma cell lines, JEG-3 and Jar. They also hint that the effect of insulin may be mediated through the IGF-IR.

Oberbauer and associates working with JAR choriocarcinoma cells identified FGF as a potential regulator of hCGβ secretion, using a different pathway to that of EGF (Oberbauer *et al.* 1988). They also suggest that FGF's ability to stimulate hCGβ is independent of its mitogenic properties. FGF appears to be produced by the placenta and works via an autocrine or paracrine pathway to modulate hCGβ synthesis.

Sawai and co-workers (1995a) investigated the effect of LIF on the differentiation of cytotrophoblasts. Differentiation was induced by both LIF and hCG in a dose-dependent manner and blocked by the addition of anti-hCG antibody to the culture system. Thus their results indicate that hCG production is stimulated by LIF enhanced cytotrophoblast differentiation. Another study by the same group, examined the role of LIF at the implantation site (Sawai *et al.* 1995b). Using RT-PCR and immunohistochemistry, they localised gp130, the signal transducer receptor component shared by cytokines such as LIF and IL-6, to trophoblasts. Both LIF and IL-6 were shown to stimulate hCG secretion from trophoblasts and this IL-6 stimulated secretion was blocked by the addition of anti-gp130 and/or anti-IL-6 receptor antibodies. LIF stimulated secretion was significantly inhibited by the anti-gp130 antibody. Thus, they conclude that as CG itself is capable of stimulating trophoblast growth and differentiation (Shi *et al.* 1993; Yagel *et al.* 1989a), the production of both LIF and IL-6 at the fetomaternal interface stimulates trophoblasts to produce CG and this may contribute to the maintenance of placental function. Another potential function of LIF in CG expression has been investigated by Nachtigall and associates (1996). Using a human cytotrophoblast culture system, they found that LIF modulates differentiation of cytotrophoblasts to anchoring trophoblasts, thereby increasing fibronectin secretion and decreasing hCG secretion.

Matsuzaki *et al.* (1992) found that TGF-β produced by the trophoblast and not Gonadotrophin Releasing Hormone (GnRH), induced release of hCG from normal human trophoblasts. Using a TGF-β sensitive cell line, Mv1Lu, they suggest that trophoblasts convert latent TGF-β to active TGF-β at the site of implantation and thus TGF-β acting in a paracrine manner, may modulate hCG and implantation. Morrish *et al.* (1991) also investigated the effect of TGF-β on placental differentiation, finding that TGF-β inhibits the secretion of hCG and hPL and inhibits cytotrophoblast differentiation.

Imakawa and co-workers (1993) cultured day 17 ovine conceptuses with various doses of GM-CSF and found that production of oTP-1 (the analogue of CG in the sheep) mRNA and protein was enhanced by GM-CSF.

1.5 *In Vitro* Culture of Pre-implantation Embryos

A number of detailed reviews have been published on the topic of culturing pre-implantation embryos (Bavister 1995; and references therein). Comprehensive reviews also discuss the metabolism of pre-implantation embryos (Leese 1991). Two of the main aspects of these reviews are 1) the use of endpoints in evaluating embryonic development and 2) whether or not to use serum in the culture medium.

Most studies examine the yield of blastocysts (as a percentage), however if embryos are collected as blastocysts, this is a bit pointless. Other endpoints studied are: 1) timing of embryonic development, 2) hatching and attachment/'implantation' into the plastic, 3) metabolism and output of hormones/growth factors, or 4) the ultimate endpoint, embryo transfer and production of a viable foetus. Of the four, the latter, embryo transfer, has the most constraints, both ethically and monetary.

Culture medium and the use of serum has been discussed at length, especially in mail groups (EmbryoMail) on the internet. Three types of medium are used for culture: 1) 'simple' medium, derived from early mouse culture work, 2) medium based on the composition of oviductal fluid and 3) complex medium. These three types of medium have all been used for culturing embryos of rodents and domestic animals and human IVF, however, the use of any particular type of medium is largely a matter of personal preference. The addition of serum to culture medium may provide many beneficial factors, including growth factors, amino acids, vitamins and energy substrates, however, there are many reports of serum being toxic or having no effect on outcome (Bavister 1995; and references therein), also serum is expensive and can vary quite substantially from batch to batch. Serum albumin can be used instead of serum, however, this still varies dramatically and most serum albumin preparations are crude lyophilised fractions of serum. A study by Caro and Trounson (1986), using protein-free medium for human IVF embryos, found no differences in

embryonic development and pregnancy outcome, compared to medium with serum. However, for handling and transfer of embryos and sperm, a carrier protein of some form is required in the medium. Also, serum provides growth factors, vitamins and amino acids, which are important for embryonic development.

Embryonic development *in vitro* is slower than *in vivo*, which implies maternal factors are involved in regulating the rate of embryonic development. In culture, the absence of maternal signals probably contributes to the slower development and reduced viability of embryos. Also, factors secreted by the embryo could possibly 'prime' the uterine epithelium for implantation of the embryo (Hartshorne and Edwards 1991; Hearn *et al.* 1988a). Chorionic gonadotrophin is the best-known example of an embryo-derived signal.

In vitro studies involving culturing of morula and blastocysts from the rhesus monkey and hatched and intrazonal human and marmoset blastocysts, show that CG is secreted by the embryo before the time it can be detected in the peripheral blood (Dimitriadou *et al.* 1992; Dokras *et al.* 1991; Hay and Lopata 1988; Hearn *et al.* 1991; Lenton *et al.* 1982; Lopata and Hay 1989a; Seshagiri and Hearn 1993). The zona pellucida may be a barrier for the secreted CG by allowing the retention within the perivitelline space, or blastocoelic cavity. Thus, when hatching occurs, just before the time of implantation, CG is released and finds its way into the maternal peripheral blood. Moreover, the levels of CG rise rapidly once hatching and attachment takes place (Hay and Lopata 1988; Lopata and Hay 1989a;

Lopata and Hay 1989b). Alternatively, an efficient fetal-maternal communication channel must be established before the secreted CG can reach the maternal circulation (Lenton *et al.* 1982).

For the human embryo, the levels of CG measured in culture are lower than the amount found in the maternal plasma during gestation (Lopata and Hay 1989b). Human CG secretion actually declines after about day 10 *in vitro*, which may suggest that there is a maternal factor that influences the continuing secretion of CG from the embryo. Or perhaps the daily changing of media depletes an embryonic factor which stimulates CG secretion via an autocrine pathway (Dokras *et al.* 1991).

In the Common Marmoset, mCG levels increase rapidly once attachment has occurred. Within 3-4 days after attachment, each embryo produces up to 240 mIU/day (Hearn 1986).

Secretion of hCG by human blastocysts *in vitro* is stimulated by the presence of serum in the medium (Lopata and Hay 1989a). However, blastocyst formation and hatching occurs in the absence of serum and the secretion of hCG can be delayed for a few days.

Lopata and Oliva (1993) cultured 'abnormal' human blastocysts in serum-free medium, and then added various supplements, to determine the influence on CG secretion. They observed great variability between individual blastocysts. Between Days 7 and 8 after fertilisation, the addition of PDGF induced a marked increase in hCG production. The addition of an insulin, transferrin and selenium mixture (ITS), resulted in a distinct increase in hCG output from Day 7 after fertilisation. A

concentration of 1% human cord serum was found to be the most effective stimulant, resulting in an initial rapid rise in hCG output. The levels either plateaued, or continued to increase, reaching between 1000 and 4000 mIU per 24 hours by Day 14 after fertilisation.

The development of a culture system to investigate the role of various cytokines and growth factors on CG expression and secretion will potentially benefit *in vitro* fertilisation technology. That is, the knowledge of the factors regulating CG expression and secretion may be used to develop new serum-free fully defined medium for the *in vitro* culture of human embryos prior to transfer. These factors may improve CG output from the cultured embryos, improve their viability and thereby increase the rate of successful implantation of transferred embryos.

Due to the ethical constraints governing the use of human embryos, a number of non-human primate species have been utilised to obtain embryos for culture. These species include the baboon, rhesus monkey and the marmoset monkey, which is the species utilised in this thesis.

1.6 Hypothesis and Aims of the Thesis

The female reproductive tract produces various cytokines that affect:

a) the implantation processes and

b) embryo development.

Similar factors are also known to influence chorionic gonadotrophin expression in human tumour cell lines and cultured trophoblast tissue.

Thus the **hypothesis** is that the primate embryo and maternal uterine epithelium also produce cytokines and growth factors that affect CG production and secretion, as well as development and implantation of the conceptus.

Using the common marmoset (*Callithrix jacchus*) as a model, the research **aims** are:

1. Characterise the onset and subsequent expression and secretion of mCG within the primate embryo at various stages of early embryonic development.

2. Develop and characterise assays for the detection and quantitation of mCG expression and secretion in embryonic tissues.

3. Use the mCG assays to determine the effect of various cytokines and growth factors on the gene expression and secretion of mCG, and the development of the pre-implantation embryo.

Chapter Two

Materials and Methods

2.1 Reagents and Solutions

All reagents were of analytical grade and were obtained from the following distributors: Ajax Chemicals (Regency Park, South Australia), Amersham Australia (North Ryde, New South Wales), Amrad-Pharmacia Biotech (Australia), BDH Laboratory Supplies (Poole, England), Beckmann Instruments (Sydney, New South Wales), Becton Dickinson (Franklin Lakes, New Jersey, USA), Bio-Rad Laboratories Pty Ltd. (North Ryde, New South Wales), Boehringer-Mannheim Australia (North Ryde, New South Wales), Bresatec (Thebarton, South Australia), CSL Biosciences (Parkville, Victoria), Delta West Pty. Ltd. (Bentley, Western Australia), Disposable Products (Ingle Farm, South Australia), DNA International Inc. (Lake Oswego, Oregon, USA), DuPont (Wilmington, Delaware, USA), Gibco-BRL Life Technologies Inc. (Gaithersberg, Maryland, USA), Jurox Pty. Ltd. (Silverwater, New South Wales), Merck (Kilsyth, Victoria), Nunc (Roskilde, Denmark), Photographic Wholesalers (Adelaide, South Australia), Pitman-Moore Australia Limited (North Ryde, New South Wales), Polaroid (U.K.) Ltd. (St. Albans, Hertfordshire, England), Professional Disposables Inc. (Orangeburg, New York, USA), Promega Corporation (Madison, Wisconsin, USA), Qiagen Inc. (Chatsworth, California, USA), Sigma Chemical Co. (St Louis, Missouri, USA), Silenus Laboratories (Hawthorn, Victoria), Terumo (Elkton, Maryland, USA), Trace Biosciences Pty. Ltd. (Castle Hill, New South

Wales), Treff Lab (Degersheim, Switzerland), Whatman (Maidstone, England).

All items ending in an asterix appear in the Appendix.

2.2 Animals

2.2.1 *Maintenance*

Breeding pairs of adult Common Marmosets, *Callithrix jacchus*, of proven fertility were housed and maintained as described by Hearn and Lunn (1975). Oestrus cycles and ovulation were detected by measuring levels of plasma progesterone, twice weekly. Females (non-sedated) were placed in a restraint tube between 0800h and 1100h (Hearn 1977) and up to 0.3 ml of blood was removed from the upper region of the femoral vein using a 1 ml syringe with a 27- or 29-gauge needle (Terumo). The blood was centrifuged in a microtainer tube with heparin (Brand plasma separator tube with Lithium Heparin; Becton Dickinson) at 3,700 rpm for 10 minutes. Plasma progesterone was subsequently measured by an Automated Chemiluminescence System (ACS; Ciba-Corning). The remaining plasma was stored at -20°C for ELISA analysis (*see Section 2.7*).

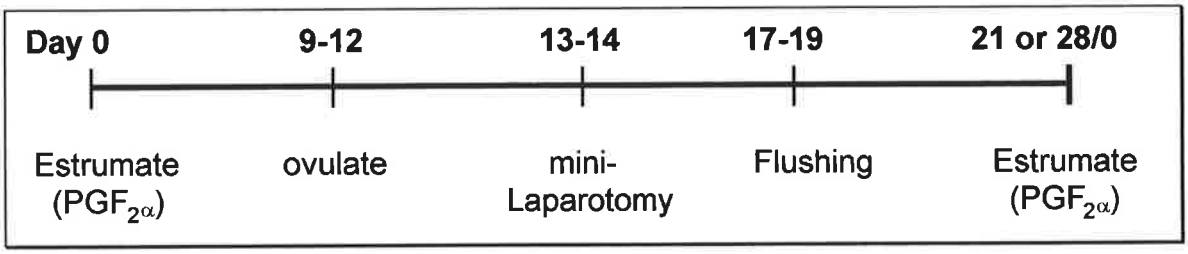
Ovulation (Day 0) represented the day before the rise in plasma progesterone above 10 ng/ml. Pregnancy was monitored by extended high plasma progesterone levels, past Day 40 after ovulation. Once a female was of proven fertility, *that is* the female

could get pregnant, it was assumed she was able to get pregnant every cycle.

Female marmosets were routinely given an injection of 0.5 g Estrumate* (Prostaglandin $F_{2\alpha}$ analogue; Jurox Pty. Ltd.) on Day 21 or 28 of their cycle, to maintain them on a constant 21- or 28-day cycle. Ovulation occurred naturally between 9 and 12 days after Estrumate, or ovulation could be induced by giving an injection of 75 IU of hCG at the end of Day 7 following Estrumate injections, after which ovulation then occurred within 2 days.

Two methods of embryo collection were employed: In Melbourne, mini-laparotomies were performed by Associate Professor Alex Lopata, or alternatively, in Adelaide, a non-surgical flushing technique was used to obtain embryos (*see Figure 2.1*). On Day 21 or 28, Estrumate was re-administered to all the females to recommence the cycle.

Figure 2.1 Diagram showing experimental protocol to synchronise the cycles of the marmoset monkeys.



2.2.2 *Mini-laparotomy Procedure*

Day 13-14 after Estrumate, female marmosets, housed in Melbourne, underwent mini-laparotomies to obtain 8-cell through to morula stage embryos. This procedure has been described previously by Summers *et al.* (1987b). Briefly, the female monkeys were anaesthetised and the ovaries and uterus were exposed through a small excision. Whilst the ovaries and cervix were occluded, the uterine cavity was irrigated with 1 ml of Alpha Modification of Eagles Medium (α-MEM) buffered with HEPES* (Trace Biosciences Pty. Ltd. and Sigma) and the washings collected through a fine cannula inserted into the fundus.

2.2.3 *Flushing Procedure*

After 6-8 days of pregnancy (Days 17-19 after Estrumate), female marmosets in Adelaide underwent a non-surgical procedure for the recovery of uterine stage pre-implantation embryos. This procedure has been described by Thomson, Kalishman and Hearn (1994). Briefly, females were lightly anaethetised by an intramuscular injection of alphaxalone and alphadolone (Saffan*; Pitman-Moore Australia Limited). Their legs and tail were secured by velcro straps and the perineal region cleaned with a solution of Chlorhexidine 0.015%, Cetrimide 0.15% aqueous irrigation (chlorhexidine gluconate 0.15 g/L, cetrimide B.P. 1.5 g/L; Delta West Pty. Ltd.). The vaginal opening was dilated with the insertion and removal of progressively larger lubricated (PDI sterile

lubricating jelly; Professional Disposables Inc.) glass tubes (3 mm to 6 mm diameters). A glass speculum (6 mm outside diameter) was then inserted and the cervix observed with the aid of a light source. A sterile 22-gauge metal stylet was then carefully guided through the cervical os into the uterus. This was removed and replaced by a 19-gauge cannula. A blunted 28-gauge needle was introduced through the cannula into the uterus. A 5 ml plastic syringe containing 5 ml of pre-warmed medium (α-MEM, buffered with HEPES*) was attached to the needle and the uterus was flushed slowly with the medium. The medium was collected in a 5 ml polystyrene tube (Disposable Products) as it returned through the cannula. Recovered ovulation products were observed and classified with an Olympus dissecting microscope. Embryos were usually at the morula through to hatching blastocyst stage of development.

2.2.4 *Ethics*

Experimental procedures involving the marmosets in this thesis had approval from the Queen Elizabeth Hospital (QEH) Animal Ethics Committee and from the University of Adelaide Animal Ethics Committee.

2.3 Culture of Trophoblastic Vesicles and Marmoset Embryos

2.3.1 *Culture of Marmoset Embryos to Trophoblastic Vesicles*

Embryos were cultured in Alpha Modification of Eagles Medium (α-MEM culture medium*) at 37·5°C in a humidified atmosphere of 5% (v/v) CO_2 and 95% (v/v) air. The culture medium was ready made by Trace Biosciences Pty. Ltd. with Earles salts and sodium bicarbonate and without L-glutamine and nucleosides. The medium was supplemented with L-glutamine (Gibco-BRL Life Technologies), 5,000 U/ml penicillin G and 5,000 µg/ml streptomycin sulphate (combined - penstrep*; CSL Biosciences), 10% heat inactivated fetal calf serum (FCS; Trace Biosciences Pty. Ltd.), 25 µg/ml transferrin* (Sigma) and 25 µg/ml insulin* (Sigma). The embryos were cultured in 1 ml of medium in 4-well plates (Nunc), allowed to hatch and attach to the plastic. The medium was changed and collected at three-day intervals. The collected medium was stored at -20°C until analysed by ELISA. Once attachment took place, a trophoblast monolayer formed and outgrowth of the trophoblast occurred (vesicle formation). Vesicles were allowed to grow and expand, changing the medium at three-day intervals. The conditioned medium was stored at -20°C for analysis by ELISA (*see Chapter 5*). During their period of growth,

vesicles can become detached from the plastic and float in the culture medium.

Once the vesicles attained a size of more than 3 mm in diameter, they were micro-dissected. Micro-dissection involved detaching the vesicle, if it was attached to the plastic and transferring to α-MEM, buffered with HEPES* to wash. The vesicles were then cut into pieces using a 27- or 29-gauge insulin syringe (Terumo), to hold the vesicle and cutting with a long beveled 23-gauge needle (Terumo) attached to a 1 ml syringe (Terumo). The fragments were then transferred to α-MEM culture medium and allowed to culture. Over the following weeks, the fragments attached to the plastic and/or reformed vesicles and grew. This is a primary vesicle culture. Once the primary vesicles were large enough to micro-dissect (>3 mm), secondary cultures were established.

Trophoblastic vesicle fluid (fluid from within the vesicle) was obtained by micropunching the vesicle with a 29 gauge needle (Terumo) attached to a 50 µl glass re-usable syringe (SGE syringe perfection, supplied by Adelab). The fluid was stored at -20°C for analysis by ELISA (*see Chapter 5*).

2.3.2 *Marmoset Trophoblastic Vesicle Fragment and Intact Trophoblastic Vesicle Culture*

Marmoset trophoblastic vesicle fragments from a secondary culture were cultured with selected cytokines for a period of six

days. At the time of micro-dissection, the vesicle fragments were cultured overnight in serum-free α-MEM medium* (Day 0), before being transferred to medium containing one of the cytokines (*discussed in Chapter Six*). The medium was changed, collected and stored at -20°C every day (Days 1-6), adding fresh cytokine into the culture system every 24 hours. At the end of the culture period, the medium was collected and stored at -20°C until analysed by ELISA and the vesicle fragments lysed for RNA extraction and subsequent RT-PCR analysis.

Intact marmoset trophoblastic vesicles were also cultured with selected cytokines for a period of 11 days. Vesicles of similar size were transferred to serum-free α-MEM medium* to culture overnight (Day 0), before being transferred to medium containing one of the cytokines (*discussed in Chapter Six*). The medium was changed, collected and stored at -20°C every 24 hours for the first three days (Days 1, 2 and 3) and then changed, collected and stored at -20°C every 2 days (Days 5, 7 and 9). At Day 11, the medium was collected and stored at -20°C until analysed by ELISA and the vesicles lysed for RNA extraction and subsequent RT-PCR analysis.

2.3.4 *Marmoset Embryo Culture*

Embryos of various stages were collected by the non-surgical flushing technique (*as described in Section 2.2.3*). Morulae were allowed to develop to the blastocyst stage in culture medium with serum. Blastocyst stage embryos were transferred to 1 ml serum-

free α-MEM culture medium, overnight and then to serum-free α-MEM containing cytokines. The embryos were observed every day and the medium collected and changed every two days. Two days after attachment, the embryos were lysed for RNA extraction and subsequent RT-PCR analysis and the medium collected and stored at -20°C for ELISA analysis (*see Chapter 6*). If the embryo compacted or did not hatch or develop, it was still lysed for RNA extraction and subsequent RT-PCR analysis. The medium was collected and stored at -20°C for ELISA analysis (*see Chapter 6*).

2.4 RNA Extraction

Total RNA was extracted from tissues and cells by the method of Chomczynski and Sacchi (1987). All tissues and embryos were either snap frozen with liquid nitrogen and stored at -80°C, or immediately lysed with Solution D and stored until required at -20°C.

2.4.1 *Tissue*

Tissue (up to 50 mg) was pulverised in liquid nitrogen using a mortar and pestle and transferred to an RNase-free 1.5 ml centrifuge tube (Treff Lab), with the addition of 500 µl of solution D* to the mortar. Then sequentially, 50 µl of 2 M sodium acetate* (Sigma), pH 4, 500 µl of water-saturated phenol* and 100 µl of chloroform-isoamyl alcohol mixture*, were added to the mixture

with vortexing after the addition of each reagent. The samples were cooled on ice for 20 minutes and then centrifuged at 13,000 rpm for 15 minutes. The aqueous phase, containing the RNA, was transferred to a fresh tube and mixed with 500 µl of isopropanol (BDH) and then placed at -20°C or -80°C overnight, to precipitate the RNA.

The RNA was pelleted by centrifugation for 20 minutes. The supernatant was discarded and the pellet dissolved in 200 µl of solution D, followed by a re-precipitation with 200 µl of isopropanol. The pelleted RNA was washed in 75% ethanol (BDH) twice and finally dissolved in diethyl pyrocarbonate (DEPC)-treated water*.

RNA was quantitated by recording the optical density (OD) at a wavelength of 260 nm. The quality of the RNA was determined by running a 1 µl sample with RNA loading buffer* on a 1% RNase-free agarose/TAE* horizontal slab gel stained with ethidium-bromide* (Boehringer-Mannheim). RNase-free gels were run in a small gel tank (gel electrophoresis apparatus GNA-100, Pharmacia) connected to a LKB-GPS 200/400 power pack (Pharmacia). Visualisation was made with an ultra-violet (254 nm) light source. Photographs were taken with Polaroid film 667.

A similar protocol was followed for the isolation of RNA from cultured adherent cell lines. Briefly, cells (5-8 x 10^6 cells) were washed twice with DEPC-treated phosphate buffered saline (PBS*) and then lysed with the addition of 2 x 250 µl of solution D, *in situ*. The remaining steps are as described above.

2.4.2 *Embryos, Trophoblastic Vesicle Fragments and Trophoblastic Vesicles*

RNA extraction from embryos, trophoblastic vesicle fragments and trophoblastic vesicles required the addition of 100 μg of carrier baker's yeast tRNA* (Boehringer Mannheim), with the addition of 500 μl of solution D to lyse the cells. The remaining steps were as described in *Section 2.4.1.*

2.5 Reverse Transcriptase -Polymerase Chain Reaction (RT-PCR) Protocols

2.5.1 *Reverse Transcription*

Two different reverse transcriptase (RT) enzymes were used for first strand complementary DNA (cDNA) synthesis: 1) Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT: Promega Corporation) and 2) Superscript™II RNaseH- Reverse Transcriptase (Superscript™II: Gibco-BRL Life Technologies).

2.5.1.1 AMV-RT:

Complementary DNA was synthesised from the total RNA from Chinese hamster ovary (CHO) cells expressing mCG, marmoset placenta, skeletal muscle and pituitary. A master mix of 5x first strand buffer, together with 500 µg/ml oligo (dT)15 primer* (Boehringer-Mannheim), 10 mM dNTP (Promega Corporation), components required by the enzyme and 400 units of AMV-RT in a final volume of 18 µl. Two microlitres of total RNA was added last to the reaction tubes, which were incubated at 42°C for 50 minutes and then heated to 95°C for 5 minutes using a thermal cycler (MJ Research, Bresatec Limited). After cooling on ice, 2 µl of cDNA was added to a 23 µl PCR reaction.

Component	**Volume (in µl)**	**Final Concentration**
5x RT buffer†	4.0	1x RT buffer
dNTP mix* (10 mM)	2.0	1 mM
oligo $(dT)_{15}$ primer* (500 µg/ml)	1.0	25 µg
RNasin (36 U/ml)	0.5	0.75 U
DTT (100 mM)	2.0	10 mM
Acetylated BSA (1 mg/ml)	2.0	100 µg
AMV-RT (200 U/µl)	2.0	20 U
water§	4.5	
RNA§	2.0	
total volume	20.0 µl	

†5x RT buffer (250 mM Tris-HCl, pH 8.3 (42°C), 250 mM KCl, 50 mM $MgCl_2$, 50 mM DTT, 2.5 mM spermidine) supplied with the enzyme.
§The volume of RNA can be increased, this is compensated for by reducing the amount of DEPC-treated water.

RNasin (recombinant RNasin ribonuclease inhibitor; Promega Corporation) has broad spectrum RNase inhibitory properties,

including the inhibition of common eukaryotic RNases. DTT (dithiothreitol; Promega Corporation) is an antioxidant used to stabilise enzymes and other proteins containing sulphydryl groups, it is also required for RNasin activity. Acetylated BSA (bovine serum albumin; Promega Corporation) is also used as an enzyme stabiliser.

2.5.1.2 Superscript™II:

Complementary DNA was synthesised from the total RNA from single embryos or trophoblastic vesicles or trophoblastic vesicle fragments. Five or ten microlitres of total RNA and 500 μg/ml oligo $(dT)_{15}$ primer* (Boehringer-Mannheim) were hybridised at 70°C for 10 minutes and quickly chilled to 4°C using a thermal cycler (MJ Research). The 5x first strand buffer, together with 100 mM DTT (supplied with the Superscript™II), 10 mM dNTP mix* (Promega Corporation) and 200 units of Superscript™II were added to a final volume of 20 μl. The reaction was incubated at 42°C for 52 or 92 minutes, heated to 70°C for 15 minutes to inactivate the enzyme and then quickly chilled to 4°C.

Component	Volume (in µl)	Final Concentration
RNA	5.0 or 10.0	
oligo dT primer* (500 µg/ml)	1.0	25 µg
water	6.0 or 1.0	
5x RT buffer†	4.0	1x RT buffer
DTT (100 mM)	2.0	10 mM
dNTP mix* (10 mM)	1.0	500 µM
Superscript™II	1.0	200 U
total volume	20.0 µl	

†5x RT buffer (250 mM Tris-HCl, pH 8.3 (25°C), 375 mM KCl, 15 mM $MgCl_2$) supplied with the enzyme.

2.5.2 *Polymerase Chain Reaction*

Three different DNA polymerase enzymes were used for the polymerase chain reaction (PCR): 1) *Pwo* DNA Polymerase, 2) *Taq* DNA Polymerase and 3) Expand™ High Fidelity PCR System.

2.5.2.1 *Pwo* DNA Polymerase:

Pwo DNA polymerase (from *Pyrococcus woesei*; Boehringer-Mannheim) is a highly processive 5'-3' DNA polymerase with proof-reading activity, thus this enzyme was used if fidelity/specificity of the sequence was important. A master mix was prepared containing buffer, dNTPs, primers and enzyme, as set out below:

Component	Volume (in µl)	Final Concentration
10x PCR buffer† (supplied with $MgSO_4$)	2.5	1x with 2.0 mM $MgSO_4$
dNTP mix* (10 mM)	0.5	200-250 µM
forward primer‡	1.0	15-70 pmoles
reverse primer§	1.0	15-70 pmoles
Pwo	0.125	2.5 U
DEPC-treated water	17.875	
template/cDNA	2.0	0.1 - 1 µg
total volume	25 µl	

†10x PCR buffer (100 mM Tris-HCl, pH 8.85 (20°C), 250 mM KCl, 50 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$).
‡forward primer - *described in Chapters 3 and 4*
§reverse primer - *described in Chapters 3 and 4*

The mixture was overlaid with sterile mineral oil (Sigma), the cDNA was added and then the reaction was amplified with a thermal cycler (MJ Research) with the following parameters:

LOUPWO35 or 40

Number of cycles	Step	Temperature	Time
1	denaturation	94°C	5 minutes
35 or 40	denaturation	94°C	30 seconds
	annealing	60°C	30 seconds
	elongation	72°C	1 minute 30 seconds
1	final extension	72°C	5 minutes

2.5.2.2 *Taq* DNA Polymerase:

Taq DNA polymerase (from *Thermus aquaticus* BM; Boehringer-Mannheim) is highly processive but lacks the proof-reading activity. An amplification master mix was prepared containing buffer, dNTPs, primers and enzyme, as set out below:

Component	**Volume (in µl)**	**Final Concentration**
10x PCR buffer† (supplied with $MgCl_2$)	2.5	1x with 1.5 mM $MgCl_2$
dNTP mix* (10 mM)	0.5	200-250 µM
forward primer‡	1.0	15-70 pmoles
reverse primer§	1.0	15-70 pmoles
Taq	0.125	2.5 U
DEPC-treated water¶	17.875	
template/cDNA¶	2.0	0.1 - 1 µg
total volume	25 µl	

†10x PCR buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3 (20°C), 15 mM $MgCl_2$).
‡forward primer - *described in Chapters 3 and 4*
§reverse primer - *described in Chapters 3 and 4*
¶The volume of cDNA can be increased, this is compensated for by reducing the amount of DEPC-treated water.

The mixture was overlaid by mineral oil, the cDNA added and the reaction amplified using a thermal cycler (MJ Research) with the following parameters:

LOUTAQ30 or 35

Number of cycles	Step	Temperature	Time
1	denaturation	94°C	5 minutes
30 or 35	denaturation	94°C	30 seconds
	annealing	55°C	30 seconds
	elongation	72°C	1 minute
1	final extension	72°C	5 minutes

2.5.2.3 Expand™ High Fidelity PCR System:

Expand™ High Fidelity PCR System (thermostable DNA polymerase mixture; Boehringer-Mannheim) is a mixture of the highly processive *Taq* DNA polymerase and the proof-reading *Pwo* DNA polymerase. Two master mixes are prepared, one with dNTPs, primers and template and the second with buffer and enzyme, as set out below.

The preparation of two master mixes avoids the enzyme mix interacting with the primers or template without dNTPs, thus preventing the partial degradation of the primer and template through the 3'-5' exonuclease activity of *Pwo*. Master Mix 1 minus the template was prepared, aliquotted out into the tubes, which were then overlaid with mineral oil before the addition of the template. Master Mix 2 was prepared and fifty microlitres was pipetted into the tubes. The tubes were mixed well and then amplified with a thermal cycler (MJ Research). The amplification parameters were as listed below (*EXHF*).

Component	Volume (in µl)	Final Concentration
master mix 1:		
dNTP mix* (10 mM)	2.0	200 µM
forward primer‡	1.0	15-30 pmoles
reverse primer§	1.0	15-30 pmoles
DEPC-treated water	44.0	
template/cDNA	2.0	
total volume (MM1)	50.0 µl	
master mix 2:		
10x Expand™ buffer	10.0	1x with 15 mM $MgCl_2$
Expand™ enzyme mix	0.75	2.6 U
DEPC-treated water	39.25	
total volume (MM2)	50.0 µl	
total volume (MM1 + MM2)	100.0 µl	

‡forward primer - *described in Chapters 3 and 4*
§reverse primer - *described in Chapters 3 and 4*

EXHF

Number of cycles	Step	Temperature	Time
1	denaturation	94°C	5 minutes
10	denaturation	94°C	15 seconds
	annealing	60°C	30 seconds
	elongation	72°C	45 seconds
30	denaturation	94°C	15 seconds
	annealing	60°C	30 seconds
	elongation	72°C	65 seconds + 20 seconds/cycle
1	final extension	72°C	11 minutes

2.5.3 *Analysing PCR Products*

2.5.3.1 Gel Electrophoresis:

Aliquots (10-15 µl) of the PCR reaction containing 3 µl of 6x DNA loading buffer* were subjected to electrophoresis in a 2% agarose (Promega Corporation) 1 x TAE* horizontal slab gel, containing ethidium bromide* (10 ng/ml; Boehringer-Mannheim). Gels were electrophoresed in a Jordan Scientific gel tank connected to an electrophoresis power supply (EPS 500/400; supplied by Pharmacia). Visualisation was made with an ultra-violet (254 nm) light source. Photographs were taken using Polaroid 667 film.

Two types of molecular weight markers were electrophoresed with the samples: 1 KB DNA ladder and DNA Mass ladder (both supplied by Gibco-BRL Life Technologies).

2.5.3.2 Purifying DNA from Agarose:

Two kits from two different companies were used to purify DNA from 2% agarose gel: Bresa-Clean™ DNA Purification Kit (Bresatec) and QIAquick Gel Extraction Kit (QIAgen). The Bresatec kit was used when required for the majority of the experiments in this thesis; the QIAquick kit was only used when the PCR product was to undergo restriction enzyme digestions (*see Section 2.5.3.3*).

Bresa-Clean™ DNA Purification Kit:

The relevant bands were excised and transferred to an eppendorf tube. The volume of DNA-containing agarose gel was

determined, assuming a mass of 1 mg to be equivalent to a volume of 1 µl. Three volumes of BRESA-SALT™ solution was added and the agarose melted by heating the tubes at 55°C. After vortexing, 5 µl of BRESA-BIND™ (glass powder) was added and the tubes vortexed and left on ice for 15 minutes. The tubes were centrifuged for 15 seconds and the supernatant discarded. The DNA-containing pellet was washed twice by resuspension in 500 µl of BRESA-WASH™. After the second wash, DNA was recovered from the glass powder with the addition of 20-50 µl of 1 x TE*, by heating the resuspended glass powder for 5 minutes at 55°C followed by centrifugation for 30 seconds, before removing the resuspended DNA to a fresh tube.

QIAquick Gel Extraction Kit:

The relevant bands were excised from the agarose gel and transferred to an eppendorf tube. The volume of DNA was determined by again assuming a mass of 1 mg to be equivalent to a volume of 1 µl. Three volumes of Buffer QG was added and the tubes incubated at 50°C for 10 minutes, or until the agarose had completely dissolved. One gel volume of isopropanol was added to the sample and mixed. The sample was then applied to a QIAquick column that was placed in a 2 ml collection tube (both supplied with the kit). The column was centrifuged for 1 minute to bind the DNA. The flow-through was discarded and 500 µl of Buffer QG was added to the column and centrifuged for 1 minute, to remove all

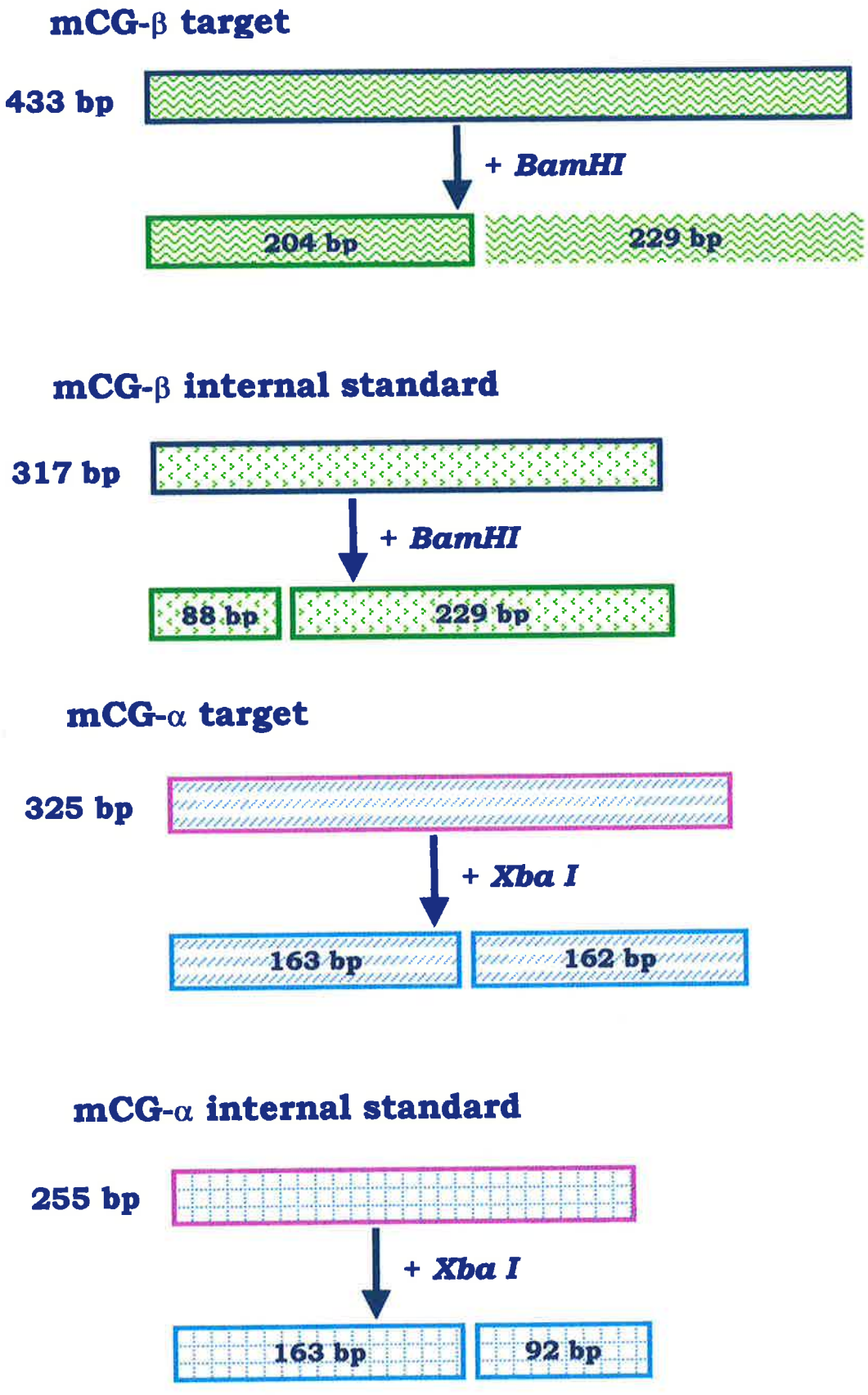
traces of the agarose. The flow-through was discarded. The DNA was washed by adding 750 µl of Buffer PE to the column and allowing the column to stand for 2-5 minutes. The column was then centrifuged for 1 minute, the flow-through discarded and the column centrifuged for a further minute, to remove any residual ethanol from Buffer PE. The column was then placed in a fresh 1.5 ml eppendorf tube and the DNA eluted from the column by adding 50 µl of Buffer EB to the centre of the column and centrifuging for 1 minute.

2.5.3.3 Restriction Enzyme Digestions:

Purified PCR products were also analysed for appropriately sized cDNA fragments by restriction enzyme cleavage. Digestions were performed with 0.5 - 1 µg of purified PCR product containing the appropriate restriction enzyme(s) (2 - 10 units of enzyme per µg of DNA at 37°C, for 2 hours) in the enzyme buffer supplied by the manufacturer. The restricted products were then analysed by electrophoresis (*as described in Section 2.5.3.1*).

Analytical digests of mCG-β fragments was performed using *BamHI* (Promega Corporation), which resulted in the 433 bp target fragment being cut into two fragments of 229 and 204 bp, whilst the 317 IS fragment was cut into 2 fragments of 229 and 88 bp (*see Figure 2.2*). Restriction enzyme digests of mCG-α with *Xba I* (Promega Corporation) resulted in the 325 bp target fragment being cut into 2 fragments of 163 and 162 bp, whilst the 255 bp IS

Figure 2.3 Diagram showing the result of enzyme digesting mCG-β target and internal standard with *BamHI* and mCG-α target and internal standard with *Xba I*.



fragment was cut into 2 fragments of 163 and 92 bp (*see Figure 2.2*).

2.5.3.4 Sequencing:

Purified PCR products and purified clones were either sequenced by an ABI model 373A automatic sequencer (Applied Biosystems) by the Haematology Department at Flinder's Medical Centre, Bedford Park, or manually by cycle sequencing. The sequence was read by the software supplied with the sequencer (version 2.0.1S) or manually.

Cycle Sequencing:

Cycle sequencing was performed using the AmpliCycle™ Sequencing kit (supplied by Perkin-Elmer), with α-^{33}P-dATP (Bresatec). For each sample to be sequenced, a 30 µl Reaction Mix was prepared, containing 10x cycling mix, primer, α-^{33}P-dATP and template.

Component	**Volume (in µl)**
water	23.0
primer (20 µM)	1.0
α-^{33}P-dATP (10 µCi/µl)	1.0
10x cycling mix	4.0
template (100 fmol)	1.0
total volume	30.0 µl

Six microlitres of the Reaction Mix was dispensed into 4 tubes containing 2 µl of each of the Termination Mixes (G, A, T, C; supplied with the kit) which had been dispensed and stored on ice. Each Termination Reaction mix was overlaid with 20 µl of mineral oil (Sigma) and placed in a preheated to 95°C thermal cycler (Corbett). Cycle sequencing proceeded according to the parameters below.

CycleSeq

Number of cycles	Step	Temperature	Time
1	denaturation	95°C	2 minutes
25	denaturation	95°C	1 minute
	annealing	68°C	1 minute
	elongation	72°C	1 minute
1		4°C	<45 minutes

After cycling, 50 µl of chloroform (BDH) was added to each tube, to allow the aqueous phase to float to the top. The aqueous phase was transferred to a fresh tube containing 4 µl of Stop Solution (provided with the kit) and the samples stored at -20°C or analysed immediately. The samples were denatured by incubating at 95°C for 3 minutes before being loaded into separate adjacent wells of the sequencing gel, which is described below.

Sequencing Gels:

Sequencing reactions were resolved on 8% vertical polyacrylamide, 1x TBE* gels containing 7 M urea. The gel was

moulded between two glass plates separated by spacers and held together with tape (this is described in more detail in the AmpliCycle Sequencing kit booklet). A sequencing gel mix* was prepared, containing acrylamide:bis (37.5:1 ratio; BioRad) and urea (BDH), which was stored at 4°C. To prepare the gel, 70-75 mls of the sequencing gel mix was mixed well with 450 µl of 10% ammonium persulphate (APS; Merck) and 75 µl of TEMED (Sigma). The acrylamide solution was poured between the sequencing plates which were held at a 45° angle to the horizontal. The flat edge of the Sharkstooth combs were slid 0.5 cm into the gel, 'bulldog' clips secured around the comb and the gel allowed to polymerise for 1 to 2 hours. After polymerisation of the gel, the bulldog clips, tape, combs and spacers were removed and the gel mould attached to the electrophoresis apparatus. The two buffer reservoirs were filled with 1x TBE buffer and the top of the gel rinsed with buffer using a 1 ml pipettor. The Sharkstooth combs were gently pressed down into the gel so that the teeth barely penetrate the gel and form wells between the teeth. The sample was prepared as described above and loaded into the gel using 'duck-bill' pipette tips. Electrophoresis was carried out at 55 watts for 2-3 hours, after which time the gel mould was removed from the electrophoresis apparatus. The gel plates were separated, leaving the gel on the longer plate, which was soaked in a solution of 10% acetic acid and 10% methanol, to remove the urea. The gel was then transferred to blotting paper (Whatman 2 mm Chromatography Paper) and dried

under vacuum at 80°C. The dried gel was exposed to x-ray film (DuPont Cronex Video Imaging Film, DuPont), overnight and the sequence read manually over a light box.

2.5.4 *Image Analysis*

Image analysis was performed using NIH Image, image analysis system (National Institute of Health, USA), combined with a Hewlett Packard ScanJet IIc desktop scanner.

2.5.5 *In vitro Transcription*

Two kits, both supplied by Bresatec were used for *in vitro* transcription, that is to make DNA into RNA: Bresatec Message Maker Kit (MMK-1) and Ambion MAXIscript™ kit. The Bresatec MMK-1 kit was initially used, however, Bresatec suggested to use the Ambion MAXIscript™ kit for higher yields of RNA. Both kits *in vitro* transcribe purified PCR product into rcRNA using T7 RNA polymerase.

2.5.5.1 Bresatec Message Maker Kit:

The reaction was prepared in at least duplicate, as set out below:

Component	Volume (in µl)
template§ (1.0 µg)	*20.0*
10x Nucleotide/Buffer Cocktail (Tube1)	4.0
DTT (100mM; 2)	4.0
RNase Inhibitor (7)	2.0
UTP (5mM; 3)	4.0
T7 RNA polymerase (5)	2.0
DEPC-treated water§ (8)	*2.0*
total volume	40.0 µl

§The volumes of *template* and *water* are dependent on the amount of cDNA present in the sample.

The *in vitro* transcription reaction was mixed gently and incubated at 37°C for 1 hour. Two microlitres of RNase-free DNAse I (tube 6) was added to remove the cDNA template and the reaction mixed and incubated a further 10 minutes. The reaction was diluted with DEPC-treated water to 100 µl (51 µl), 100 µl of phenol:chloroform* was added and the tubes vortexed and centrifuged for 10 minutes. The aqueous layer was transferred to a fresh tube and 100 µl of chloroform was added. The tubes were vortexed and re-centrifuged for 10 minutes. The aqueous layer transferred to a fresh tube, acidified with the addition of 20 µl of 3 M sodium acetate* and then ethanol precipitated by adding 2.5 volumes of 97% ethanol and placing at -20°C overnight. The RNA was pelleted by centrifuging for 15 minutes and resuspended in 20 µl of DEPC-treated water. The amount of RNA was calculated by an OD reading on a spectrophotometer (Department of Rheumatology, QEH).

2.5.5.2 Ambion MAXIscript™:

The reaction was prepared in at least duplicate, as set out below:

Component	Volume (in µl)
template (2.5 µg)	*x*
DTT (200 mM)	2.5
ATP	2.5
CTP	2.5
GTP	2.5
UTP	2.5
10x buffer	5.0
RNase Inhibitor	2.5
T7 RNA polymerase	2.5
DEPC-treated water	*y*
total volume	50 µl

The volumes of *x* & y are dependent on the amount of cDNA present in the sample.

The reaction was mixed gently and incubated for 1 hour at 37°C. To remove the cDNA template, 2 µl of RNase-free DNase I (supplied with the kit) was added and the reaction incubated a further 10 minutes at 37°C.

The reaction was then diluted with DEPC-treated water to a final volume of 100 µl and an equal volume of phenol:chloroform was added. After vortexing, the tubes were centrifuged for 10 minutes, the aqueous layer was transferred to a fresh tube and 100 µl of chloroform was added. The tubes were re-centrifuged for 10 minutes, the aqueous layer again transferred to a fresh tube and 20 µl of 3 M sodium acetate and 300 µl of 97% ethanol was added.

After an overnight precipitation at -20°C, the tubes were centrifuged for 15 minutes. The supernatant was removed and the pellet allowed to dry before being re-suspended in 20 µl of DEPC-treated water. The amount of RNA present was calculated by an OD reading on a spectrophotometer (Department of Rheumatology, TQEH).

2.6 Cloning of DNA Fragments

2.6.1 *Preparation of Plasmid DNA*

In a total volume of 20 µl, 10 µl of purified Bluescript SK+ vector, 2 µl of *EcoR I* restriction enzyme and 2 µl of 10x buffer recommended for *EcoR I* (Promega Corporation) were incubated for 2 hours at 37°C. The sample was electrophoresed on an agarose gel and stained with ethidium bromide (*as described in Section 2.5.3.1*). After visualisation with a UV light source, the restricted vector was excised from the agarose and purified using the Bresa-Clean™ kit (Bresatec; *as described in Section 2.5.3.2*). The restricted vector was eluted from the BRESA-BIND™ with 20 µl (2 x 10 µl) of TE.

2.6.2 *Preparation of DNA Insert*

2.6.2.1 Purification of DNA Insert:

The insert PCR product was electrophoresed on a 2% agarose gel, stained with ethidium bromide (*as described in Section 2.5.3.1*). The expected band was excised and purified using Bresa-Clean™ (*as described in Section 2.5.3.2*), the DNA being eluted from the BRESA-BIND™ by 40 µl (2 x 20 µl) of DEPC-treated water.

2.6.2.2 Digestion of DNA Insert:

The purified insert (40 µl) was incubated with *EcoR I* (3 µl) and 5 µl of 10x buffer at 37°C overnight. Two microlitres of the cut insert was electrophoresed alongside a 2 µl sample of the uncut insert (*as described in Section 2.5.3.1*), to check that the insert was restricted.

The remainder of the cut insert was electrophoresed (*as described in Section 2.5.3.1*), excised and purified using Bresa-Clean™ (*as described in Section 2.5.3.2*), eluting off the BRESA-BIND™ with 20 µl (2 x 10 µl) of TE.

2.6.3 *Ligation of DNA Fragment into Plasmid DNA*

The restricted Bluescript vector (1 µl) and 5 µl of purified insert were incubated at room temperature over the weekend in ligase buffer (2 µl) with one Weiss unit of T4 DNA ligase enzyme (2 µl; Promega Corporation) in a total volume of 10 µl. A negative

control was also set up with DEPC-treated water instead of the insert.

2.6.4 *Transformation of* E.coli *JM109 cells*

The *E.coli* JM109 cells were incubated at 37°C in 10 ml of 2x TY broth* under constant shaking overnight. One millilitre of the overnight culture was added to 20 ml of fresh 2x TY broth and the cells incubated under constant shaking a further 2-3 hours to make competent cells.

The log-phase cells were then pelleted (5,000 rpm, 10 minutes at RT) and re-suspended in 20 ml of 80 mM calcium chloride ($CaCl_2$*) and incubated on ice for 0.5-1 hour. The cells were then re-pelleted and re-suspended in fresh 80 mM $CaCl_2$, ready for transfection with plasmid DNA or ligated DNA.

One hundred microlitres of the competent cells were mixed with the ligations (*described in Section 2.7.1.1*). The cells were incubated on ice for 1 hour and then "heat-shocked" by placing them at 37°C for 5 minutes before incubating on ice.

Seven hundred microlitres of L-broth* was added to each tube, containing the competent cells and ligations. The tubes were then incubated at 37°C for 1 hour, under constant shaking. The cells were pelleted, 500 µl of the supernatant was removed and the cells re-suspended in the remainder (~300 µl). This resuspension was plated out onto LB agar plates containing 0.5 mM IPTG* and 80 µg/ml X-gal* and incubated at 37°C overnight.

2.6.5 *Selection of Transformants*

Standard colour selection allowed white (recombinant) colonies to be differentiated from colonies containing the parent vector (blue). The white colonies were picked from the plate, using sterile toothpicks and streaked over an LB agar plate containing 50 μg/ml ampicillin, 0.5 mM IPTG and 80 μg/ml X-gal. The plate was incubated overnight at 37°C.

2.6.6 *Screening for Positive Clones*

Overnight cultures were set-up by inoculating 3 ml of T-broth* containing 50 μg/ml ampicillin with white colonies picked from the plate with sterile toothpicks. The cultures were grown overnight to saturation with constant shaking at 37°C. The plasmid was purified using the QIAprep-spin Plasmid Miniprep kit.

2.6.6.1 QIAprep-spin Plasmid Miniprep:

The 3 ml culture was divided into two 1.5 ml eppendorf tubes and centrifuged for 60 seconds to pellet the bacteria. The supernatant was discarded and one of the pellets was re-suspended in 250 μl of buffer P1. This re-suspension was transferred to the second tube and the pellet was re-suspended, before adding 250 μl of buffer P2 to lyse the bacteria and release the plasmid. The lysate was mixed by inversion and then incubated at room temperature for 5 minutes. To neutralise the lysate, 350 μl of chilled buffer N3 was added, the tube mixed by inversion and incubated on ice for 5

minutes. The precipitated debris was removed by centrifugation for 10 minutes, leaving a clear lysate to load onto the QIAprep-spin column. The lysate was applied to the column, which was in an eppendorf tube to collect the flowthrough. The column was centrifuged for 30-60 seconds and the flowthrough discarded. The column was washed with 500 µl of buffer PB, centrifuged and the flowthrough discarded. The column was then washed with 700 µl of buffer PE, centrifuged and the flowthrough discarded. To remove any residual wash buffer, the column was re-centrifuged and flowthrough discarded. The column was placed in a fresh eppendorf tube and the DNA eluted from the column by adding 50 µl of TE. The purified DNA was collected by centrifuging the column.

2.6.6.2 Restriction Endonuclease Analysis:

A small sample of purified plasmid (10 µl) was incubated with *EcoR I* (1.7 µl) and 10x buffer (1.3 µl) for 2 hours at 37°C. The restricted plasmid was electrophoresed (*as described in Section 2.5.3.1*).

2.6.7 *Large Scale Plasmid Preparations*

For the large-scale isolation of DNA of the desired plasmid, a large overnight culture was set up using 150 ml of T-broth with 50 µg/ml of ampicillin. The T-broth was inoculated with bacteria from the appropriate colony by picking with sterile toothpicks. The

culture was incubated overnight at 37°C with constant shaking. The plasmid was purified by using the QIAgen Plasmid Midiprep.

2.6.7.1 QIAgen Plasmid Midiprep:

The 150 ml suspension was divided into three 50 ml Falcon tubes (Becton-Dickinson) and centrifuged to harvest the bacterial cells. All traces of supernatant was removed by inverting the tubes. The pellet was re-suspended in 4 ml of buffer P1. The cells were lysed by the addition of 4 ml of buffer P2, mixed by inversion and incubated at room temperature for 5 minutes. The suspension was transferred to a Nalgene tube and 4 ml of chilled buffer P3 was added. The cells were mixed by inversion and incubated on ice for 15 minutes. The cellular debris was separated from the plasmid by centrifuging at 30,000xg for 30 minutes at 4°C. The clear lysate was transferred to a fresh tube. Whilst the lysate was centrifuging, the QIAgen-tip was equilibrated with 4 ml of buffer QBT, allowing the column to empty by gravity flow into a falcon tube. The clear lysate was applied to the column and the plasmid allowed to enter the resin by gravity flow. The column was washed twice with 10 ml of buffer QC and the DNA eluted from the tip into a Nalgene tube with 5 ml of buffer QF. The DNA was precipitated with 0.7 volumes of isopropanol and centrifuged at 15,000 xg for 30 minutes at 4°C. The supernatant was discarded and the tube inverted for 30 minutes to allow the pellet to dry. The pellet was re-dissolved in 200 µl of TE. The amount of DNA was quantitated by OD reading using a spectrophotometer (Department of Rheumatology, QEH).

For the preparation of stocks for frozen storage, a 10 ml culture of L-broth with ampicillin was inoculated with a colony from the struck plate with a sterile toothpick. The suspension was grown overnight at 37°C with constant shaking. The bacterial cells were pelleted by centrifugation and resuspended in 1 ml L-broth and 1 ml glycerol (ICN). After thorough mixing, the mix was transferred to cryovials and frozen at -80°C together with a sample of the template.

2.7 ELISA

Enzyme-linked immunosorbent assay (ELISA) is a powerful technique applied routinely to the detection and quantitation of a wide variety of analytes. A double antibody sandwich assay was developed to determine the amount of antigen (mCG) in a sample.

A microtitre plate was coated with anti-rabbit immunoglobulin (anti-rabbit IgG) which captured the primary antibody raised in the rabbit. The standard/sample and enzyme-conjugated secondary antibody were added. After an overnight incubation, the unbound conjugate was washed away and streptavidin/horse-radish peroxidase (HRP), followed by the substrate were added. The amount of colour development was determined by reading the absorbance on a spectrophotometer.

2.7.1 *Antibodies*

2.7.1.1 Primary Antibody:

The primary antibody, R64, is a polyclonal rabbit antibody raised to recombinant mCG tagged with 6 histidines (mCG-6His). This recombinant mCG-6His protein was expressed in Chinese Hamster Ovary cells and purified using immobilised metal affinity chromatography (IMAC) in our laboratory (Amato *et al.* 1998; Simula *et al.* 1995). A New Zealand White rabbit was immunised by sub-cutaneous multiple-site injection. Approximately 3,000 picomoles of immunogen in complete Freund's adjuvant was injected into the rabbit, followed by booster injections of approximately 1,000 picomoles of protein in incomplete adjuvant every 3-4 weeks. The rabbit was bled two weeks after each immunisation and the antibody titres were determined by testing serial dilutions of each serum for their ability to bind radiolabelled purified mCG-β.

The primary antibody was diluted 1/2,000 in assay buffer for use in the ELISA.

2.7.1.2 Secondary Antibody:

The secondary antibody is a monoclonal anti-bovine Luteinising Hormone-β antibody (anti-bLH-β; $518B_7$), kindly donated by Dr. Jan Roser, Department of Animal Science, University of California, Davis, California, USA (Matteri *et al.* 1987; Simula *et al.* 1995).

This secondary antibody was biotinylated using a Biotin Labelling kit from Boehringer Mannheim. Briefly, the antibody and D-biotinoyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (biotin-7-NHS) were mixed in a molar ratio of 1:10 and incubated at room temperature, with mixing, for two hours. Non-reacted biotin was separated from the biotinylated anti-bLH-β antibody by gel filtration on a Sephadex® G-25 column (provided in the kit).

The secondary antibody was diluted 1/2,000 in assay buffer after biotinylation for use in the ELISA.

2.7.2 *Procedure*

2.7.2.1 Coating and Blocking Plates:

One hundred microlitres of coating buffer* with affinity-purified anti-rabbit immunoglobulin {1/1,250 dilution of anti-rabbit IgG (0.9 μg; Silenus Laboratories) in coating buffer} was pipetted into each well of a 96-well microtitre plate (Maxisorp: Nunc). The plate was covered with plastic wrap and incubated overnight at 4°C. The coating buffer was decanted and 260 μl of blocking buffer* was added and the plate incubated at room temperature for 1 hour. The plate was then washed three times with 260 μl wash buffer*.

2.7.2.2 Primary & Secondary Antibodies and Standards & Samples:

After washing, 100 µl of R64, the primary antibody, was added and the plate incubated at room temperature for 2 hours, covered with plastic wrap. After washing with wash buffer (as above), 50 µl of sample or standard: recombinant mCG dimer or rmCG-β ranging in concentration from 0.01 to 24 ng/ml was added, as well as 50 µl of the secondary antibody: biotinylated anti-bLH-β. The plates were covered with plastic wrap and incubated overnight at room temperature.

2.7.2.3 Colour Development and Detection:

After washing with wash buffer, 100 µl of streptavidin/horse radish peroxidase (HRP) enzyme conjugate was added and the plate incubated under alfoil at room temperature for 30 minutes. The plate was washed three times with wash buffer, followed by deionised water. Washing was followed by the addition of 100 µl of freshly prepared substrate. The plate was incubated at room temperature for approximately 10 minutes, carefully observing the colour development. The colour reaction was stopped by the addition of 100 µl of stop solution and the absorbance was measured at dual wavelengths with a spectrophotometer.

Chapter Three

Qualitative

RT-PCR

3.1 Introduction

Chorionic gonadotrophin (CG) secreted from the embryo is detected in the human peripheral blood between Days 8-11 after ovulation, corresponding to the expanded blastocyst stage of development in the human (Lenton *et al.* 1982). *In vitro* studies, however, have shown that CG is secreted by the embryo before it can be detected in the peripheral blood (Hay and Lopata 1988; Hearn *et al.* 1988b). The zona pellucida may be a barrier for the secreted CG by allowing retention within the perivitelline space or the blastocoelic cavity. Thus, when hatching and implantation occurs, CG is released and finds its way into the maternal peripheral blood, causing CG levels to rise rapidly (Hay and Lopata 1988). Alternatively, hatching may allow rapid expansion of the trophoblastic tissues with enhance CG production.

In situ hybridisation data of tripronucleate (abnormal) human embryos has suggested the presence of hCG-β mRNA at the eight-cell stage (Bonduelle *et al.* 1988). Nevertheless, whether the mRNA is being translated into protein and what role the CG protein could be playing at this early stage is yet to be determined.

The marmoset monkey has been used for many years to study primate reproductive physiology (Hearn 1983 and references therein). Our group has cloned and sequenced marmoset CG alpha and beta (Simula *et al.* 1995) and an *in situ* hybridisation study by our group (Lopata *et al.* 1995) has investigated the differential distribution of mRNA for mCG-alpha and beta in implantation stage embryos of

the marmoset monkey. Messenger RNA for mCG-β was localised to the syncytiotrophoblast at the embryonic pole and the mural trophoblast, whilst the mRNA for mCG-α was more uniformly distributed in the trophoblast, in particular at the embryonic pole, as well as in much lower levels in the inner cell mass and early endoderm.

Currently, it is not known when CG expression and/or secretion is first detected in the primate embryo, thus the aim of the experiments described in this chapter was to determine the time of onset of expression of the mCG alpha and beta subunits in the pre-implantation embryo. This information will assist us in determining the role of cytokines and growth factors in the regulation of CG expression and secretion. In turn, this knowledge will aid in improving culture conditions and viability of human *in vitro* fertilised embryos, as well as enhancing our understanding of primate reproductive physiology.

3.2 Collection of Embryos and Other Tissues

Marmoset embryos of various stages (4-cell through to hatching blastocyst stage; *Table 3.1*) were collected by the mini-laparotomy method (*as described in Section 2.2.2*) or by the non-surgical flushing method (*as described in Section 2.2.3*). Marmoset placental tissue was collected from three different animals at between day 55-60 of pregnancy. Marmoset skeletal muscle and

pituitary tissue was kindly donated to us by CSIRO - Human Nutrition. Marmoset oocytes, either denuded or with attached cumulus cells and pieces of ovarian tissue were a kind gift of Dr. R.B. Gilchrist and empty zona pellucida were provided by Associate Professor Alex Lopata.

Embryos, trophoblastic vesicles, oocytes, zona pellucida and marmoset tissues were placed into RNase-free centrifuge tubes (in minimal media, in the case of the embryos, trophoblastic vesicles and oocytes) and snap-frozen in liquid nitrogen before storing at -80°C.

3.3 RNA Extraction of Embryos and Other Tissues

Total RNA was isolated from marmoset embryos of different developmental stages and from marmoset trophoblastic vesicles, oocytes and zona pellucida by the Chomczynski and Sacchi method (*as described in Section 2.4.2; Table 3.1*). Total RNA was extracted from other marmoset tissues by the same method, after being pulverised in the presence of liquid nitrogen (*as described in Section 2.4.1*). Total RNA extracted from the marmoset tissues was quantitated using a spectrophotometer. In addition, total placental, pituitary and skeletal muscle RNA was electrophoresed through a 1% RNase-free agarose gel in 1 x TAE buffer, stained with ethidium bromide and the 18S and 28S ribosomal RNA bands

visualised by viewing under ultraviolet illumination (*as described in Section 2.5.3*) to verify both the concentration of the RNA and its integrity.

Table 3.1 Stage of development and number of marmoset embryos extracted for RNA and other tissues extracted.

Stage of Development	**Number**
four-cell	1
eight-cell	2
morula	5
early blastocyst	4
expanded blastocyst	5
hatching blastocyst	3
trophoblastic vesicle	5
placenta	4
denuded oocyte	3
cumulus-oocyte complex	3
cumulus	3
degenerate blastocyst	3
zona pellucida	2*

*pooled together

3.4 Qualitative RT-PCR

3.4.1 *Oligonucleotide Primers*

3.4.1.1 mCG-α:

Marmoset CG-α oligonucleotide primers were chosen from the mCG-α sequence, which was previously sequenced in our laboratory (Simula *et al.* 1995). The forward primer (αFP) corresponds to nucleotides 22-39, whilst the reverse primer (αRP) is complimentary to nucleotides 329-346 (*see Table 3.2 for sequences*). Marmoset CG-α primers were synthesised and purified by DNA International Inc. and Gibco-BRL Life Technologies Inc. A concentration of approximately 50 picomoles of each mCG-α primer was used in PCR reactions. The target sequence for mCG-α was 325 base pairs in length.

Table 3.2 Oligonucleotide sequences used for amplifying mCG-α target.

Direction	Oligonucleotide sequences	Concentration
αFP	5'-GCAGCTATCATTCTGATC-3'	50 pmoles
αRP	5'-AACAAGTACTGCAGTGGC-3'	50 pmoles

3.4.1.2 mCG-β:

Oligonucleotide primers for amplifying mCG-β were chosen from the mCG-β sequence, which was previously sequenced in our laboratory (Simula *et al.* 1995). The forward primer (βFP) corresponds

to nucleotides 60-77, whilst the reverse primer (βRP) is complimentary to nucleotides 478-495 (*see Table 3.3 for sequences*). Marmoset CG-β primers were synthesised and purified by Beckmann Instruments, DNA International Inc. and Gibco-BRL Life Technologies Inc. A concentration of approximately 50 picomoles of each mCG-β primer was used in PCR reactions. The mCG-β target fragment was 433 base pairs in length.

Table 3.3 Oligonucleotide sequences used for amplifying mCG-β target.

Direction	Oligonucleotide sequences	Concentration
βFP	5'-ATCCAAGGAGCCACTTCG-3'	50 pmoles
βRP	5'-TTGTGGGACTAATGGAGG-3'	50 pmoles

3.4.1.3 mGAPDH:

Glyceraldehyde-phosphate-dehydrogenase (GAPDH), a house-keeping gene, was used as an endogenous standard to establish the integrity of the recovered cellular RNA from the embryos and tissues. The sequence of mGAPDH is unknown, thus from alignments of the human, mouse and rat sequences of GAPDH, forward and reverse primers (GEcoFP & GEcoRP) were designed, with an added *EcoRI* restriction site (*see Table 3.4 for sequences and Figure 3.2 for the alignment*).

Table 3.4 Oligonucleotide sequences used for amplifying the 716 base pair mGAPDH amplicon.

Direction	Oligonucleotide sequences	Concentration
GEcoFP	5'-GATCGAATTC-CTTCACCACCATGGAGAA-3'	77 pmoles
GEcoRP	5'-ATGGCCTCCAAGGAGTAA-GAATTCGATC-3'	77 pmoles

The 716 base pair amplicon generated from marmoset skeletal muscle by PCR amplification, using *Pwo* DNA polymerase with the GEcoFP and GEcoRP primers (*as described in Section 2.5.2.1*) was then digested with the restriction enzyme *EcoRI* (*see Section 2.5.3.3*), to confirm that it was GAPDH, before cloning it into linearised Bluescript SK+ (Stratagene Cloning Systems, *see Section 2.6*). *E.coli* JM109 cells were transformed with the ligated products (*see Section 2.6.4*). Positive transformants (*see Section 2.6.5*) were analysed by performing small scale plasmid preparations (*see Section 2.6.6*), followed by restriction endonuclease digestion (*as described in Section 2.6.6.2*).

Large scale plasmid preparations (*see Section 2.6.7*) were performed on colonies containing the insert and plasmid DNA purified by the QIAgen Plasmid Midiprep kit (QIAgen; *see Section 2.6.7.1*) before partial sequencing (*see Section 2.5.3.4*).

From the sequencing results, forward and reverse primers (mGAPFP & mGAPRP) were determined and used for subsequent PCR reactions at a concentration of approximately 70 picomoles (*See Table 3.5 for sequences*).

Figure 3.2 Alignment of human, mouse and rat sequences of GAPDH, showing the position of the Geco oligonucleotide primers (*Table 3.4*) underlined in red and the position of the mGAP oligonucleotide primers (*Table 3.5*) underlined in green. The position of the internal 18 base pair sequence (*Chapter Four*) is underlined in pink.

```
          0                    20                   40                   60
human     GTTCGACAGTCAGCCGCATCTTCTTTTGCGTCGCCAGCCGAGCCACATCGCTCAGACACC
mouse     ---------ACAGCCGCATCTTCTTGTGCAGTGCCAGCC-----TCGTCCCGTAGACAAA
rat       -------------------------GGTCAGTGCCGGCC-----TCGTCTCATAGACAAG
                                    *   *** ***       * ** *  ******

                              80                   100                  120
human     ATGGGGAAGGTGAAGGTCGGAGTCAACGGATTTGGTCGTATTGGGCGCCTGGTCACCAGG
mouse     ATG------GTGAAGGTCGGTGTGAACGGATTTGGCCGTATTGGGCGCCTGGTCACCAGG
rat       ATG------GTGAAGGTCGGTGTCAACGGATTTGGCCGTATTGGCCGCCTGGTCACCAGG
          ***       ************ ** *********** ******** ****************

                             140                  160                  180
human     GCTGCTTTTAACTCTGGTAAAGTGGATATTGTTGCCATCAATGACCCCTTCATTGACCTC
mouse     GCTGCCATTTGCAGTGGCAAAGTGGAGATTGTTGCCATCAACGACCCCTTCATTGACCTC
rat       GCTGCCTTCTCTTGTGACAAAGTGGACATTGTTGCCATCAACGACCCCTTCATTGACCTC
          *****  *       **  ******** ************** ********************

                             200                  220                  240
human     AACTACATGGTTTACATGTTCCAATATGATTCCACCCATGGCAAATTCCATGGCACCGTC
mouse     AACTACATGGTCTACATGTTCCAGTATGACTCCACTCACGGCAAATTCAACGGCACAGTC
rat       AACTACATGGTCTACATGTTCCAGTATGATTCTACCCACGGCAAGTTCAATGGCACAGTC
          *********** *********** ***** ** ** ** ***** *** * ***** ***

                             260                  280                  300
human     AAGGCTGAGAACGGGAAGCTTGTCATCAATGGAAATCCCATCACCATCTTCCAGGAGCGA
mouse     AAGGCCGAGAATGGGAAGCTTGTCATCAACGGGAAGCCCATCACCATCTTCCAGGAGCGA
rat       AAGGCTGAGAATGGGAAGCTGGTCATCAATGGGAAACCCATCACCATCTTCCAGGAGCGC
          ***** ***** ******** ******** ** ** ************************

                             320                  340                  360
human     GATCCCTCCAAAATCAAGTGGGGCGATGCTGGCGCTGAGTACGTCGTGGAGTCCACTGGC
mouse     GACCCCACTAACATCAAATGGGGTGAGGCCGGTGCTGAGTATGTCGTGGAGTCTACTGGT
rat       GATCCCGCTAACATCAAATGGGGTGATGCTGGTGCTGAGTATGTCGTGGAGTCTACTGGC
          ** *** * ** ***** ***** ** ** ** ******** *********** *****

                             380                  400                  420
human     GTCTTCACCACCATGGAGAAGGCTGGGGCTCATTTGCAGGGGGGAGCCAAAAGGGTCATC
mouse     GTCTTCACCACCATGGAGAAGGCCGGGGCCCACTTGAAGGGTGGAGCCAAACGGGTCATC
rat       GTCTTCACCACCATGGAGAAGGCTGGGGCTCACCTGAAGGGTGGTGCCAAAAGGGTCATC
          ********************** ***** **  ** **** ** ******* ********
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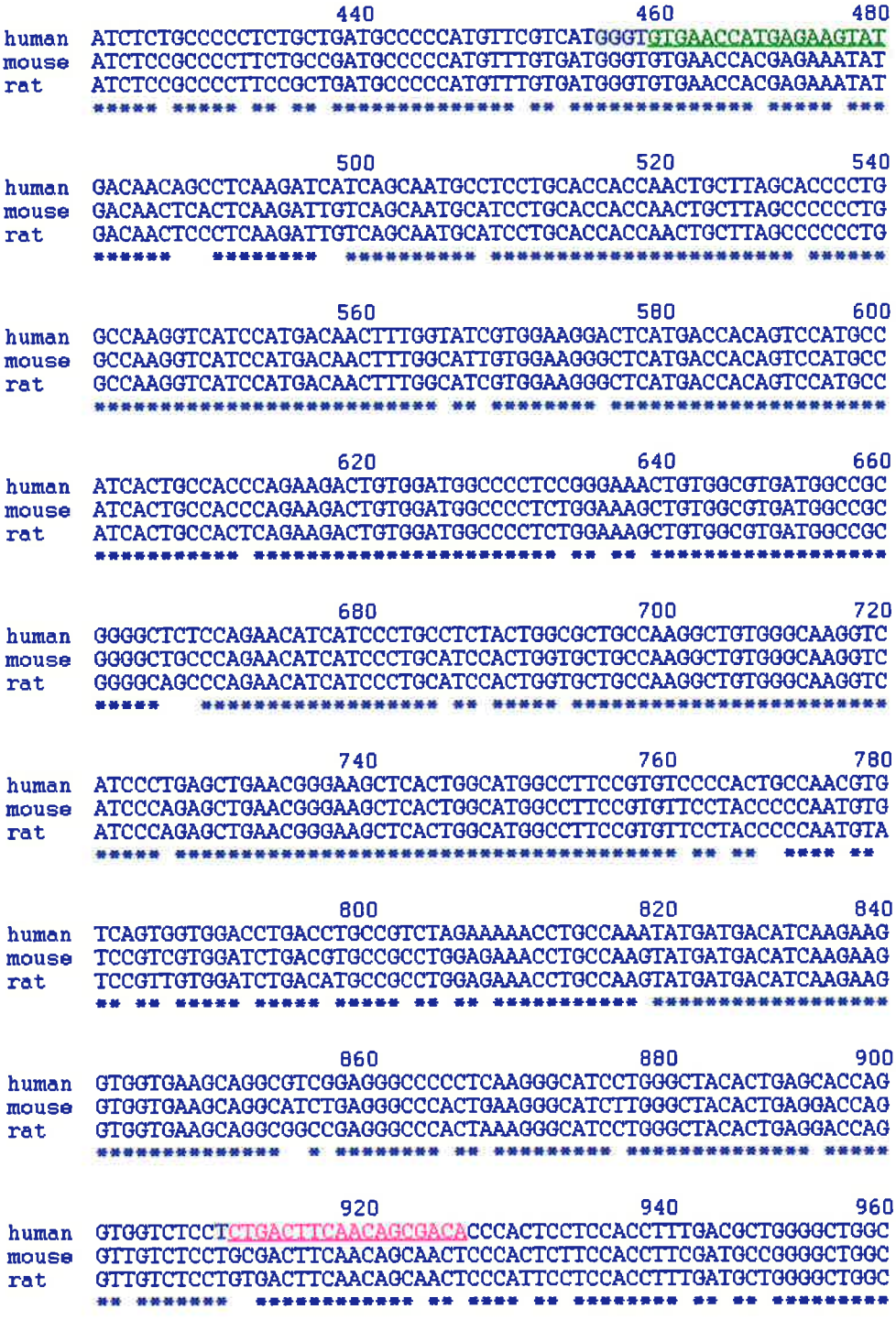

440 460 480
human ATCTCTGCCCCCTCTGCTGATGCCCCCATGTTCGTCATGGGTGTGAACCATGAGAAGTAT
mouse ATCTCCGCCCCTTCTGCCGATGCCCCCATGTTTGTGATGGGTGTGAACCACGAGAAATAT
rat ATCTCCGCCCCTTCCGCTGATGCCCCCATGTTTGTGATGGGTGTGAACCACGAGAAATAT
500 520 540
human GACAACAGCCTCAAGATCATCAGCAATGCCTCCTGCACCACCAACTGCTTAGCACCCCTG
mouse GACAACTCACTCAAGATTGTCAGCAATGCATCCTGCACCACCAACTGCTTAGCCCCCCTG
rat GACAACTCCCTCAAGATTGTCAGCAATGCATCCTGCACCACCAACTGCTTAGCCCCCCTG
560 580 600
human GCCAAGGTCATCCATGACAACTTTGGTATCGTGGAAGGACTCATGACCACAGTCCATGCC
mouse GCCAAGGTCATCCATGACAACTTTGGCATTGTGGAAGGGCTCATGACCACAGTCCATGCC
rat GCCAAGGTCATCCATGACAACTTTGGCATCGTGGAAGGGCTCATGACCACAGTCCATGCC
620 640 660
human ATCACTGCCACCCAGAAGACTGTGGATGGCCCCTCCGGGAAACTGTGGCGTGATGGCCGC
mouse ATCACTGCCACCCAGAAGACTGTGGATGGCCCCTCTGGAAAGCTGTGGCGTGATGGCCGC
rat ATCACTGCCACTCAGAAGACTGTGGATGGCCCCTCTGGAAAGCTGTGGCGTGATGGCCGC
680 700 720
human GGGGCTCTCCAGAACATCATCCCTGCCTCTACTGGCGCTGCCAAGGCTGTGGGCAAGGTC
mouse GGGGCTGCCCAGAACATCATCCCTGCATCCACTGGTGCTGCCAAGGCTGTGGGCAAGGTC
rat GGGGCAGCCCAGAACATCATCCCTGCATCCACTGGTGCTGCCAAGGCTGTGGGCAAGGTC
740 760 780
human ATCCCTGAGCTGAACGGGAAGCTCACTGGCATGGCCTTCCGTGTCCCCACTGCCAACGTG
mouse ATCCCAGAGCTGAACGGGAAGCTCACTGGCATGGCCTTCCGTGTTCCTACCCCCAATGTG
rat ATCCCAGAGCTGAACGGGAAGCTCACTGGCATGGCCTTCCGTGTTCCTACCCCCAATGTA
800 820 840
human TCAGTGGTGGACCTGACCTGCCGTCTAGAAAAACCTGCCAAATATGATGACATCAAGAAG
mouse TCCGTCGTGGATCTGACGTGCCGCCTGGAGAAACCTGCCAAGTATGATGACATCAAGAAG
rat TCCGTTGTGGATCTGACATGCCGCCTGGAGAAACCTGCCAAGTATGATGACATCAAGAAG
860 880 900
human GTGGTGAAGCAGGCGTCGGAGGGCCCCCTCAAGGGCATCCTGGGCTACACTGAGCACCAG
mouse GTGGTGAAGCAGGCATCTGAGGGCCCACTGAAGGGCATCTTGGGCTACACTGAGGACCAG
rat GTGGTGAAGCAGGCGGCCGAGGGCCCACTAAAGGGCATCCTGGGCTACACTGAGGACCAG
920 940 960
human GTGGTCTCCTCTGACTTCAACAGCGACACCCACTCCTCCACCTTTGACGCTGGGGCTGGC
mouse GTTGTCTCCTGCGACTTCAACAGCAACTCCCACTCTTCCACCTTCGATGCCGGGGCTGGC
rat GTTGTCTCCTGTGACTTCAACAGCAACTCCCATTCCTCCACCTTTGATGCTGGGGCTGGC

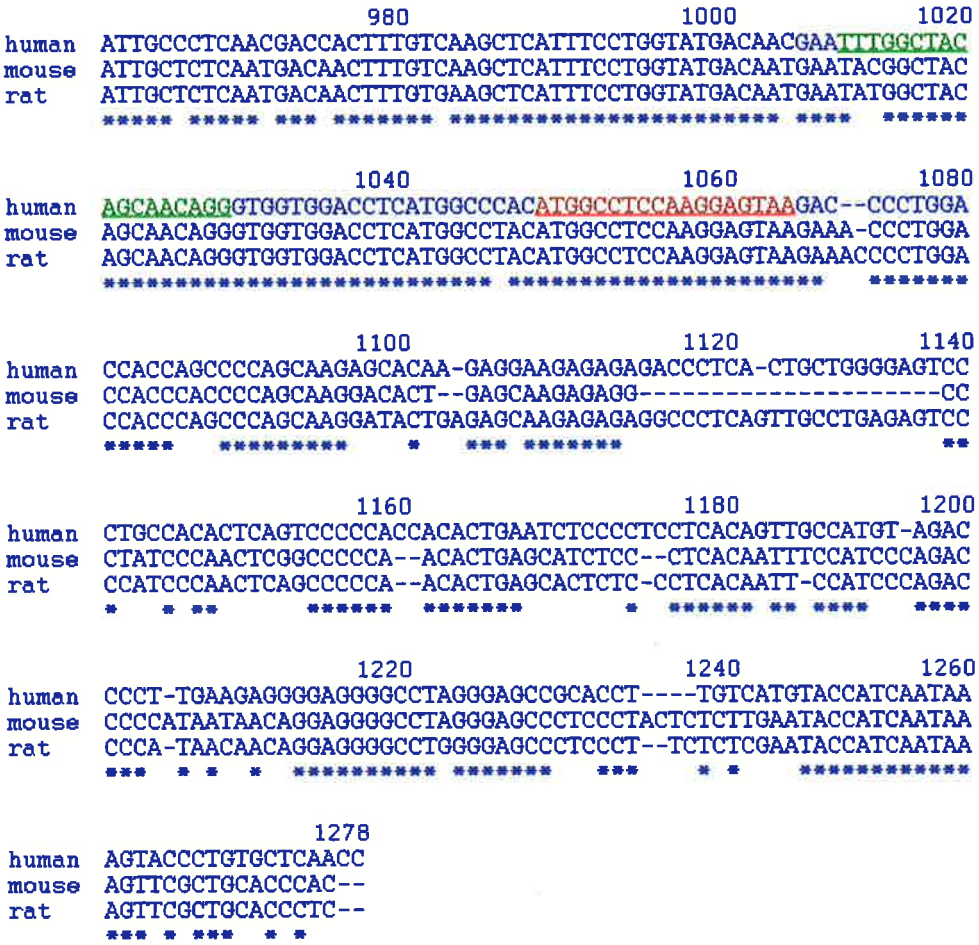

980 1000 1020
human ATTGCCCTCAACGACCACTTTGTCAAGCTCATTTCCTGGTATGACAACGAATTTGGCTAC
mouse ATTGCTCTCAATGACAACTTTGTCAAGCTCATTTCCTGGTATGACAATGAATACGGCTAC
rat ATTGCTCTCAATGACAACTTTGTGAAGCTCATTTCCTGGTATGACAATGAATATGGCTAC
1040 1060 1080
human AGCAACAGGGTGGTGGACCTCATGGCCCACATGGCCTCCAAGGAGTAAGAC--CCCTGGA
mouse AGCAACAGGGTGGTGGACCTCATGGCCTACATGGCCTCCAAGGAGTAAGAAA-CCCTGGA
rat AGCAACAGGGTGGTGGACCTCATGGCCTACATGGCCTCCAAGGAGTAAGAAACCCCTGGA
1100 1120 1140
human CCACCAGCCCCAGCAAGAGCACAA-GAGGAAGAGAGAGACCCTCA-CTGCTGGGGAGTCC
mouse CCACCCACCCCAGCAAGGACACT--GAGCAAGAGAGG----------CC
rat CCACCCAGCCCAGCAAGGATACTGAGAGCAAGAGAGAGGCCCTCAGTTGCCTGAGAGTCC
1160 1180 1200
human CTGCCACACTCAGTCCCCCACCACACTGAATCTCCCCTCCTCACAGTTGCCATGT-AGAC
mouse CTATCCCAACTCGGCCCCCA--ACACTGAGCATCTCC--CTCACAATTTCCATCCCAGAC
rat CCATCCCAACTCAGCCCCCA--ACACTGAGCACTCTC-CCTCACAATT-CCATCCCAGAC
1220 1240 1260
human CCCT-TGAAGAGGGGAGGGGCCTAGGGAGCCGCACCT----TGTCATGTACCATCAATAA
mouse CCCCATAATAACAGGAGGGGCCTAGGGAGCCCTCCCTACTCTCTTGAATACCATCAATAA
rat CCCA-TAACAACAGGAGGGGCCTGGGGAGCCCTCCCT--TCTCTCGAATACCATCAATAA
1278
human AGTACCCTGTGCTCAACC
mouse AGTTCGCTGCACCCAC--
rat AGTTCGCTGCACCCTC--

Table 3.5 Oligonucleotide sequences used for amplifying mGAPDH target.

Direction	Oligonucleotide sequences	Concentration
mGAPFP	5'-GTGAACCATGAGAAGTAT-3'	70 pmoles
mGAPRP	5'-GGACAACGACATCGGTTT-3'	70 pmoles

The initial oligonucleotide primers, GAPEcoFP and GAPEcoRP correspond to nucleotides 363-380 and 1050-1068, respectively, of the human GAPDH sequence and were synthesised and purified by DNA International Inc. The mGAPDH forward and reverse assay primers (mGAPFP & mGAPRP) correspond to nucleotides 462-480 and 1012-1029 of the human GAPDH sequence, respectively and were synthesised and purified by Gibco-BRL Life Technologies Inc. The mGAPDH target fragment was 567 base pairs in length.

3.4.2 *Reverse Transcription*

Total RNA was reverse transcribed, using oligo d(T) (*as described in Section 2.5.1.2*). Briefly, 5 or 10 µl of total RNA was hybridised at 70°C for 10 minutes together with 500 µg/ml oligo $d(T)_{15}$ primer and then quickly chilled to 4°C. A master mix of 5x first strand buffer, 100 mM DTT, 10 mM dNTPs and 200 units of Superscript™II RNaseH$^-$ Reverse Transcriptase was prepared and pipetted to the tubes. The reaction was incubated at 42°C for 92 minutes, before being heated to 70°C for 15 minutes, to inactivate the enzyme and then quickly chilled to 4°C using a thermal cycler. The reaction was used immediately for PCR or stored at -20°C.

3.4.3 *Polymerase Chain Reaction*

Marmoset CG-α and -β and mGAPDH gene expression was determined by amplifying the prepared cDNA from above using *Taq* DNA polymerase and specific primers (*as described in Section 2.5.2.2*). A master mix was prepared, containing 10x PCR buffer, 10 mM dNTPs, primers (*see Section 3.4.1*) and *Taq* DNA polymerase. The 23 µl reaction was overlaid with mineral oil, before 2 µl cDNA was added. PCR was performed with negative controls (no cDNA template) under the parameters described in *Section 2.5.2.2* (*LOUTAQ30*).

The primary PCR product was then diluted 10-fold and a 2 µl aliquot was subjected to a further 35 cycles of amplification (*LOUTAQ35*) in a fresh tube with the same primers and conditions as described above.

3.4.4 *Gel Electrophoresis*

Fifteen microlitres of the primary and secondary PCR reactions were subjected to agarose gel electrophoresis (*as described in Section 2.5.3*). After electrophoresis a polaroid photograph was taken using UV illumination (*as described in Section 2.5.3.1*).

The PCR products were excised from the agarose, purifed (*see Section 2.5.3.2*) and their identity verified by sequencing (*see*

Section 2.5.3.4) or by restriction enzyme digestion (*see Section 2.5.3.3*).

3.5 Results

Messenger RNA expression was analysed by RT-PCR of the total RNA from embryos of different developmental stages using oligonucleotide primers specific for mCG-α, mCG-β and mGAPDH (*see Tables 3.2, 3.3 and 3.5*). Amplification with these primers resulted in PCR products of 325, 433 and 567 base pairs in size, for mCG-α, mCG-β and mGAPDH, respectively. The identity of the resulting products was verified by sequencing and/or restriction enzyme digestion of the purified product.

Transcription of mCG-α (*lanes marked α in Figure 3.2*) was detected in most samples, although very faintly in the 4- and 8-cell and morula (*panel A*). Neither the denuded oocytes (*panel D*), nor the pooled zona pellucida (*panel C*) showed any mCG-α expression.

Messenger RNA for mCG-β (*lanes marked β in Figure 3.2*) was detected from the morula stage of development, although there is variation between samples (the morulae, early blastocyst 2 and hatched blastocyst shown in *Figure 3.2* do not show expression for mCG-β). The negative control sample, pooled zona pellucida (*panel*

Figure 3.2 Qualitative RT-PCR of marmoset monkey embryos, from the 4-cell stage through to the hatching blastocyst stage of development and other marmoset tissues.

The mCG-α, -β and mGAPDH products are shown in lanes α, β and g, respectively. Molecular weight markers (M) were the 1Kb DNA ladder.

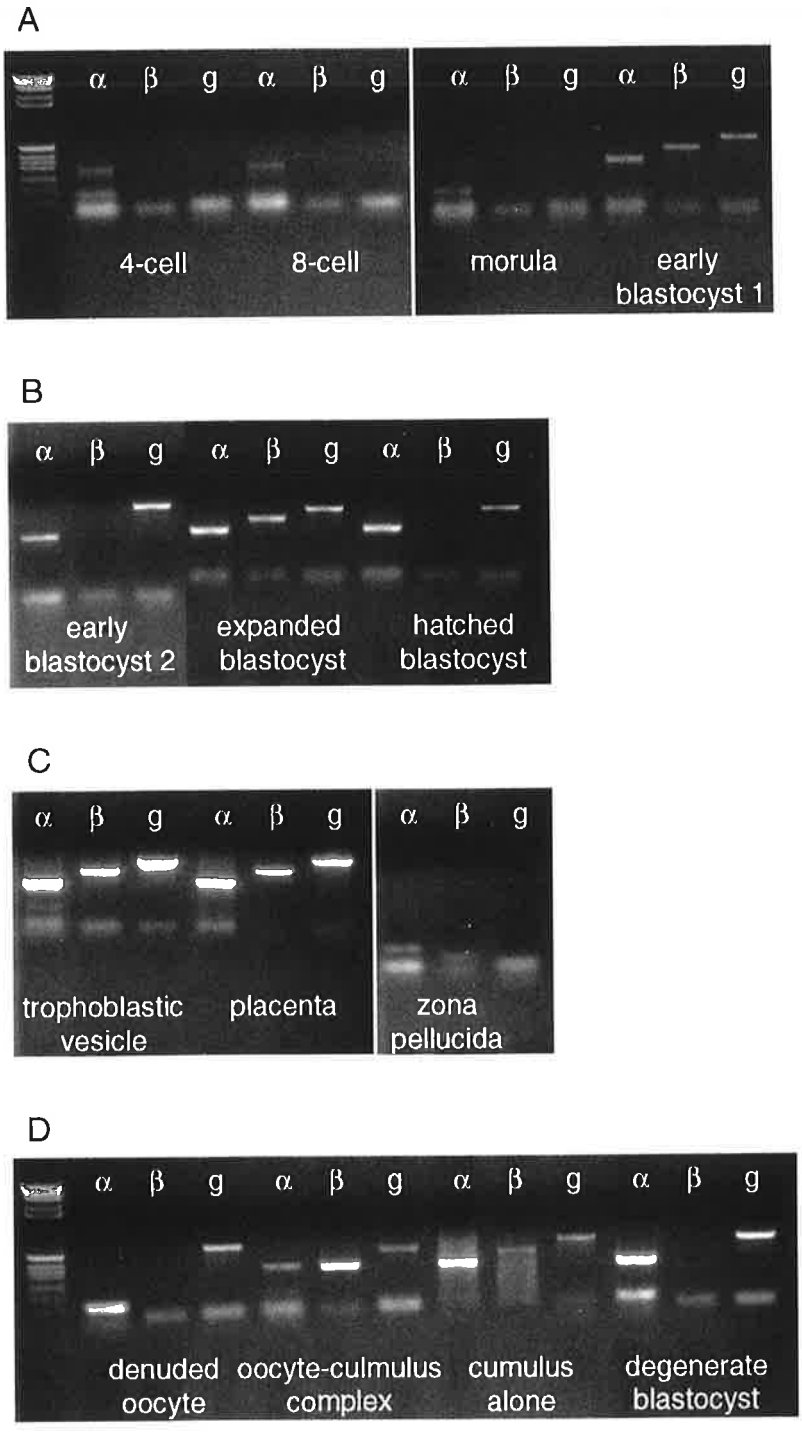
Panel A: four-cell, eight-cell, morula and early blastocyst 1.

Panel B: early blastocyst 2, expanded blastocyst, hatching blastocyst.

Panel C: trophoblastic vesicle, placenta and zona pellucida.

Panel D: denuded oocyte, cumulus-oocyte complex, cumulus alone and degenerate blastocyst.

Figure 3.2


A
α β g α β g
4-cell
8-cell
α β g α β g
morula
early blastocyst 1
B
α β g α β g α β g
early blastocyst 2
expanded blastocyst
hatched blastocyst
C
α β g α β g
trophoblastic vesicle
placenta
α β g
zona pellucida
D
α β g α β g α β g α β g
denuded oocyte
oocyte-culmulus complex
cumulus alone
degenerate blastocyst

C) does not show expression for mCG-β. Messenger RNA for mCG-β was also not detected in denuded oocytes, nor degenerate blastocysts (*panel D*). The expected product of 433 base pairs was not detected in the cumulus-oocyte complexes, instead, a product approximately 300 base pairs was amplified.

The positive control house-keeping gene, mGAPDH (*lanes marked g in Figure 3.2*), was detected in all samples, except the 4- and 8-cell and the negative control, zona pellucida. Other morula have shown mGAPDH expression.

All results obtained from analysing embryos of different developmental stages and other marmoset tissues are summarised in *Table 3.6*. Only one of two eight-cells showed expression for mCG-α. Expression of mCG-β was detected in four of five morula, three of four early blastocysts, two of three hatched blastocysts and three of five trophoblastic vesicles.

Table 3.6 Summary of results, showing stage of development and number of embryos and other tissues and pattern of expression.

Stage of Development	No	Expression of		
		mCG-α	mCG-β	mGAPDH
Four-cell	1	+	-	-
Eight-cell	2	+ (1/2)	-	-
Morula	5	+	+ (4/5)	+
Early Blastocyst	4	+	+ (3/4)	+
Expanded Blastocyst	5	+	+	+
Hatching Blastocyst	3	+	+ (2/3)	+
Trophoblastic Vesicle	5	+	+ (3/5)	+
Placenta	4	+	+	+
Denuded Oocyte	3	-	-	+
Cumulus-oocyte complex	3	+	+	+
Cumulus alone	3	+	+	+
Degenerate Blastocyst	3	+	-	+
Zona Pellucida	2	-	-	-

3.6 Discussion

The results presented in this chapter have shown, for the first time, the presence of mRNA encoding chorionic gonadotrophin in early pre-implantation stage marmoset embryos. A recent study (Sharkey *et al.* 1995) investigated the stage-specific expression of cytokine and receptor mRNA in human pre-implantation embryos. They used a nested RT-PCR protocol, allowing the detection of mRNA species as early as the 2-cell stage. The experiments in this chapter use the Common Marmoset as a

primate model to determine the pattern of expression of mCG in the marmoset pre-implantation embryo, using an RT-PCR protocol. A previous study (Bonduelle *et al.* 1988) examined the onset of expression of hCG-β in polyploid human embryos after *in vitro* fertilisation (IVF), using *in situ* hybridisation. They suggested that the hCG-β gene family was prematurely activated and that their polyploid embryos were grossly abnormal in their patterns of transcriptional activity. However, they then suggested that tripronucleate embryos would be a good model for studying pre-implantation development and that the products seen were as a result of *de novo* transcription from the embryonic genome, which is consistent with the observations of Tesarik *et al.* (1986) and Braude *et al.* (1987), who suggest that transcription only commences after the late four-cell stage in the human.

CG-β expression is thought to be trophoblast-specific and *in vivo* expression is normally limited to differentiated cells (Gaspard *et al.* 1980) However, it is well established that all blastomeres of 8-cell mouse embryos retain their totipotency (Balakier and Pedersen 1982), as do some of the cells of mouse blastocysts (Dyce *et al.* 1987; Pedersen *et al.* 1986). Thus strict cell lineage specificity of gene expression may not occur until the establishment of two distinct cell types at the blastocyst stage (Bonduelle *et al.* 1988).

Unfortunately, only one 4-cell and two 8-cell embryos have been analysed. This is due to the scarcity of embryos at these stages of development. The four-cell and one of the eight-cells showed mCG-α expression, but no mCG-β or mGAPDH expression.

This expression of mCG-α is rather precocious and has been shown to be produced in abundance early in pregnancy in the human before the beta subunit (Daniels-McQueen *et al.* 1978; Vaitukaitis 1974). Furthermore, it's expression is consistent with the study by Lopata *et al.* (1995), in which mCG-α expression was observed in almost all cell types in the implanting marmoset blastocyst, whereas, mCG-β was only observed in a restricted number of cells. The inability to amplify a GAPDH product in the 4- and 8-cell stage embryos, may be due to the recovery of very low levels of RNA from these embryos and the results may only reflect their relative abundance at this stage of development. Hence, it may be possible that mCG-β is also expressed at this stage.

Expression of mCG-β and mGAPDH were detected at the morula stage and were present throughout the subsequent developmental stages. Marmoset GAPDH expression appears to increase as the embryo grows, which is consistent with increasing cell numbers. The presence of mCG at these early pre-implantation stages may indicate a possible local function for CG at peri-implantation. Such an effect has been reported previously by Reshef and coworkers (Reshef *et al.* 1990) in which they describe local effects of CG on the endometrium, influencing white blood cells and altering local blood flow via receptors in the uterine tissue.

Chorionic gonadotrophin-beta has also been detected by immunohistochemistry and *in* situ hybridisation in secretory phase normal cyclic endometrium (Wolkersdörfer *et al.* 1998), however, some of the endometrial specimens utilised could be classified as

abnormal and so the patterns of transcriptional activity and protein secretion could be aberrant. Nevertheless, the authors suggest a paracrine role for CG in endometrial physiology. Human CG-β has also been shown to exist in immunologically active tissues, including respiratory tract mucosa, intestinal mucosa and urinary tract mucosa (Braunstein *et al.* 1979; Chen *et al.* 1976) Yoshimoto *et al.* 1979). Mononuclear cells are capable of secreting hCG (Nisula and Bartocci, 1984), suggesting an immunomodulatory function for CG (Harbour-McMenamin *et al.* 1986).

Other tissues investigated, included denuded oocytes, cumulus-oocyte complexes and cumulus cells alone. All showed expression for mGAPDH, however expression for mCG-α and -β was not seen in the denuded oocytes, which indicates that CG is an embryonic transcript. Amplification of mCG-β in the oocyte-cumulus complexes resulted in a smaller sized PCR product than expected. This could possibly be an alternate transcript, but this needs further investigation.

The absence of expression of mCG-β in *in vitro* cultured, degenerate blastocysts may suggest that mCG-β is an indicator of viability. Moreover, low hCG levels are an indicator of abnormal human pregnancy (Lenton *et al.* 1981; Shepherd *et al.* 1990).

In conclusion, the results in this chapter have shown that mCG is expressed in early pre-implantation marmoset embryos before the time that CG protein is detected within the peripheral blood. Thus this may indicate a possible local function for CG at peri-implantation.

Chapter Four

Quantitative

RT-PCR

4.1 Introduction

Although chorionic gonadotrophin is known to be essential for implantation and a viable pregnancy in primates, an understanding of the factors regulating expression is lacking. The lack of knowledge of CG expression at peri-implantation is due to a number of factors, including (i) a paucity of tissue from suitable primate models, with appropriate probes and (ii) the ethical restrictions with the use of human embryos. The findings reported in the previous chapter established the presence of mCG-β and –α gene expression at developmental stages well before implantation, which may indicate a local mechanism of action for mCG.

The limited number of studies investigating the production rates of CG *in vitro* from human and marmoset embryos, suggest that secreted levels are lower than those predicted from *in vivo* circulating levels, indicating that culture conditions for mammalian embryos are suboptimal (Lopata and Hay 1989a; Lopata and Hay 1989b). In the mouse, cultured 1-cell embryos fail to progress beyond the two cell stage, which can be overcome when co-cultured with cells from the maternal reproductive tract. Similar blocks have also been observed in other mammals, and indicate possible deficiencies in the culture systems used. These data strongly suggest that some factor(s), which may be maternally-derived, may be lacking from the culture media necessary for correct expression of CG. Endogenous factors such as cytokines

and other growth factors have been shown to be produced by the maternal reproductive tissues in a spatial and temporal manner.

Various cytokines and growth factors have been studied in human placental cell lines and shown to alter expression of CG (Li *et al.* 1992; Masuhiro *et al.* 1991; Matsuzaki *et al.* 1992; Morrish *et al.* 1991; Sawai *et al.* 1995a; Sawai *et al.* 1995b). A limited study has been carried out in human embryos (Lopata and Oliva 1993), in which a mixture of insulin, transferrin and selenium (ITS), platelet-derived growth factor (PDGF) and 8-bromo-cyclic-3'-5'-adenosine mono-phosphate (8-bromo-cAMP) were shown to influence the secretion of CG.

While the marmoset monkey has been used for many years to study primate reproductive physiology (Hearn, 1983; and references therein), only recently have the DNA sequences for the mCG subunits been determined (Simula *et al.* 1995), thus, providing the tools and information to study the genetic expression of CG in this particular species. Therefore, the aim of this chapter is to establish a sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) assay suitable for the assessment of CG expression in the marmoset embryo at peri-implantation. These assays will be used in further experiments (*see Chapter 6*) to determine the pattern of expression of mCG of marmoset trophoblastic vesicles and embryos cultured in the presence or absence of cytokines and growth factors.

RT-PCR is a very sensitive extension of the PCR technique for detecting low levels of mRNA and can be quantitative or semi-quantitative by the inclusion of an appropriate competing internal

standard. A competitive RT-PCR assay requires that a known amount of an appropriate exogenous recombinant RNA (rcRNA) internal standard (IS) be added to a constant amount of target RNA. The IS and target sequences are then reverse transcribed and amplified in the same reaction mixture using the same primers. The IS is distinguished from the target by its size difference and the relative amounts of the two products can be compared. Alternatively, an exogenous recombinant DNA (rcDNA) internal standard can be added to a constant amount of reverse transcribed target cDNA in a competitive PCR assay. The IS and target are then co-amplified, under the same conditions and with the same primers. Once again, the IS and target are distinguished from each other by size and the relative amounts of the two can be compared.

Under competitive conditions, the absolute amount of IS added to the reaction is equal to the amount of target, when the molar ratios of products becomes equal for IS and target. Thus, an equivalency point can be determined. The molar ratios of products can be determined by UV illumination of an ethidium bromide-stained agarose gel, or based on scintillation counting of excised PCR-radiolabelled DNA bands from an agarose gel.

4.2 Construction of Internal Standards

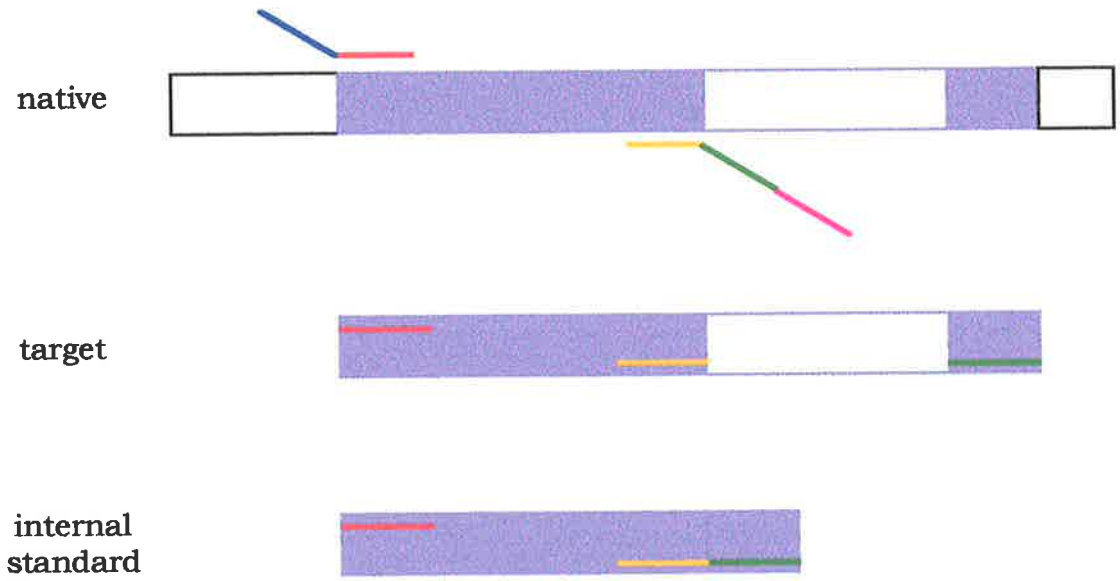
The three internal standards for mCG-β, -α and mGAPDH were all constructed following the same protocol. Construction of the

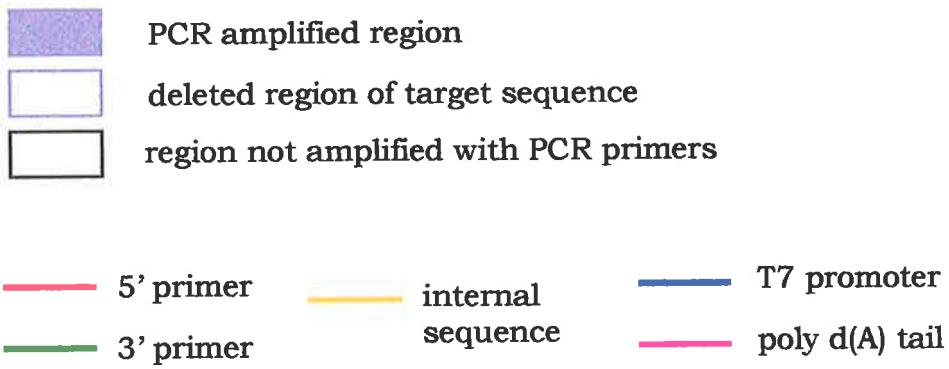
recombinant RNA internal standard (rcRNA IS) was achieved by first amplifying the cDNA template using the modified forward and reverse oligonucleotide primers (Celi *et al.* 1993; Förster 1994; Van den Heuvel *et al.* 1993) shown in *Table 4.1*. The forward primer, ISFP, consisted of the T7 promoter (T7 RNA polymerase promoter) and the 18 base pair forward assay primer (*see Chapter Three*), whilst the reverse primer, ISRP, was composed of an 18 base pair poly d(T) tail, an internal 18 base pair sequence and the 18 base pair reverse assay primer (*see Chapter Three*). Thus, this initial PCR incorporated a T7 promoter region and a poly d(T) tail to allow for the synthesis of a rcRNA with its own poly d(A) tail, suitable for the competitive RT-PCR assay. Amplification with these modified primers produced an amplicon which when amplified using the forward and reverse assay primers (*Chapter Three*) would give a product smaller than the target cDNA (*Figure 4.1*).

Table 4.1 Oligonucleotide sequences used for constructing the recombinant RNA internal standards, as described in the text.

Direction	Oligonucleotide sequences
βISFP	5'-TAATACGACTCACTATAGG-ATCCAAGGAGCCACTTCG-3'
βISRP	5'-TTTTTTTTTTTTTTTTTT-TTGTGGGACTAATGGAGG-AGTCAGAATAGCTTCTGC-3'
αISFP	5'-TAATACGACTCACTATAGG-GCAGCTATCATTCTGATC-3'
αISRP	5'-TTTTTTTTTTTTTTTTTT-AACAAGTACTGCAGTGGC-AGTGGACTCTGAGGTGAC-3'
mGAPISFP	5'-TAATACGACTCACTATAGG-GTGAACCATGAGAAGTAT-3'
mGAPISRP	5'-TTTTTTTTTTTTTTTTTT-AAACCGATGTCGTTGTCC-TGTCGCTGTTGAAGTCAG-3'

Figure 4.1 Schematic diagram of the construction strategy for the internal standard cDNAs for quantitative RT-PCR (not drawn to scale). The positions where the modified forward and reverse oligonucleotide primers anneal, are shown.





For **mCG-β**, the internal standard was 116 base pairs smaller than the target sequence of 433 base pairs. The **mCG-α** internal standard was 245 base pairs in length, compared to the mCG-α target of 325 base pairs in length. The internal standard for **mGAPDH** was 83 base pairs smaller than the mGAPDH target, which was 567 base pairs in length.

The IS was amplified in a 100 µl PCR reaction, using *Pwo* DNA polymerase and 15 pmoles of each primer (*as described in Section 2.5.2.1*). A master mix was prepared, containing 10x PCR buffer, 10 mM dNTPs, 15 pmoles of each primer (ISFP & ISRP) and *Pwo* DNA polymerase. The 99 or 97 µl reaction (mCG-β & -α and mGAPDH, respectively) was overlaid with mineral oil before 1 or 3 µl of diluted template (mCG-β: β5 500 ng/µl; mCG-α: α/pAX-8(+) 300 ng/µl (Simula *et al.* 1995); mGAPDH: pKS+/mGAPDH, 1035 ng/ml, diluted ten-fold) was added. The amplification was performed in duplicate with a negative control (no template) for 35 cycles at 60°C annealing (*LOUPWO35: as described in Section 2.5.2.1*).

The Expand™ High Fidelity system from Boehringer Mannheim (ExHF system) was also used in some instances, to amplify the IS sequence. Two master mixes were prepared, one (Master Mix 1) containing 10 mM dNTPs, 15 pmoles of each primer (ISFP & ISRP) and template and the other (Master Mix 2) consisting of 1x ExHF buffer and 2.6 units of ExHF enzyme mix (*as described in Section 2.5.2.3*). Master Mix 1 (minus the template) was prepared and aliquotted to the tubes and the mixture overlaid with mineral oil before the addition of the template. Master Mix 2 was

then prepared and added to the tubes and PCR was performed in duplicate with a negative control (water instead of template) under the parameters described in *Section 2.5.2.3* (*EXHF*).

The PCR products (both from *Puo* and *ExHF*) were then analysed by 2% agarose gel electrophoresis (*described in Section 2.5.3*). The relevant bands were excised and purified by BresaClean (*described in Section 2.5.5.1*) and a small portion of the purified products was re-electrophoresed with a DNA Mass ladder (*described in Section 2.5.3*), to estimate the amount of product present. Five microlitres of the purified product was re-amplified with the assay primers (*described in Chapter Three*), to determine that the purified product was the correct product. To obtain more IS product, 5 µl of the purified product was re-amplified with the IS primers (*Table 4.1*). Both of these assays were performed using a 50 µl PCR reaction, in duplicate with *Puo* DNA polymerase (*as described in Section 2.5.2.1*). A master mix was prepared, consisting of 10x PCR buffer, 10 mM dNTPs, 50 or 15 pmoles of each primer (assay primers or IS primers, respectively) and *Puo* DNA polymerase. The 45 µl reaction was overlaid with mineral oil, 5 µl of the purified PCR product added and then the tubes amplified under the same conditions as above (*LOUPWO35*).

The resultant PCR products were electrophoresed, the IS PCR products were excised from the gel and purified, as above. A small portion of the purified IS product was re-electrophoresed with a DNA Mass ladder, as above, to determine an approximate quantity for the *in vitro* transcription reaction.

The purified IS PCR product was then *in vitro* transcribed into rcRNA using T7 RNA polymerase (*as described in Section 2.5.6*). The quantity of rcRNA IS was determined by an OD reading using a spectrophotometer.

A dilution series (10^{-1}-10^{-11}) of rcRNA IS was reverse transcribed (*as described in Section 2.5.1*) and amplified (*as described in Section 2.5.2.2*) to check the reverse transcription and amplification efficiency of the rcRNA IS fragment. The reaction products were electrophoresed on a 2% agarose ethidium bromide-stained gel (*as described in Section 2.5.3*). Once the rcRNA IS fragment was known to amplify and dilute out, the next step was to add a constant amount of total RNA to a dilution series of rcRNA IS. This is described below in *Section 4.3*.

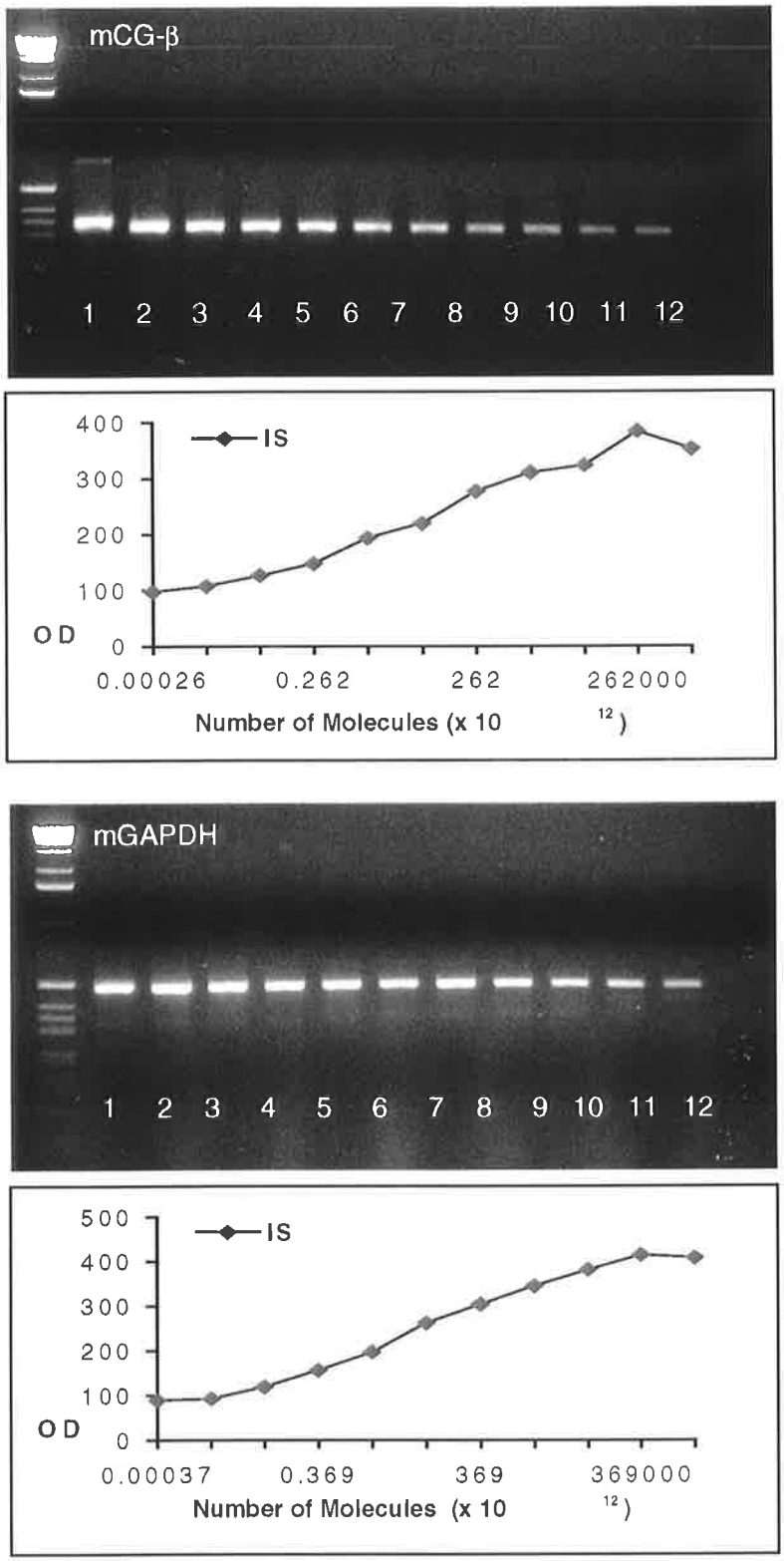
Some of the purified IS PCR product was not *in vitro* transcribed, but kept as rcDNA for the competitive PCR assay. The amount of rcDNA present was calculated by an OD reading using a spectrophotometer. A dilution series (10^{0}-10^{-10}) of the rcDNA IS was amplified (*as described in Section 2.5.2.2*) to check the amplification efficiency of the rcDNA IS fragment. The PCR products were electrophoresed and photographed (*as described in Section 2.5.3*; *Figure 4.2*). Once the rcDNA IS fragment was known to amplify and dilute out, the next step was to add a constant amount of cDNA to a dilution series of rcDNA IS. This is described below in *Section 4.3*.

Figure 4.2 mCG-β (a) and mGAPDH (b) rcDNA internal standard dilution PCR.

Figure 4.2a: Lanes 1-11, ten-fold serial dilutions of mCG-β rcDNA IS (from 2.62 x 10^{18} to 2.62 x 10^{8} copies); lane 12 contains water instead of cDNA. Lane M contains a 1Kb DNA ladder.

Figure 4.2b: Lanes 1-11, ten-fold serial dilutions of mGAPDH rcDNA IS (from 3.69 x 10^{18} to 3.69 x 10^{8} copies); lane 12 contains water instead of cDNA. Lane M is a 1Kb DNA ladder.

Figure 4.2


mCG-β
1 2 3 4 5 6 7 8 9 10 11 12
IS
400
300
200
100
0
OD
0.00026
0.262
262
262000
Number of Molecules (x 10 12)
mGAPDH
1 2 3 4 5 6 7 8 9 10 11 12
IS
500
400
300
200
100
0
OD
0.00037
0.369
369
369000
Number of Molecules (x 10 12)

4.3 Development of the Quantitative Assays

4.3.1 *RNA Preparation*

Target RNA:

Total RNA was isolated from marmoset placenta, pituitary and skeletal muscle and from Chinese Hamster Ovary (CHO) cell lines expressing mCG-β and mCG dimer, using the Chomczynski and Sacchi method (*as described in Section 2.4*). Each sample was quantitated using a spectrophotometer (*see Table 4.2*).

Internal Standard rcRNA:

Internal standard rcRNA was prepared for mCG-β and mGAPDH (*as described in Section 4.2*) and was quantitated using a spectrophotometer (*see Table 4.2*).

Table 4.2 Concentration of representative RNA samples.

RNA Sample	A_{260}/A_{280}	Concentration (in ng/ml)
Placenta	1.37	1568
Pituitary	1.19	620
mCG-β expressing CHO cells	1.31	456
mCG-β IS	1.50	756.4
mGAPDH IS	1.47	727.99

4.3.2 *Complementary DNA Synthesis*

4.3.2.1 Competitive RT-PCR assay

Both target (native) RNA and internal standard rcRNA were co-reverse transcribed initially by AMV-Reverse Transcriptase, in a 20 µl reaction (as described in *Section 2.5.1.1*). Briefly, a master mix was prepared containing 5x RT buffer, 10 mM dNTPs, 500 µg/ml oligo $(dT)_{15}$ primer, 36 U/ml RNasin, 100 mM DTT, 1 mg/ml acetylated BSA and 400 U AMV-RT enzyme.

To develop the assay with AMV RT, a constant amount of placental total RNA or CHO cell RNA was added as the target total RNA in 2 µl, whilst a dilution curve (for example: 10^{-6}-10^{-14}: corresponding to for mCG-β IS $1.66 \times 10^{3} \rightarrow 1.66 \times 10^{11}$ molecules) of rcRNA IS in 2 µl was added as the IS RNA. Positive and negative controls were also prepared: 1) target only, 2) highest concentration of IS only, 3) another positive control RNA (*for example* placenta) and 4) water only. The RT reaction was carried out at 42°C for 50 minutes, followed by a 5 minute period at 95°C and then quickly chilled to 4°C, ready for PCR amplification.

Chinese Hamster Ovary (CHO) cells expressing mCG-β were diluted so that the equivalent of 12 and 120 cells were added as target to a competitive RT-PCR assay. The cells were added to half of the reverse transcription (RT) reaction (RT buffer, dNTP, oligo (dT) and water) with the mCG-β IS rcRNA and heated to 100°C for 1 minute, to lyse the cells and release the RNA. The protein

components of the RT reaction (RNAsin, DTT, BSA and AMV-RT) were added and the RNA reverse transcribed, as above.

As the assay was to be used to quantitate mRNA levels from small amounts of tissue, Superscript™II RNaseH- Reverse Transcriptase was also used as the RT enzyme to develop the assay. Thus, both target RNA and IS rcRNA (mCG-β and mGAPDH) were co-reverse transcribed by Superscript™II in a 20 µl reaction (*as described in Section 2.5.1.2*). Briefly, 2 µl of target placental or pituitary total RNA (100 &/or 200 ng), 2 µl of rcRNA IS and 500 µg/ml oligo $(dT)_{15}$ primer were heated to 70°C for 10 minutes and quickly chilled to 4°C. The 5x first strand buffer, together with 100 mM DTT, 10 mM dNTP and 200 U Superscript™II were added to a final volume of 20 µls. The reaction was incubated at 42°C for 92 minutes, heated to 70°C for 15 minutes to inactivate the enzyme and then quickly chilled to 4°C, ready for PCR amplification.

Trophoblastic vesicle and embryo total RNA were also co-reverse transcribed with a ten-fold serial dilution of mGAPDH rcRNA IS.

4.3.2.2 Competitive PCR assay

Target cDNA:

Complementary DNA was synthesised from ~200 and ~270 ng of placental total RNA, as well as from two-fifths (10 µl) the total RNA of embryos, using Superscript™II as the RT-enzyme (*as described above, in Section 2.5.1.2 and Chapter Three*).

Internal Standard rcDNA:

Internal standard rcDNA was prepared for mCG-β and mGAPDH (*as described in Section 4.2*). Each rcDNA IS was quantitated using a spectrophotometer (*see Table 4.3*).

Table 4.3 Concentration of recombinant DNA internal standard samples.

rcDNA IS	A_{260}/A_{280}	Concentration (in ng/ml)
mCG-β rcDNA IS	1.53	48
mGAPDH rcDNA IS	1.69	52

4.3.3 *Polymerase Chain Reaction*

4.3.3.1 Competitive RT-PCR assay

After co-reverse transcription, 2 µl of cDNA was added to a 23 µl PCR reaction, containing buffer, dNTPs, primers and DNA polymerase (*as described in Section 2.5.2*). Briefly, a master mix was prepared containing 10x PCR buffer, 10 mM dNTPs, primers (*as described in Section 3.4.1*) and DNA polymerase. The PCR mixture was overlaid with mineral oil, the cDNA added and the reaction amplified using parameters suitable for the enzyme. Negative controls were also amplified, using water instead of cDNA.

Both *Pwo* DNA polymerase and *Taq* DNA polymerase were used for PCR amplification (*as described in Sections 4.2.1 and 4.2.2*).

4.3.3.2 Competitive PCR assay

After reverse transcription of the target RNA into cDNA, 2 µl of target cDNA and 2 µl of rcDNA IS were added to a 21 µl PCR reaction, using *Taq* DNA polymerase (*as described in Section 2.5.2*). The reaction was amplified for 30 cycles with a 55°C annealing step (*LOUTAQ30*).

In some cases, to enable the detection of the PCR products, a second round of PCR was performed (*as described in Chapter Three*). The primary PCR product was diluted ten-fold and a 2 µl aliquot subjected to a further 35 cycles of amplification (*LOUTAQ35*) in a fresh PCR tube, with the same primers and amplified with the conditions as described above.

4.3.4 *Gel Electrophoresis*

Eight to twelve microlitre aliquots of PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and photographed under UV illumination with Polaroid 667 film (*as described in Section 2.5.3*). Photographs were scanned, inverted and analysed by NIH image (*described in Section 2.5.4*).

4.3.5 *Analysis*

The number of molecules of each internal standard was determined using the following formulas:

1. Molecular Weight (MWt) of IS = 660 x number of bp of IS
2. nmoles of IS = MWt of IS ÷ ng/µl of IS
3. Number of molecules = concentration of IS (in moles) x dilution factor of IS x Avogadro's Number (6×10^{23})

A sample calculation: assuming that the concentration of IS is 300 ng/µl and that the equivalency point is at the 10^{-6} dilution.

IS	MWt	nmoles of IS	No. of molecules
mCG-β	209,220	697.4	4.18×10^{11}
mCG-α	161,700	539.0	3.23×10^{11}
mGAPDH	319,440	1064.8	6.39×10^{11}

NIH image aided in the determination of the optical density of each DNA band. The optical density of each band (target and internal standard) was plotted on a logarithmic graph against the number of molecules of internal standard added to the reaction.

4.3.6 *Results*

4.3.6.1 Competitive RT-PCR assay

Initially the competitive RT-PCR assay was developed with AMV-RT and *Pwo* DNA polymerase. However, as the assay was to be applied to small cell numbers, it was also developed with a reverse transcriptase enzyme that is more suited to small amounts of RNA: Superscript™II.

AMV-Reverse Transcriptase and *Pwo* DNA polymerase:

Initially the level of mCG-β expressed in 1 and 10 ng of mCG-β expressing CHO cell total RNA was investigated, (*see Table 4.4 and Figure 4.3*). The copy number was determined by plotting the number of molecules of internal standard added to the reaction on a logarithmic scale against the optical density of the PCR bands, which was determined by NIH image. For 1 ng, the copy number was determined to be 609,000 copies, whilst that for 10 ng was 6.16×10^{10} copies. Taking the amount of RNA added to the reaction into account, the result for the 10 ng assay is 6.16×10^{8} copies/ng of RNA.

Competitive RT-PCR assays to establish the sensitivity of the assay were also performed as the assay would be applied to RNA from small cell numbers. CHO cells expressing mCG-β were diluted so that the equivalent of 12 cells were added as target to a competitive RT-PCR assay. This was also carried out with approximately 120 CHO cells as target (*see Table 4.4 and Figure 4.4*). For both assays the bands on the gels are very faint and so NIH image could not accurately determine the optical density of the bands, thus a range of between 1.14×10^{7} and 1.14×10^{8} copies was seen for the 12 cell assay and between 1.14×10^{8} and 1.14×10^{9} copies for the 120 cell assay.

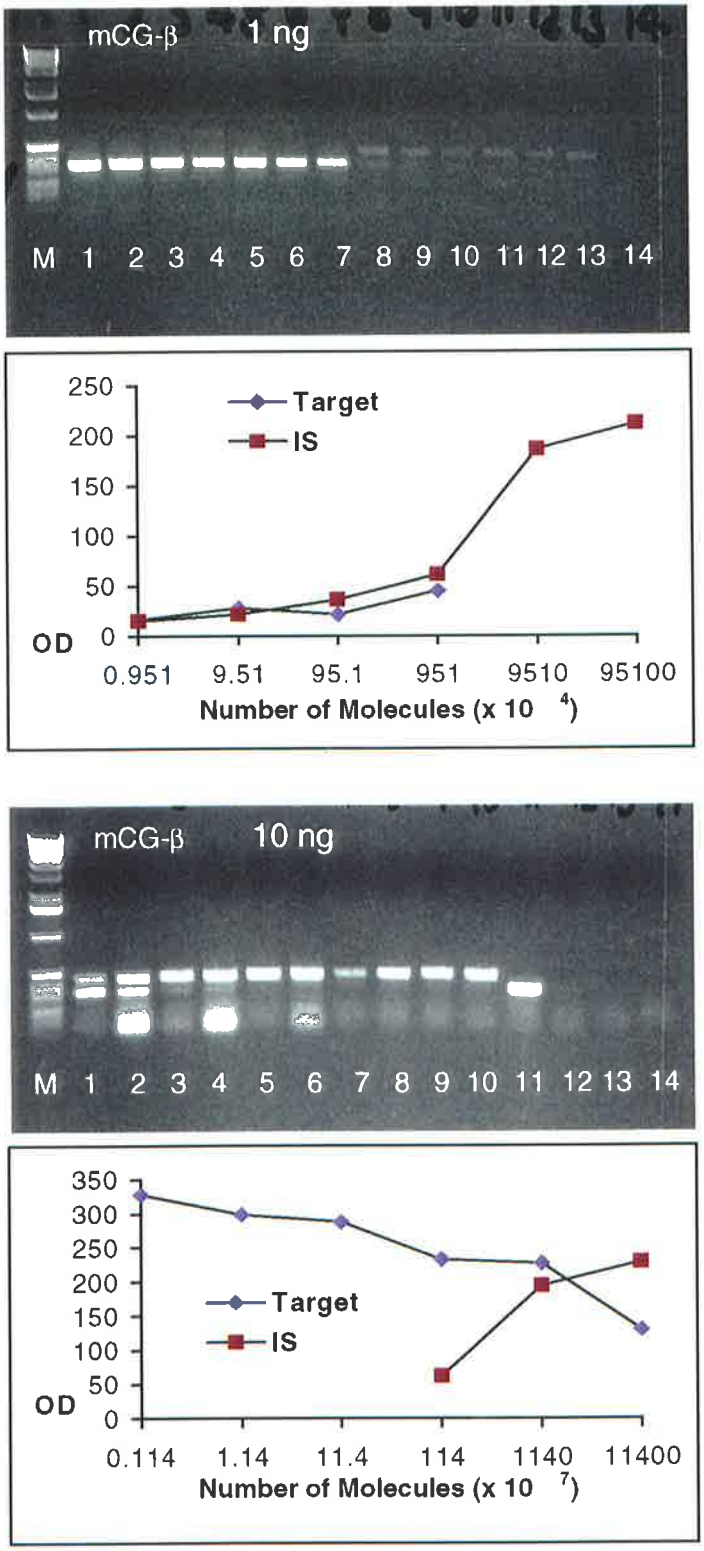
Figure 4.3 mCG-β competitive RT-PCR assays of 1 ng (a) and 10 ng (b) of CHO cell total RNA expressing mCG-β, using AMV-RT and *Pwo* DNA polymerase.

The graph below each photograph shows the number of molecules of internal standard against the optical density of the PCR bands of target and internal standard (as determined by NIH image) for both the target and internal standard.

Figure 4.3a: Lanes 1-12, ten-fold serial dilutions of rcRNA IS (from 9.51 x 10^{13} to 9.51 x 10^{2} copies); lanes 13 and 14 contain only target RNA and water, respectively. Lane M contains a 1Kb DNA ladder.

Figure 4.3b Lanes 1-9, ten-fold serial dilutions of rcRNA IS (from 1.14 x 10^{11} to 1.14 x 10^{3} copies); lanes 10 and 11 contain only target RNA and rcRNA IS, respectively. Lane M contains a 1Kb DNA ladder.

Figure 4.3


mCG-β 1 ng
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14
250
200
150
100
50
0
OD
Target
IS
0.951 9.51 95.1 951 9510 95100
Number of Molecules (x 10 4)
mCG-β 10 ng
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14
350
300
250
200
150
100
50
0
OD
Target
IS
0.114 1.14 11.4 114 1140 11400
Number of Molecules (x 10 7)

The next step was to apply the assay to RNA isolated from marmoset placenta and pituitary tissue. For both RNA samples, 100 ng of total RNA was co-reverse transcribed and amplified with mCG-β rcRNA IS (*see Table 4.4 and Figure 4.5*). For the placental RNA, the copy number was determined to be 3.14×10^9 copies and that for the pituitary RNA was 5.81×10^{12} copies. Taking the total RNA added to the reaction into account, the copy number per ng of RNA is 3.14×10^7 and 5.81×10^{10} copies for placental and pituitary RNA respectively.

Table 4.4 Results of mCG-β competitive RT-PCR assays using AMV-RT and *Pwo* DNA polymerase.

RNA	Equivalency Point*	Copy Number§
CHO cells		
1 ng	between lanes 7 & 8	6.09×10^5
10 ng	between lanes 1 & 2	6.16×10^{10}
12 cells	between lanes 7 & 8	between 1.14×10^7 & 1.14×10^{8}**
120 cells	between lanes 6 & 7	between 1.14×10^8 & 1.14×10^{9}**
Marmoset Tissue		
Placenta	between lanes 5 & 6	3.14×10^9
Pituitary	between lanes 2 & 3	5.81×10^{12}

*Equivalency point was determined by "eye-balling" the agarose gel.

§Copy Number was determined by plotting the optical density resolved from NIH image on a logarithmic graph against the number of molecules of internal standard added to the reaction.

**These results were determined from the number of molecules of internal standard added to the reaction. The bands on the gels were too faint for NIH image to determine the optical density.

Figure 4.4 mCG-β competitive RT-PCR assays of 12 (a) and 120 (b) CHO cells.

The graph below each photograph shows the number of molecules of internal standard against the optical density of the PCR bands of target and internal standard (as determined by NIH image) for both the target and internal standard.

Lanes 1-12, ten-fold serial dilutions of rcRNA IS (from 1.14×10^{14} to 1.14×10^{3} copies); lanes 13 and 14 contain only target RNA and rcRNA IS, respectively. Lane M contains a 1Kb DNA ladder.

Figure 4.4

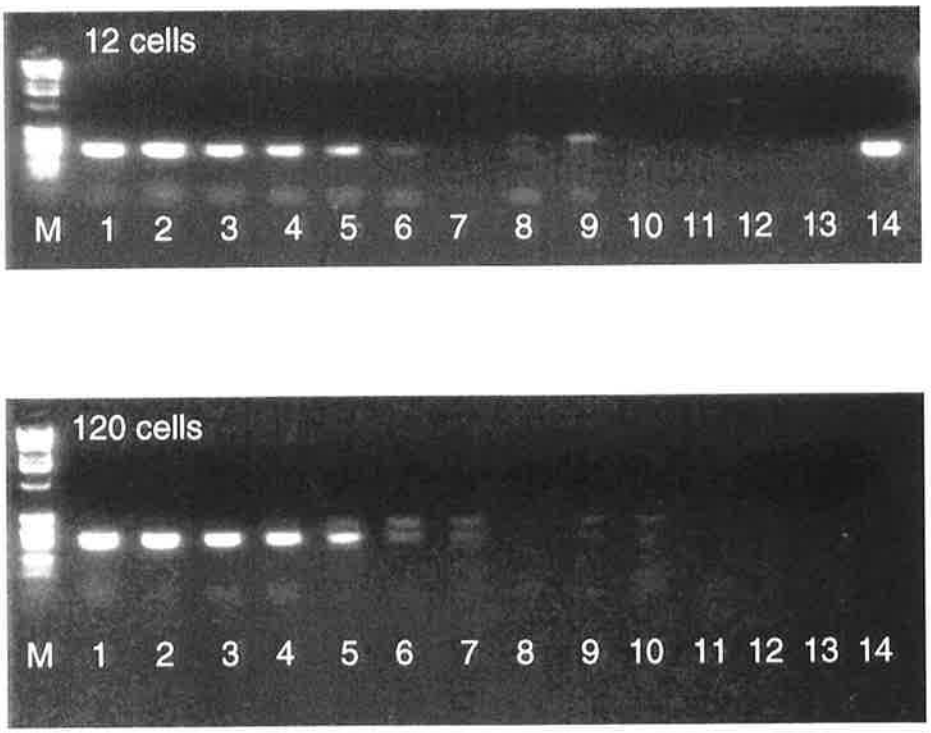
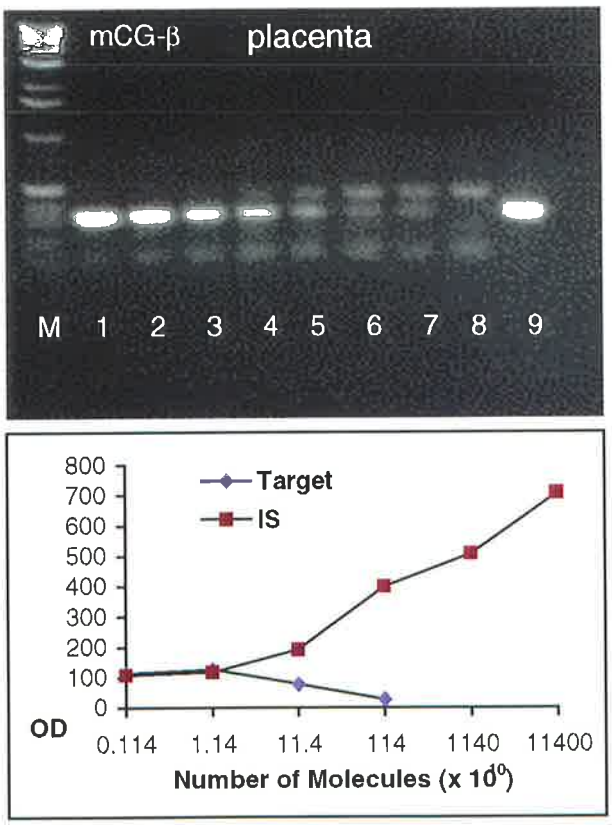

12 cells
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14
120 cells
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14

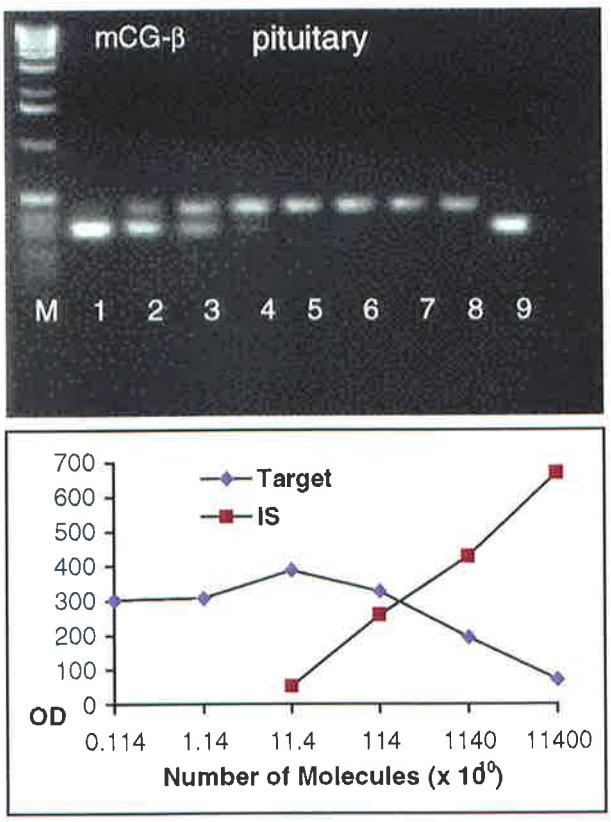
Figure 4.5 mCG-β competitive RT-PCR assays of marmoset placental (a) and pituitary (b) tissue.

The graph below each photograph shows the number of molecules of internal standard against the optical density of the PCR bands of target and internal standard (as determined by NIH image) for both the target and internal standard.

Lanes 1-7, ten-fold serial dilutions of rcRNA IS (from 1.14×10^{14} to 1.14×10^{8} copies); lanes 8 and 9 contain only target RNA and rcRNA IS, respectively. Lane M contains a 1Kb DNA ladder.

Figure 4.5


mCG-β
placenta
M 1 2 3 4 5 6 7 8 9
800
700
600
500
400
300
200
100
0
OD
Target
IS
0.114 1.14 11.4 114 1140 11400
Number of Molecules (x 10⁰)


mCG-β
pituitary
M 1 2 3 4 5 6 7 8 9
700
600
500
400
300
200
100
0
OD
Target
IS
0.114 1.14 11.4 114 1140 11400
Number of Molecules (x 10⁰)

Superscript™II and *Taq* DNA polymerase:

Initially the level of mCG-β and mGAPDH mRNA expressed in 100 and 200 ng of marmoset placental total RNA was investigated (*see Table 4.5 and Figures 4.6 and 4.7*). For mCG-β, the copy number for the 100 ng assay was determined at 1.40 x 10^{12} copies, whilst that for the 200 ng assay was 1.62 x 10^{12} copies. Taking the total RNA into account, the number of copies per ng of RNA are 1.40 x 10^{10} and 8.10 x 10^{9} copies. For mGAPDH, the copy number for the 100 and 200 ng assays was determined to be 2.57 x 10^{13} and 2.37 x 10^{14} copies, respectively. Based on the amount of RNA added to each reaction, there are 2.57 x 10^{11} and 1.185 x 10^{12} copies/ng of RNA in each assay.

This was followed by a competitive RT-PCR assay for mCG-β and mGAPDH in 100 ng of pituitary total RNA (*see Table 4.5 and Figure 4.8*). Copy number was determined at 1.51 x 10^{14} and 2.50 x 10^{12} for mCG-β and mGAPDH, respectively. Based on the total RNA, the results are 1.51 x 10^{12} and 1.25 x 10^{10} copies/ng of RNA for each assay.

The next step was to carry out mGAPDH competitive RT-PCR assays on RNA isolated from marmoset trophoblastic vesicles and embryos (*see Table 4.5 and Figure 4.9*). The equivalency point can be seen in lane 3 of Figure 4.9, which corresponds to 3.95 x 10^{11} copies of mGAPDH species/trophoblastic vesicle. Competitive RT-PCR assays were attempted with embryo RNA without success.

Figure 4.6 mCG-β competitive RT-PCR assays of 100 ng (a) and 200 ng (b) of marmoset placental RNA.

The graph below each photograph shows the number of molecules of internal standard against the optical density of the PCR bands of target and internal standard (as determined by NIH image) for both the target and internal standard.

Lanes 1-6, ten-fold serial dilution of rcRNA IS (from 1.66×10^{14} to 1.66×10^{9} copies); lanes 7, 8 and 9 contain only target RNA, rcRNA IS and water, respectively. Lane M contains a 1Kb DNA ladder.

Figure 4.6

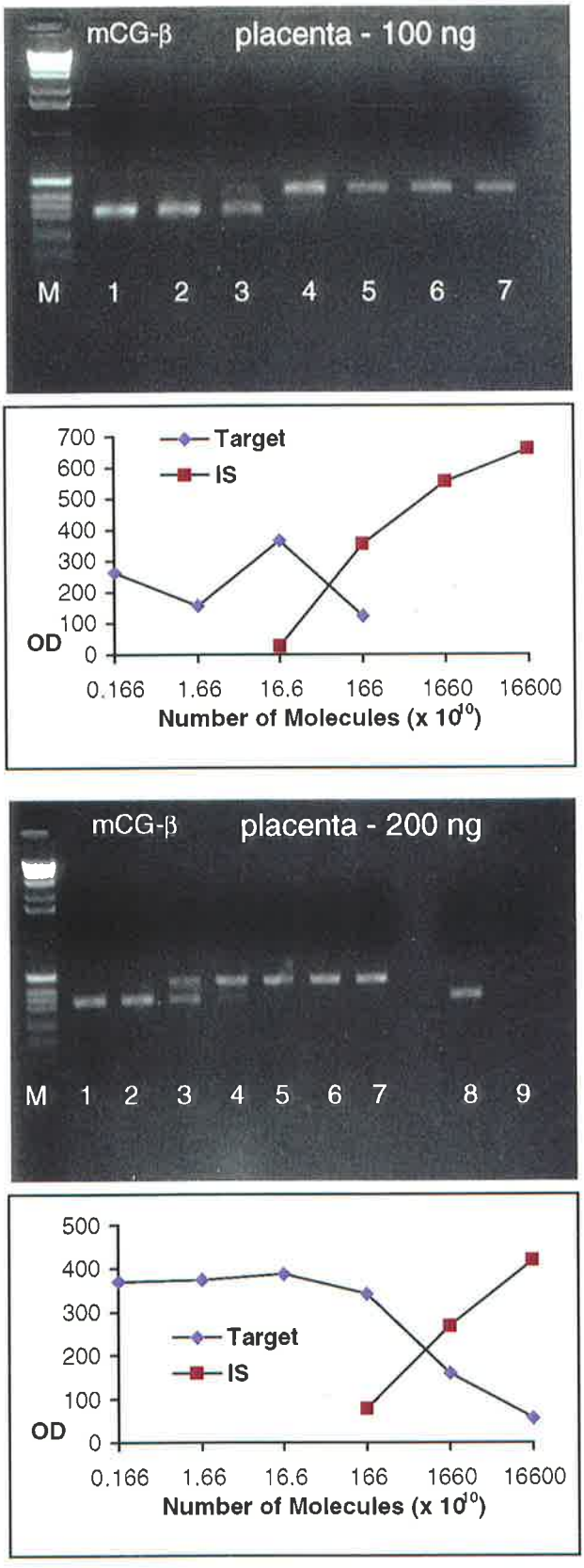
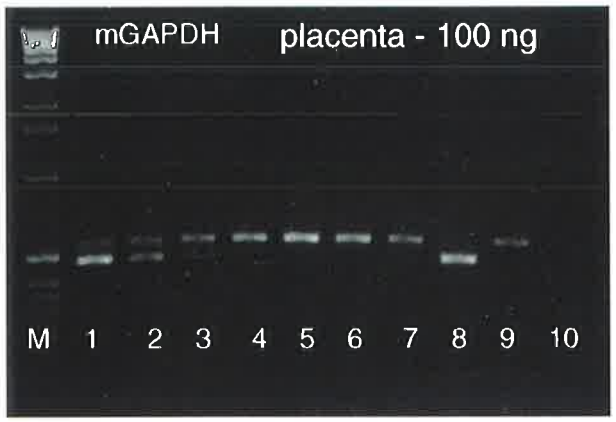

mCG-β placenta - 100 ng
M 1 2 3 4 5 6 7
Target
IS
OD
700
600
500
400
300
200
100
0
0.166 1.66 16.6 166 1660 16600
Number of Molecules (x 10^0)
mCG-β placenta - 200 ng
M 1 2 3 4 5 6 7 8 9
Target
IS
OD
500
400
300
200
100
0
0.166 1.66 16.6 166 1660 16600
Number of Molecules (x 10^0)

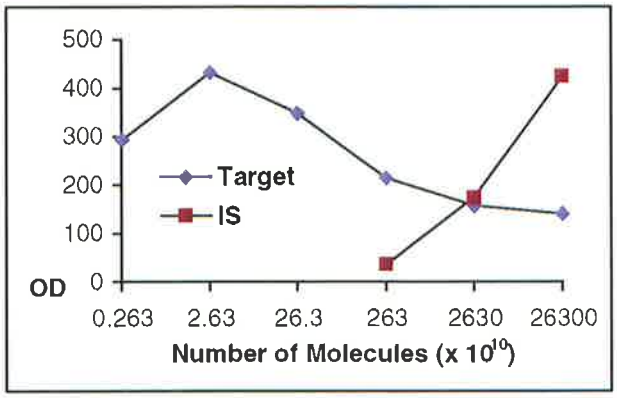
Figure 4.7 mGAPDH competitive RT-PCR assay of 100 ng (a) and 200 ng (b) of marmoset placental RNA.

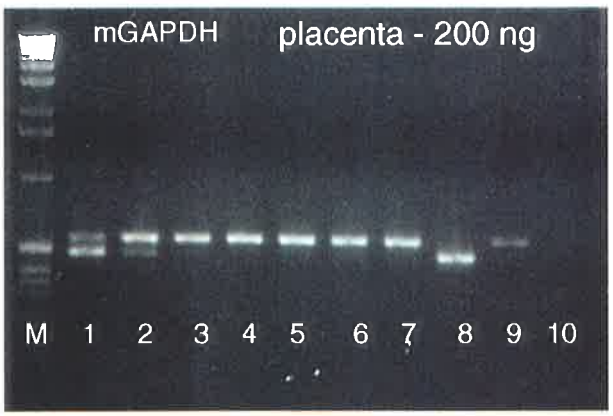
The graph below each photograph shows the number of molecules of internal standard against the optical density of the PCR bands of target and internal standard (as determined by NIH image) for both the target and internal standard.

Lanes 1-6, ten-fold serial dilution of rcRNA IS (from 2.63 x 10^{14} to 2.63 x 10^{9} copies); lanes 7 and 8 contain only target RNA and rcRNA IS, respectively. Lane 9 contains a different placental RNA sample and lane 10 contains water, instead of cDNA. Lane M contains a 1Kb DNA ladder.

Figure 4.7


mGAPDH
placenta - 100 ng
M 1 2 3 4 5 6 7 8 9 10


500
400
300
200
100
0
OD
Target
IS
0.263 2.63 26.3 263 2630 26300
Number of Molecules (x 10^10)


mGAPDH
placenta - 200 ng
M 1 2 3 4 5 6 7 8 9 10

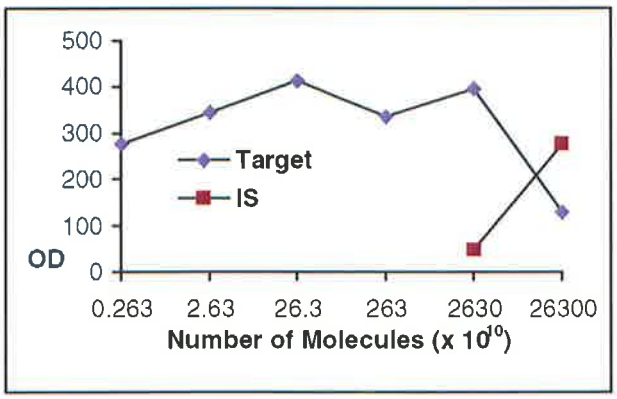
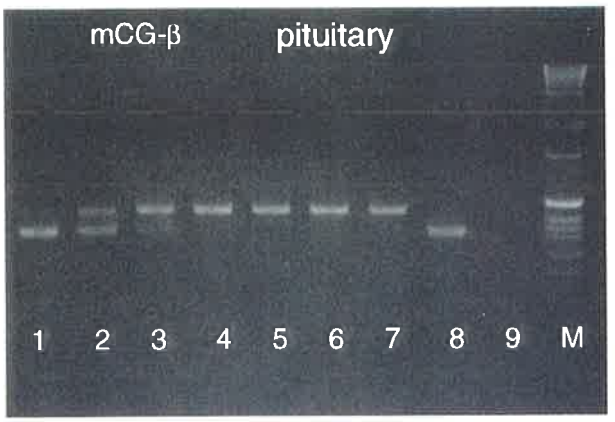

500
400
300
200
100
0
OD
Target
IS
0.263 2.63 26.3 263 2630 26300
Number of Molecules (x 10^10)

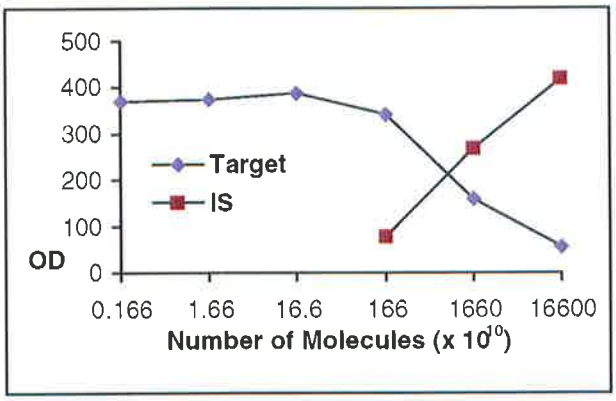
Figure 4.8 mCG-β (a) and mGAPDH (b) competitive RT-PCR assays of pituitary total RNA.

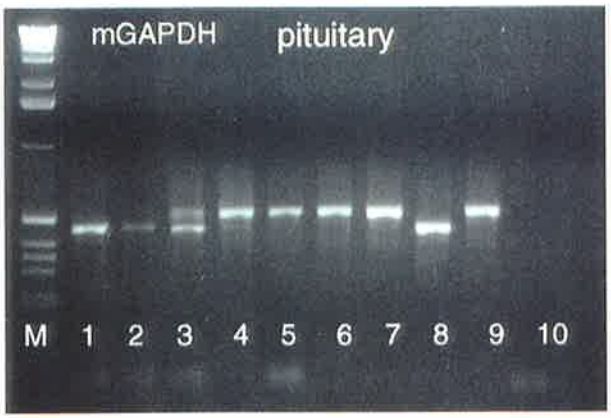
The graph below each photograph shows the number of molecules of internal standard against the optical density of the PCR bands of target and internal standard (as determined by NIH image) for both the target and internal standard.

Lanes 1-6, ten-fold serial dilutions of mCG-β or mGAPDH rcRNA IS (*mCG-β*: from 1.66 x 10^{14} to 1.66 x 10^{9} copies; *mGAPDH*: from 2.63 x 10^{14} to 2.63 x 10^{9} copies); lanes 7 and 8 contain only target RNA and rcRNA IS, respectively; lane 9 contains a different sample of placental RNA. Lanes 10 and 11 have water replacing RNA and cDNA, respectively. Lane M contains a 1Kb DNA ladder.

Figure 4.8


mCG-β
pituitary
1 2 3 4 5 6 7 8 9 M


500
400
300
200
100
0
OD
Target
IS
0.166 1.66 16.6 166 1660 16600
Number of Molecules (x 10^0)


mGAPDH
pituitary
M 1 2 3 4 5 6 7 8 9 10

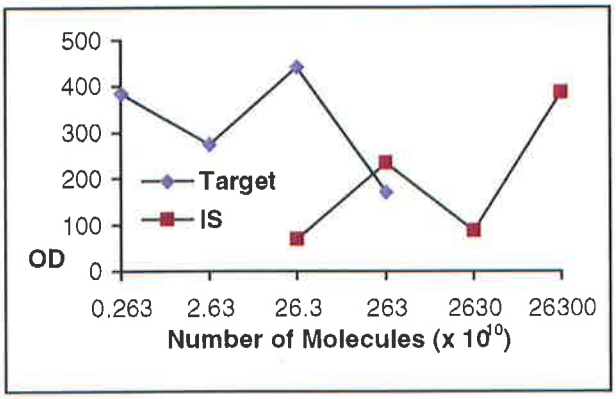
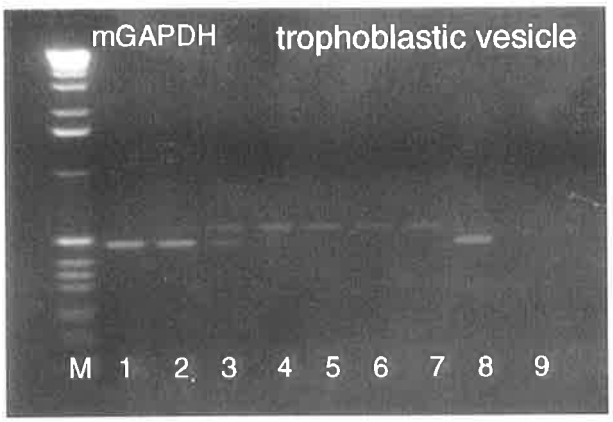

500
400
300
200
100
0
OD
Target
IS
0.263 2.63 26.3 263 2630 26300
Number of Molecules (x 10^0)

Figure 4.9 mGAPDH competitive RT-PCR assay of trophoblastic vesicle total RNA.

The graph below the photograph shows the number of molecules of internal standard against the optical density of the PCR bands of target and internal standard (as determined by NIH image) for both the target and internal standard.

Lanes 1-6, ten-fold serial dilutions of mGAPDH rcRNA IS (2.63×10^{13} to 2.63×10^{8} copies); lanes 7 and 8 contain only target RNA and rcRNA IS, respectively; lane 9 contains water replacing RNA. Lane M contains a 1Kb DNA ladder.

Figure 4.9


mGAPDH
trophoblastic vesicle
M 1 2 3 4 5 6 7 8 9

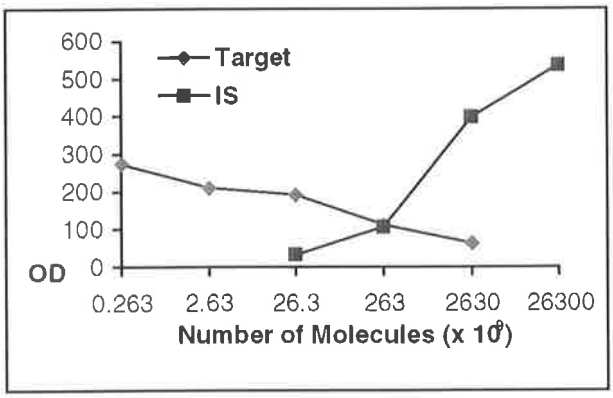

600
500
400
300
200
100
0
OD
Target
IS
0.263
2.63
26.3
263
2630
26300
Number of Molecules (x 10⁰)

Table 4.5 Results of mCG-β and mGAPDH competitive RT-PCR assays using Superscript™II and *Taq* DNA polymerase.

RNA		Equivalency Point*	Copy Number§
placenta			
mCG-β	100 ng	between lanes 3 & 4	1.4×10^{12}
	200 ng	lane 3	1.62×10^{12}
mGAPDH	100 ng	lane 2	2.57×10^{13}
	200 ng	between lanes 1 & 2	2.37×10^{14}
pituitary			
mCG-β	100 ng	lane 2	1.51×10^{14}
mGAPDH	100 ng	lane 3	2.5×10^{12}
mGAPDH only			
trophoblastic vesicle		lane 3	3.95×10^{11}

*Equivalency point was determined by "eye-balling" the agarose gel.

§Copy Number was determined by plotting the optical density resolved from NIH image on a logarithmic graph against the number of molecules of internal standard added to the reaction.

4.3.6.2 Competitive PCR Assay

The level of mCG-β cDNA was quantitated in marmoset placental cDNA, which had been reverse transcribed from ~270 ng of placental RNA, whilst mGAPDH cDNA levels were quantitated in placental cDNA which had been reverse transcribed from 200 ng of placental RNA (*see Table 4.6 and Figure 4.10*). The equivalency point for the first assay was seen between lanes 1 and 2, which equals approximately 2.45×10^{11} copies of mCG-β species; this corresponds to 9.07×10^{8} copies/ng of RNA. For the second assay, the equivalency point was seen between lanes 2 and 3, equalling

3.17 x 10^{14} copies of mGAPDH species, which corresponds to 1.585 x 10^{12} copies/ng of RNA.

The level of mCG-β was then investigated in cDNA synthesised from a single morula and a single expanded blastocyst (*from Chapter Three*). A second round of amplification was required to enable clearer visualisation of the DNA bands (*see Table 4.6 and Figure 4.11*). Marmoset CG-β was estimated at 2.10 x 10^{9} and 2.60 x 10^{9} copies in the morula and expanded blastocyst, respectively.

Table 4.6 Results of mCG-β and mGAPDH competitive PCR assays.

cDNA	**Equivalency Point***	**Copy Number§**
placenta		
mCG-β	between lanes 1 & 2	2.45 x 10^{11}
mGAPDH	between lanes 2 & 3	3.17 x 10^{14}
mCG-β only		
morula	between lanes 3 & 4	2.10 x 10^{9}
expanded blastocyst	between lanes 5 & 6	2.60 x 10^{9}

*Equivalency point was determined by "eye-balling" the agarose gel.

§Copy Number was determined by plotting the optical density resolved from NIH image on a logarithmic graph against the number of molecules of internal standard added to the reaction.

Figure 4.10 mCG-β (a) and mGAPDH (b) competitive PCR assay of placental cDNA. Below each photo is the analysis by NIH image.

The graph below each photograph shows the number of molecules of internal standard against the optical density of the PCR bands of target and internal standard (as determined by NIH image) for both the target and internal standard.

Lanes 1-6, ten-fold serial dilutions of rcDNA IS (*mCG-β*: from 2.62 x 10^{11} to 2.62 x 10^{6} copies; *mGAPDH*: from 3.69 x 10^{15} to 3.69 x 10^{10} copies); lanes 7 and 8 contain only target cDNA and rcDNA IS, respectively. Lane 9 contains a different cDNA sample (*mCG-β*: trophoblastic vesicle; *mGAPDH*: placenta). Lane M contains a 1Kb DNA ladder.

Figure 4.10

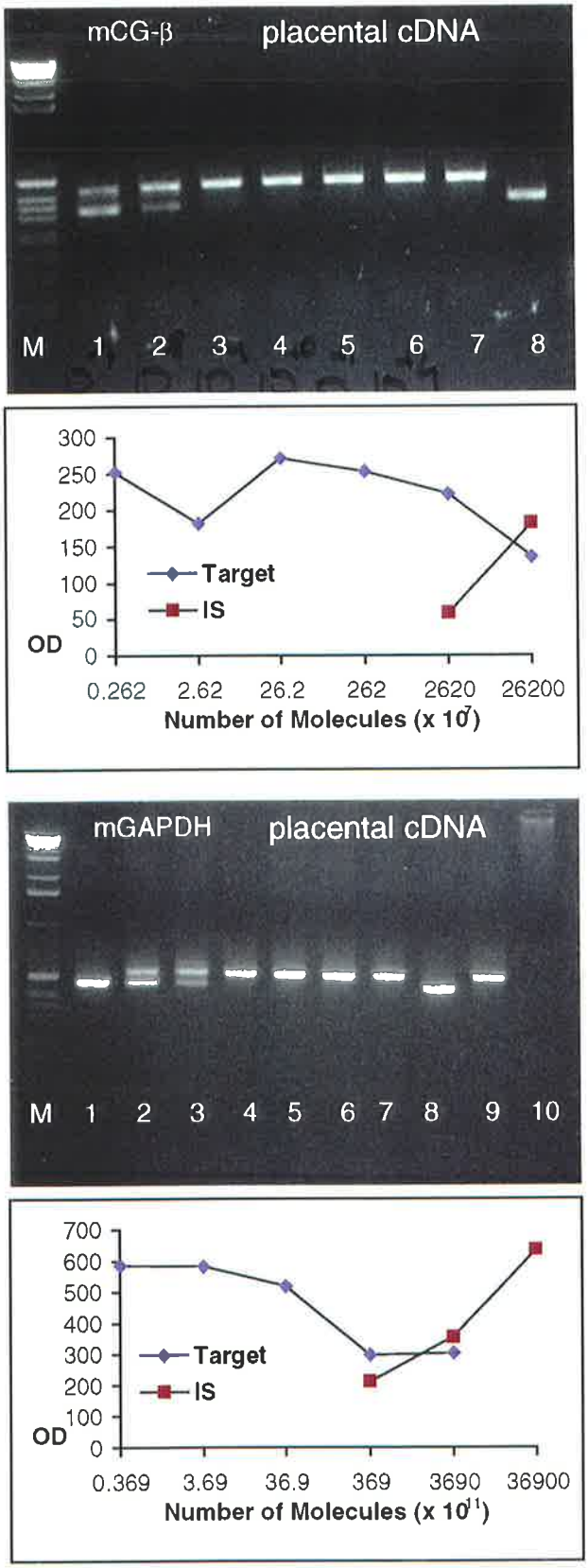
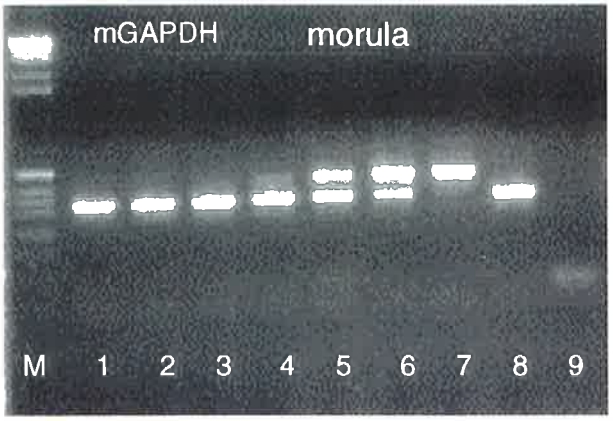

mCG-β placental cDNA
M 1 2 3 4 5 6 7 8
300
250
200
150
100
50
0
OD
Target
IS
0.262 2.62 26.2 262 2620 26200
Number of Molecules (x 10⁷)
mGAPDH placental cDNA
M 1 2 3 4 5 6 7 8 9 10
700
600
500
400
300
200
100
0
OD
Target
IS
0.369 3.69 36.9 369 3690 36900
Number of Molecules (x 10¹¹)

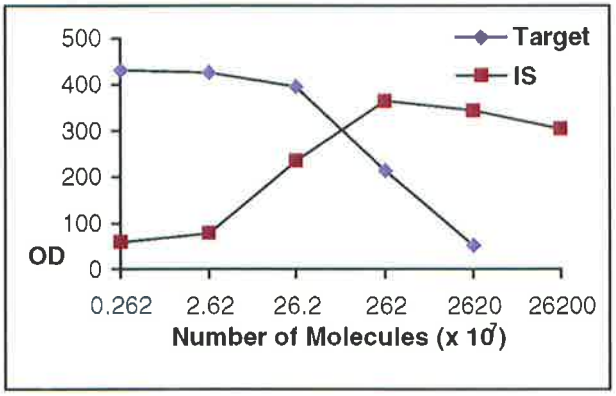
Figure 4.11 mCG-β competitive PCR assay of a morula (a) and an expanded blastocyst (b). Below each photograph is the analysis by NIH image.

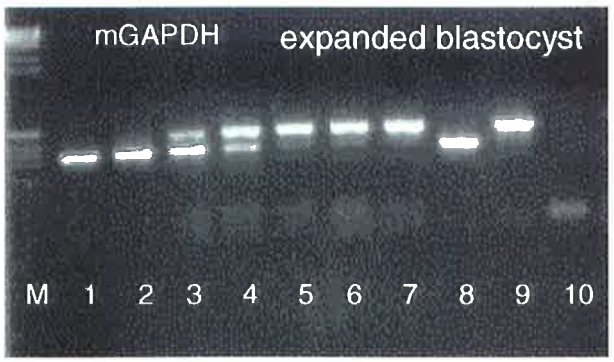
The graph below each photograph shows the number of molecules of internal standard against the optical density of the PCR bands of target and internal standard (as determined by NIH image) for both the target and internal standard.

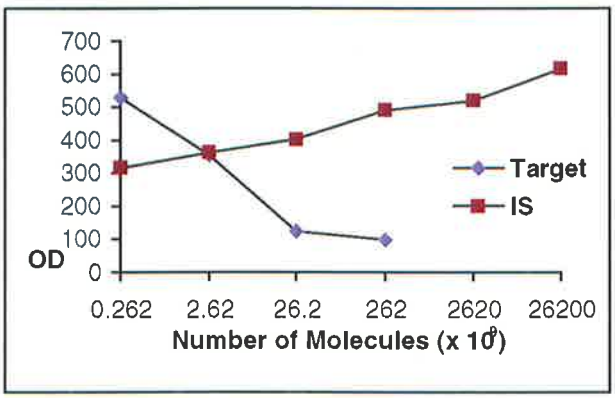
Lanes 1-6, ten-fold serial dilutions of rcDNA IS (*morula*: from 2.62 x 10^{11} to 2.62 x 10^{6} copies; *expanded blastocyst*: from 2.62 x 10^{8} to 2.62 x 10^{3} copies); lanes 7 and 8 contain only target cDNA and rcDNA IS, respectively. *Figure 4.11a*: lane 9, contains water. *Figure 4.11b*: lanes 9 and 10 contain trophoblastic vesicle cDNA and water, respectively. Lane M contains a 1Kb DNA ladder.

Figure 4.11


mGAPDH
morula
M 1 2 3 4 5 6 7 8 9


500
400
300
200
100
OD
0
Target
IS
0.262 2.62 26.2 262 2620 26200
Number of Molecules (x 10⁷)


mGAPDH
expanded blastocyst
M 1 2 3 4 5 6 7 8 9 10


700
600
500
400
300
200
100
OD
0
Target
IS
0.262 2.62 26.2 262 2620 26200
Number of Molecules (x 10⁸)

4.5 Discussion

This chapter has described the development of a competitive RT-PCR assay for quantitating mCG-β and mGAPDH mRNA expression, in a variety of marmoset tissues.

Due to potential problem with amounts of RNA recoverable from marmoset embryos an alternative, competitive PCR assay was also developed. These two assays will subsequently be used to detect any changes in the amount of mRNA being expressed by the embryo in the presence or absence of cytokines and growth factors (*Chapter Six*). Moreover, as the amount of RNA recovered from an embryo is so small, a second round of amplification was required to enable visualisation of the PCR products.

Both mCG-β and mGAPDH rcRNA internal standards reverse transcribed and amplified efficiently and diluted out when a ten-fold serial dilution of the IS was reverse transcribed and amplified. *Pwo* DNA polymerase was found to be the best DNA polymerase to do the initial amplification because it has proof-reading activity.

Unfortunately, the mCG-α rcRNA IS did not seem to amplify correctly from the template. Furthermore, when it was purified from the agarose and re-amplified with both the assay and IS primers, it amplified inconsistently. Another enzyme was tested (Expand High Fidelity), but the mCG-α IS still did not seem to amplify consistently. Moreover, the mCG-α rcDNA IS did not amplify and dilute out when a ten-fold serial dilution PCR was done, therefore a mCG-α quantitative assay was not developed. As

can be seen in *Figure 4.2*, the mCG-β and mGAPDH rcDNA IS diluted out when a ten-fold serial dilution PCR was done.

Initially, the enzymes AMV-RT and *Pwo* DNA polymerase were used to develop the competitive RT-PCR assay, however, during the qualitative RT-PCR project (*Chapter Three*), it was discovered that AMV-RT was not efficient at reverse transcribing RNA from small cell numbers and so Superscript™II was used. Also, *Taq* DNA polymerase was found to be more time- and cost-effective than *Pwo* DNA polymerase, as well as more suited to the annealing temperatures of the assay primers. Nevertheless, the results for the competitive RT-PCR assay using both sets of enzymes on two different pituitary RNA samples were consistent, as can be seen in *Tables 4.3 & 4.4 and Figures 4.5b & 4.8a.* The competitive RT-PCR using the two sets of enzymes for the placental RNA were not as consistent with an approximately 100-fold difference between the two results (*see Tables 4.3 & 4.4 and Figures 4.5a & 4.7a*). Two different placental RNA samples were used, thus assay variations could explain the difference in copy number. The results obtained from AMV-RT and *Pwo* DNA polymerase have been reported in the literature (Gameau *et al.* 1995). In that study it was reported that there was a 1000-fold higher level of mCG-β-like species in the marmoset pituitary compared to the placental RNA sample. Both products were sequenced and as the product from the pituitary had the same sequence to the published placental mCG-β sequence (Simula *et al.* 1995) and that it was consistently the only product obtained, it may indicate that the marmoset may have an LH-β subunit peptide

which is structurally similar to mCG-β with the C-terminal extension.

Changing the concentration of target CHO cell RNA changes the amount of IS required to achieve equivalency, as can be seen in *Figure 4.3*, although the increase in copy number was surprising. The CHO cells were also used to demonstrate the sensitivity of the competitive RT-PCR assay, by using the equivalent of 12 or 120 cells as target (*see Table 4.3 and Figure 4.4*). This change in cell number also proportionately increased the amount of IS required to achieve equivalency. Unfortunately, the photographs were very faint and accurate quantitation could not be performed. As can be seen in *Figures 4.6 and 4.7*, changing the concentration of placental RNA from 100 to 200 ng, as above, changed the amount of IS required to achieve equivalency in both the mCG-β and mGAPDH competitive RT-PCR assays. However, the mCG-β assays did not change the equivalency as expected (a factor of 1.16 increase in copy number compared to an expected factor of 2 increase). The copy number for the 200 ng mGAPDH assay increased by a factor of 9.22, compared to the 100 ng assay, which is substantially more than the expected factor of 2 increase. The reason for these discrepancies is unknown, it could be assay variation, but it highlights the limitations of these assays.

Marmoset trophoblastic vesicles and embryos were the next RNA samples investigated. The RNA was not quantitated by spectrophotometer, as there was so little RNA present and a second round of amplification was required, similar to the qualitative RT-

PCR (*see Chapter Three*) to enable clearer detection of the PCR products, especially the target. As trophoblastic vesicles consist of cytotrophoblast cells and do not usually express mCG-β, only the mGAPDH competitive RT-PCR was done (*see Figure 4.9a*). However, a few vesicles have been found to express mCG-β and competitive RT-PCR assays have been attempted, but the vesicles do not consistently give good results. The competitive RT-PCR assay was subsequently tested on marmoset embryos, however, under the current conditions was not sensitive enough to quantitate the limited amount of mCG-β mRNA, even though mGAPDH mRNA was able to be quantitated (*see Figure 4.9b*). Thus, a competitive PCR assay was developed with placental cDNA and tested on trophoblastic vesicle fragments, trophoblastic vesicles and finally, on marmoset embryos. This competitive PCR assay was used to quantitate the amount of mCG-β within the marmoset embryo at two stages of development (morula and expanded blastocyst). It was found that embryos have a variable level of gene expression and are thus, very individual and that gene expression is dependent on the viability of the embryo.

The competitive (RT-)PCR assay is a powerful and sensitive tool for assessing the relative abundance of mRNA in tissue from which little RNA can be obtained. Furthermore, with the analysis of an appropriate reference mRNA species by the same method, it is possible to monitor changes in the levels of expression of genes in very limited tissue samples. While these assays may not reflect an accurate measure of the level of mRNA copies in the various

tissues tested, it is possible to use the assay to assess the impact of factors on CG expression in relative terms.

Chapter Five

ELISA

5.1 Introduction

Until recently, the methods available for monitoring mCG levels throughout pregnancy of the marmoset monkey and in early cultured marmoset embryos were limited to expensive and labour intensive bioassays. One assay, developed by Ziegler and associates (1986), was an *in vitro* bioassay using mouse leydig cells. It is very sensitive and able to detect hCG levels as low as 100 pg/ml (Seshagiri and Hearn 1993). An alternative assay, an in vitro bioassay for LH/CG, developed by Ascoli (1981), uses a cell line derived from a mouse leydig cell tumour. We have used this bioassay in our laboratory, monitoring bioactive mCG (Simula *et al.* 1995).

Although sensitive, the bioassays are much more labour-intensive and can not distinguish between CG and LH. With specific immunoassays, there is the possibility of measuring CG or LH independently. Chambers and Hearn (1979) and Ziegler *et al.* (1986) have described heterologous antibody radioimmunoassays (RIA) for mCG using polyclonal antisera. Recently, a mouse monoclonal antibody, raised against the C-terminus of bovine LH-β, has been characterised and found to crossreact with both human and marmoset CG/LH (Matteri *et al.* 1987; Ziegler *et al.* 1993; Rosenbusch *et al.* 1994; Simula *et al.* 1995). This monoclonal antibody has been used to develop a simple competitive RIA (Ziegler *et al.* 1993), which is less sensitive than the mouse leydig cell bioassay (Seshagiri, and Hearn 1993). This antibody has also been the basis of the assay we developed.

Enzyme-linked immunosorbent assay (ELISA) is a powerful technique routinely applied to the detection and quantitation of a wide variety of analytes. In classical ELISA, antibodies for an antigen are detected in fluids. The ELISA we devcloped was a double antibody sandwich assay. The plastic microtitre plate was coated with anti-rabbit immunoglobulin which captured the primary antibody, a rabbit polyclonal raised against 6-Histidine-tagged recombinant mCG (R64). The sample was then added and any antigen present bound to the polyclonal antibody. After washing, which removed any unbound antigen, a biotinylated secondary antibody (monoclonal anti-bovine LH-β; 518B7) was added. A second wash, to remove any unbound labelled antibody, was followed by the addition of a substrate for development of the colour. The amount of colour was directly proportional to the amount of antigen present in the sample.

The combination of the specific monoclonal antibody for bLH-β and the polyclonal antibody for mCG makes this ELISA sensitive and specific for mCG and able to detect the small amounts of protein secreted by marmoset embryos.

Thus, the aim of the project described in this chapter was to characterise the mCG ELISA, for specificity and sensitivity, using recombinant mCG and marmoset biological fluids.

5.2 Materials and Methods

The detailed procedure for the ELISA is found in *Chapter Two, Section 2.7.* The development and characterisation of the ELISA has been described in the literature (Amato *et al.* 1998). However, some of the characteristics are included in this section.

5.2.1 *Sensitivity of the ELISA*

The sensitivity of the assay was determined by performing assays with various concentrations of the standard. The standard was either rmCG dimer or rmCG-β ranging from 0.01 to 24 ng/ml. CG levels were also measured by the ELISA in 27 medium samples collected from trophoblastic vesicle cultures and compared with CG levels measured by the bioassay (MA 10 cell assay: Simula *et al.* 1995).

5.2.2 *Recoveries from the ELISA*

Marmoset plasma, urine and embryo culture medium were spiked with three different concentrations (range 2.38 - 11.9 ng/ml) of rmCG. This was done in triplicate and the results tested against the expected value using the paired *t*-test.

5.2.3 *Crossreactivity with Other Gonadotrophins*

The cross-reactivity of related molecules was tested, on a molar basis. The following gonadotrophins were used: baboon LH (baLH) and its beta subunit (baLH-β), cynomolgus monkey LH (moLH) and its beta subunit (moLH-β), baboon FSH (baFSH), cynomolgus monkey FSH (moFSH) and hCG.

5.2.4 *Parallelism*

Extracts of placenta and pituitaries, pregnant marmoset urine and plasma, recombinant mCG (rmCG) and its beta subunit (rmCG-β), 6-histidine tagged mCG (rmCG-6His) and media collected from cultured trophoblastic vesicles were serially diluted. These dilutions were assessed for immunoactivity and the slopes of the dilution curves compared using statistical analyses. Firstly, a linear analysis of each dilution curve was performed, then, secondly the resulting β values (the slope) and its standard error were subjected to the Student's *t*-test.

5.2.5 *Collection of Biological Fluids*

5.2.5.1 Marmoset Monkey Plasma

Plasma from cycling or pregnant female marmoset monkeys was obtained by taking blood (0.2 - 0.4 ml) from the femoral vein up to three times a week (*as described in Section 2.2.1*). Plasma

was also obtained from cycling monkeys around the time of ovulation.

5.2.5.2 Trophoblastic Vesicle and Embryo Culture Medium

Embryos were collected by the non-surgical flushing technique (*as described in Section 2.2.3*) and cultured through to the trophoblastic vesicle stage. Four embryos were cultured individually in 1 ml of medium in 4-well plates (Nunc), allowed to hatch and attach to the plate. The medium was changed after the first day of culture and subsequently every three - five days. Medium was also collected from individual trophoblastic vesicle cultures. Some vesicles were micro-punctured to obtain the fluid within the vesicle, which was also investigated for mCG protein.

5.3 Results

5.3.1 *Sensitivity of the ELISA*

The sensitivity of the assay was 103 pg/ml (5.2 pg/well) for mCG-β and 476 pg/ml (24 pg/well) for mCG dimer. The inter-assay variation measured in culture medium containing mean levels of 1.6, 11.6 and 18.25 ng/ml of recombinant mCG was 16, 13.5 and 9.8 %, respectively. The CG levels measured by ELISA were also compared to levels determined by bioassay. Twenty-seven trophoblastic vesicle media samples were assayed and the results

from the ELISA correlated well with those from the bioassay (correlation coefficient 0.9672).

5.3.2 *Recoveries from the ELISA*

The mean recoveries of rmCG spiked in triplicate at three different concentrations (range 2.38 - 11.9 ng/ml) in marmoset plasma, marmoset urine and embryo culture medium were 92% (85 - 100%; $p = 0.0827$), 112% (103 - 123%; $p = 0.1499$) and 104% (89 - 118%; $p = 0.1499$), respectively.

5.3.3 *Crossreactivity with Other Gonadotrophins*

The cross-reactivity of related molecules was tested, on a molar basis, measured at 30% signal to noise ratio (*that is* the concentration of hormone which resulted in an OD reading, above background, of 30% of the maximum OD reading obtained with recombinant mCG). Shown in *Figure 5.1* is the dose-response curves of the related molecules as listed in *Section 5.2.3*. The 30% signal to noise ratio for rmCG, baLH, baLH-β, moLH, moLH-β, baFSH, moFSH and hCG compared to rmCG-β is 23, 0.07, 2.3, <0.07, 0.21, <0.07, <0.07 and <0.007%, respectively.

Figure 5.1 Crossreactivity of related gonadotrophins: recombinant mCG (rmCG), its beta subunit (rmCGb), baboon LH (baLH), its beta subunit (baLHb), cynomolgus monkey LH (moLH), its beta subunit (moLHb), baboon FSH (baFSH), cynomolgus monkey FSH (moFSH) and human CG (hCG; CR127).

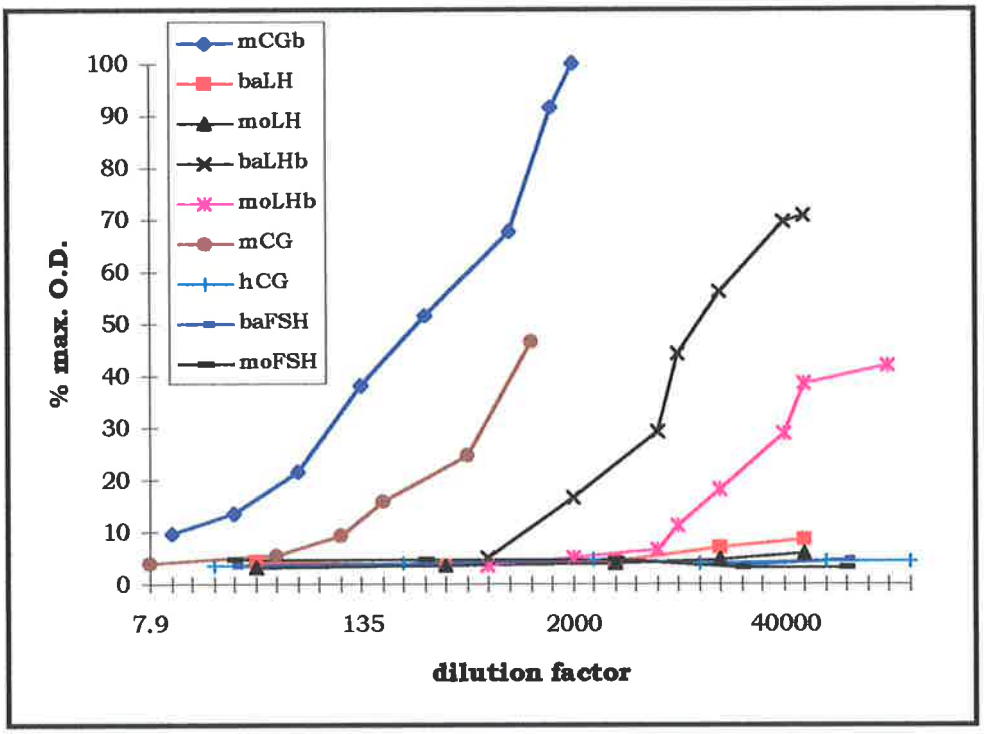
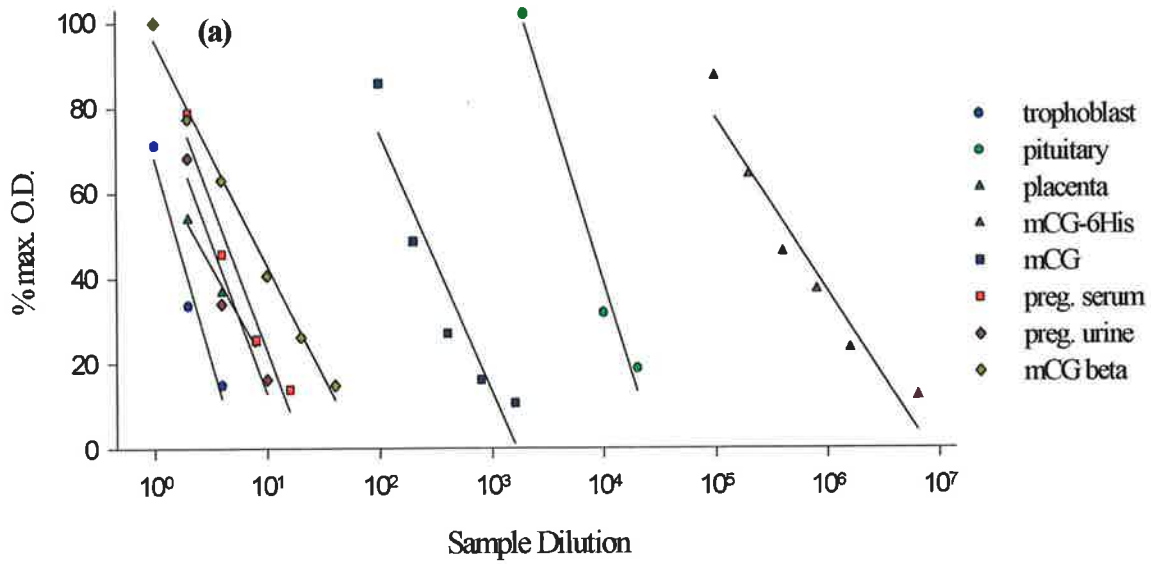


Figure 5.2 Dilution curves of marmoset pituitary and placental extracts, pregnant marmoset urine and plasma, rmCG, its beta subunit, 6-histidine tagged mCG and culture medium from trophoblastic vesicles.



5.3.4 *Parallelism*

Extracts of placenta and pituitaries, pregnant marmoset urine and plasma, recombinant mCG (rmCG) and its beta subunit (rmCG-β), 6-histidine tagged mCG (rmCG-6His) and media collected from cultured trophoblastic vesicles were serially diluted. These dilutions resulted in regression lines with slopes that were not statistically significantly different ($p > 0.05$; *see Figure 5.2*).

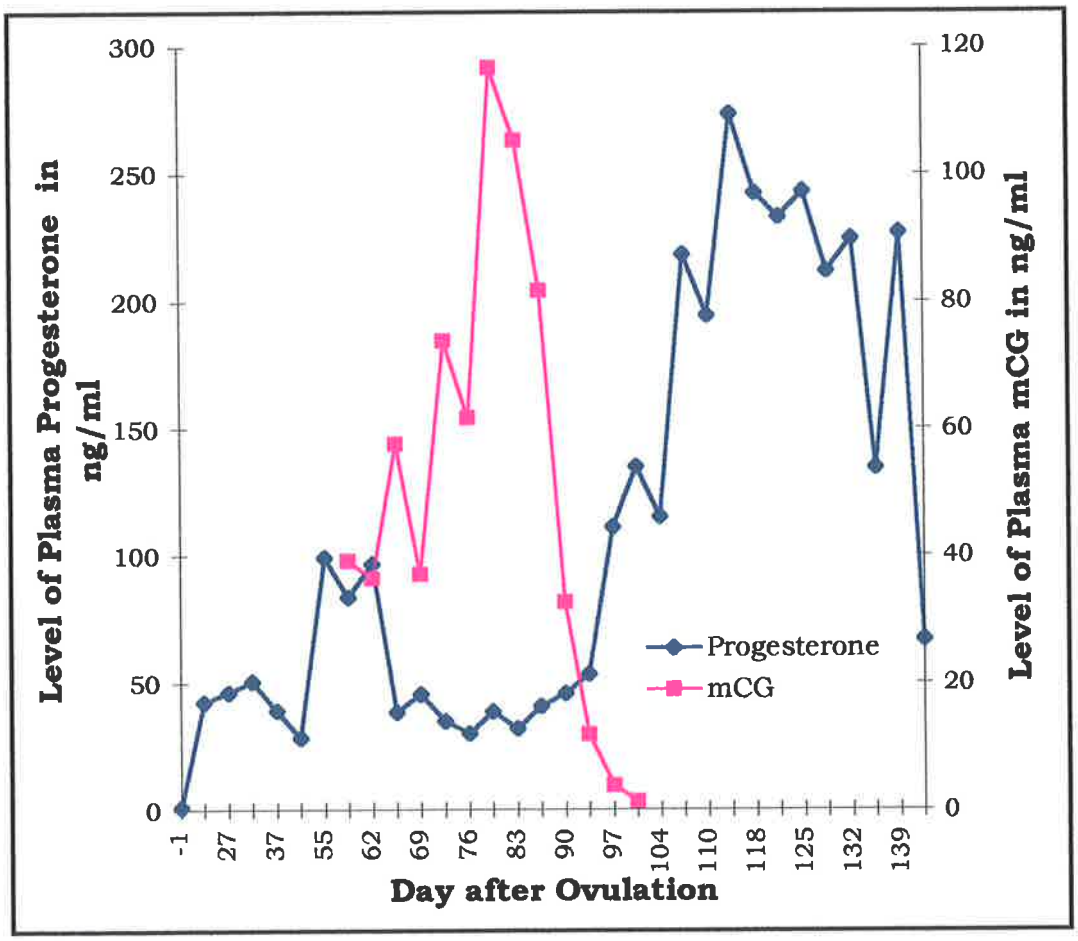
5.3.5 *mCG Protein in Monkey Plasma*

5.3.5.1 mCG Protein during Pregnancy

The ELISA measured mCG protein in the plasma of one monkey from approximately Day 58 after ovulation to Day 100 and from two monkeys from the start of pregnancy through to approximately Day 43 or 44 of pregnancy. These results are shown in *Figures 5.3 - 5.5* with the corresponding progesterone levels.

Plasma progesterone levels were measured for the entire pregnancy of the first monkey (Speedy) and as can be seen in *Figure 5.3*, it has a peak at approximately Day 57, it declines and plateaus and then steadily increases late in pregnancy. Just before parturition the plasma progesterone level decreases dramatically. Plasma mCG has a peak at approximately Day 83 and then declines over the following weeks.

Figure 5.3 mCG secretion in marmoset plasma from Speedy #38 from day 58 after ovulation through to parturition compared to the level of plasma progesterone.



The profile of plasma progesterone for the other two monkeys (Isabel: *Figure 5.4* and Tuppy: *Figure 5.5*) is similar. The level of plasma progesterone rises at ovulation, peaking at approximately Day 6-7 after ovulation. The levels then fluctuate and decline. Plasma mCG levels are slightly different between the two animals. Marmoset CG is not detected until Day 21 in plasma from Isabel, whilst mCG was detected from Day 7 in plasma from Tuppy. In both, the levels increased after first detection.

5.4.1.2 mCG Protein at the Time of Ovulation

Some monkeys were bled at around the day of ovulation (between days 9-12 of the synchronised cycle). These results are shown in *Figures 5.6 and 5.7* with the corresponding progesterone and bioassay results. In *Figure 5.6*, mCG was detected only at the time of expected ovulation in two different cycles from the same animal (*b* and *c*). Marmoset CG was detected at the time of expected ovulation in *Figure 5.6a* and at much lower levels after expected ovulation. Marmoset CG was also measured in three other animals at the time of expected ovulation only (*see Figure 5.7*).

Figure 5.4 mCG secretion in marmoset plasma from Isabel #55 during early pregnancy through to day 42 compared to the level of plasma progesterone.

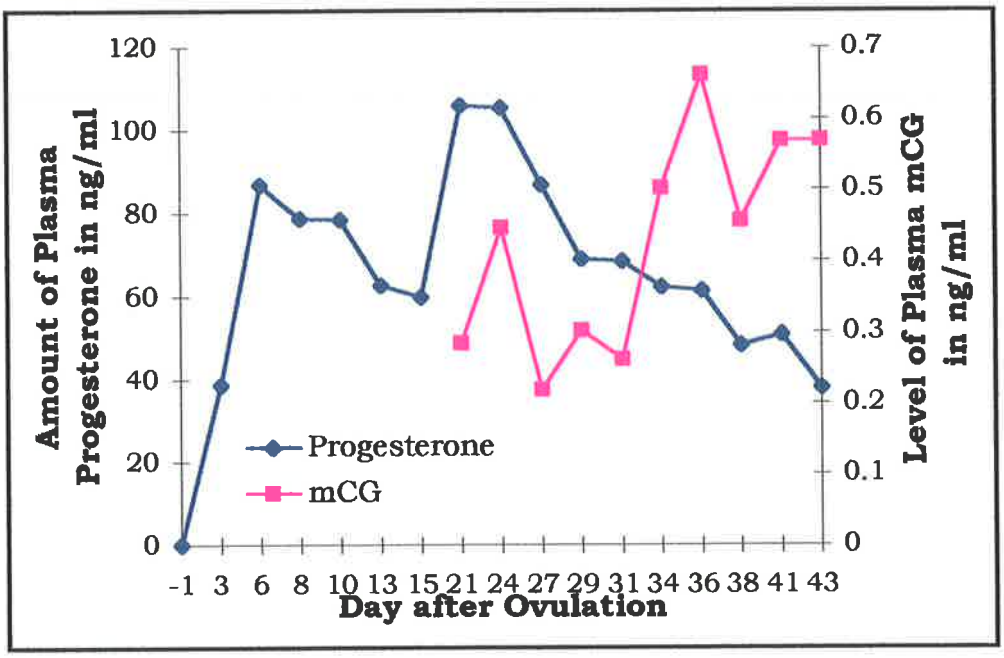


Figure 5.5 mCG secretion in marmoset plasma from Tuppy #650 during early pregnancy through to day 43 compared to the level of plasma progesterone.

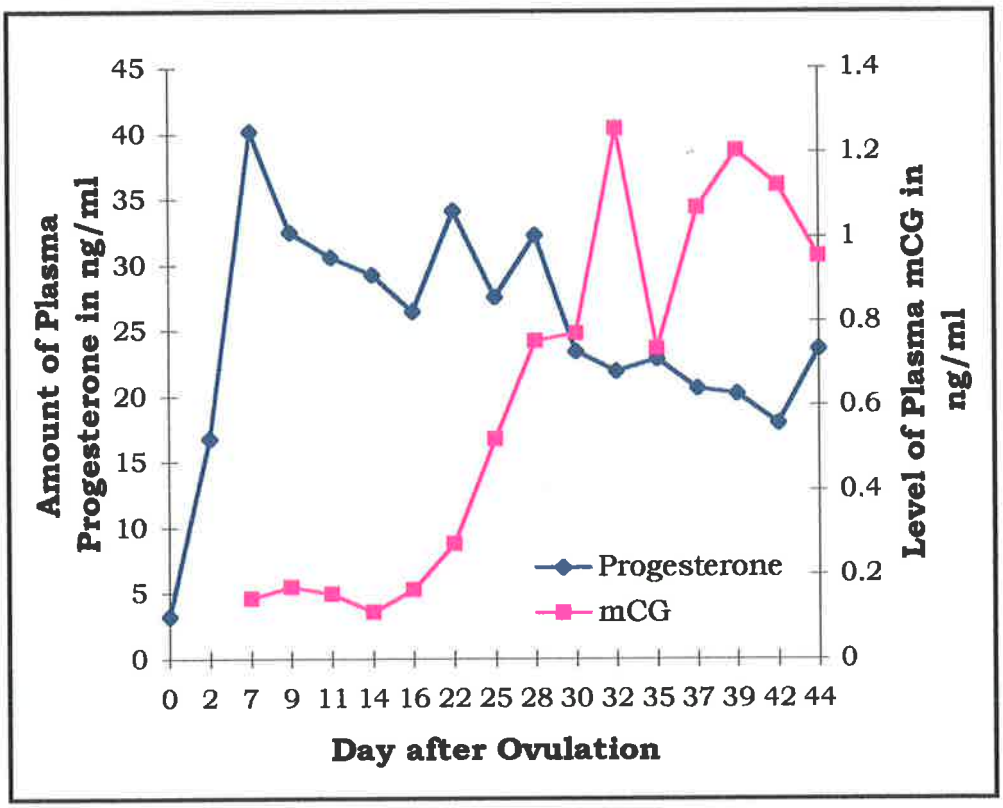
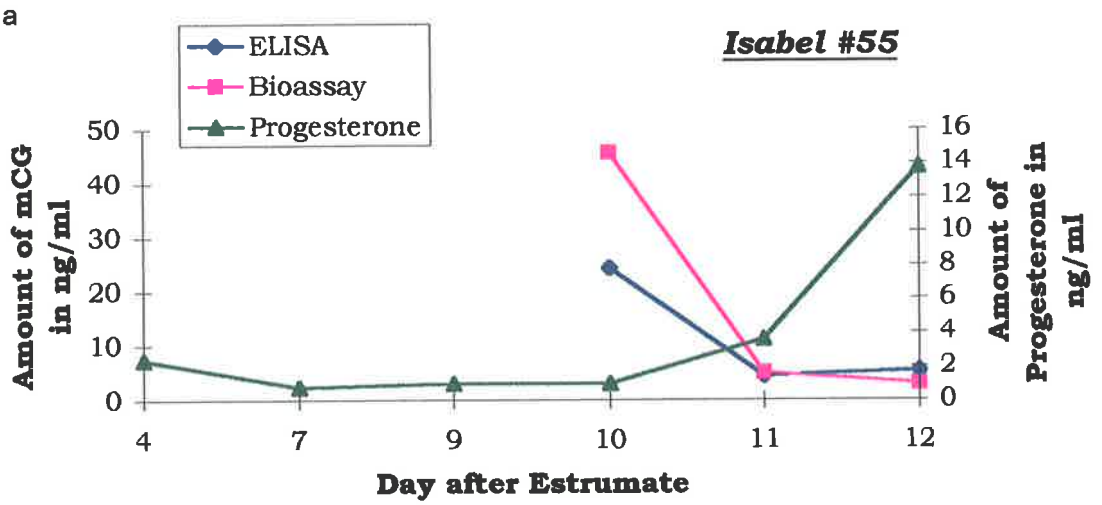
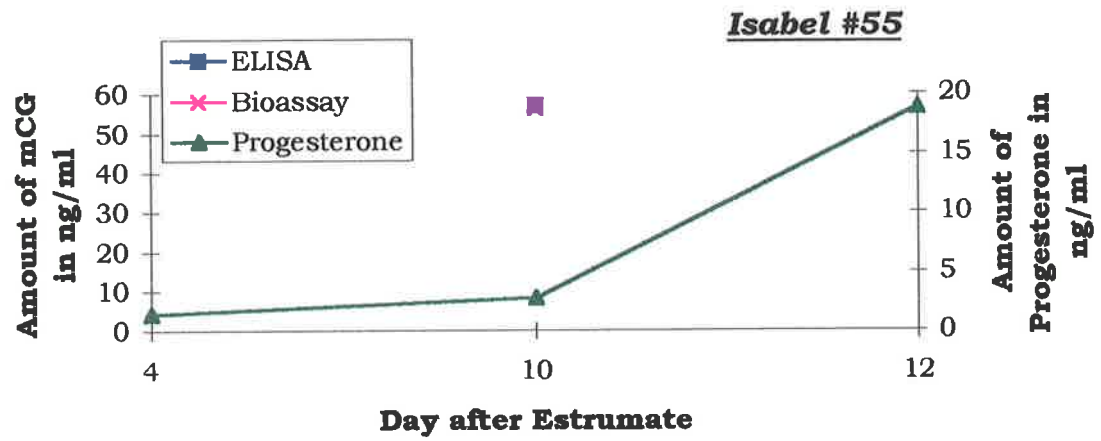


Figure 5.6 mCG/mLH in plasma from Isabel #55 at the time of ovulation, comparison between ELISA and bioassay results. The level of plasma progesterone is also shown.

a



b



c

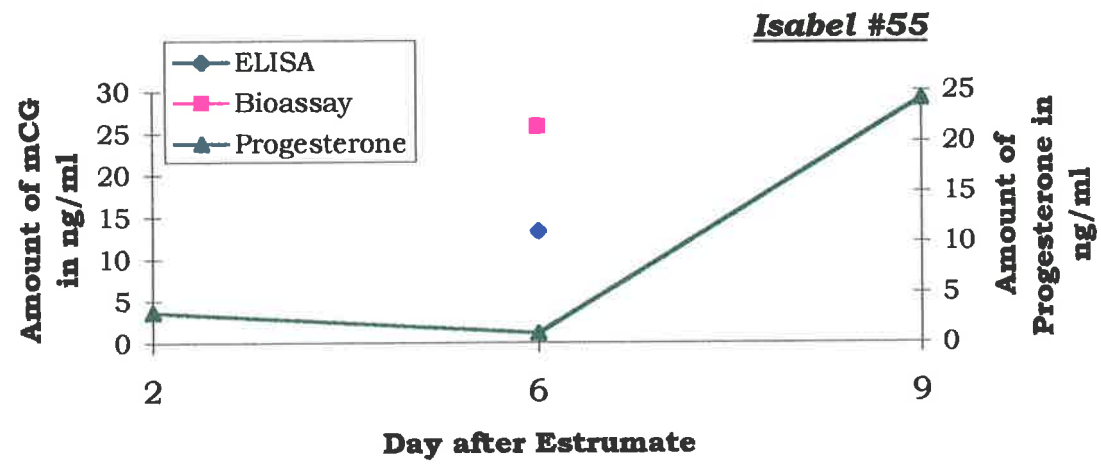
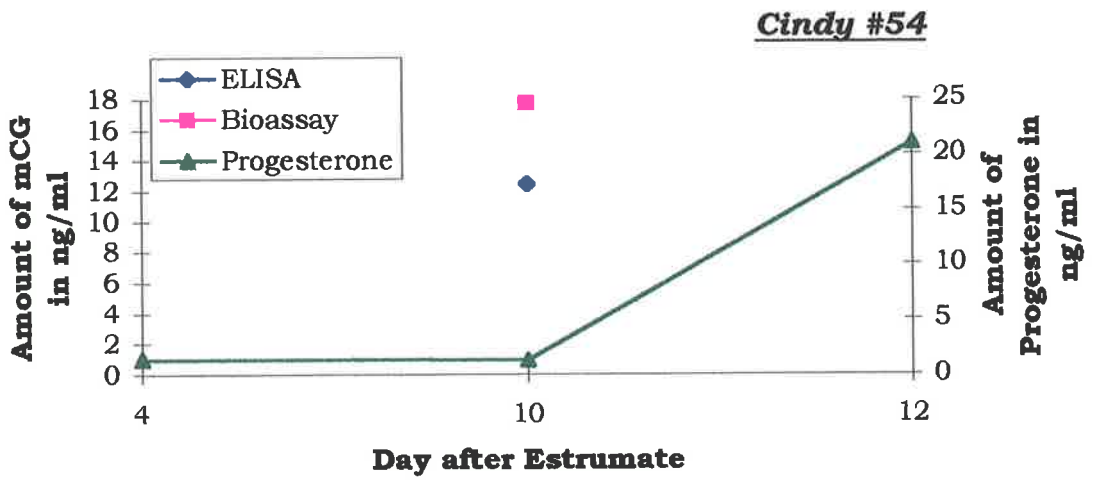
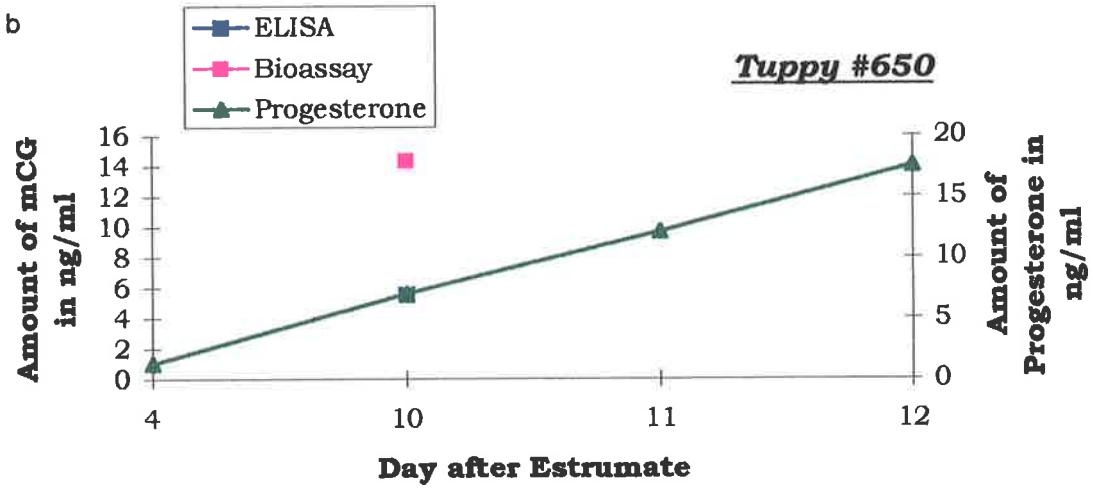


Figure 5.7 mCG/mLH in plasma from three marmosets (Cindy #54, Tuppy #650 & Sophia #657) at the time of ovulation, comparison between ELISA and bioassay results and level of plasma progesterone.

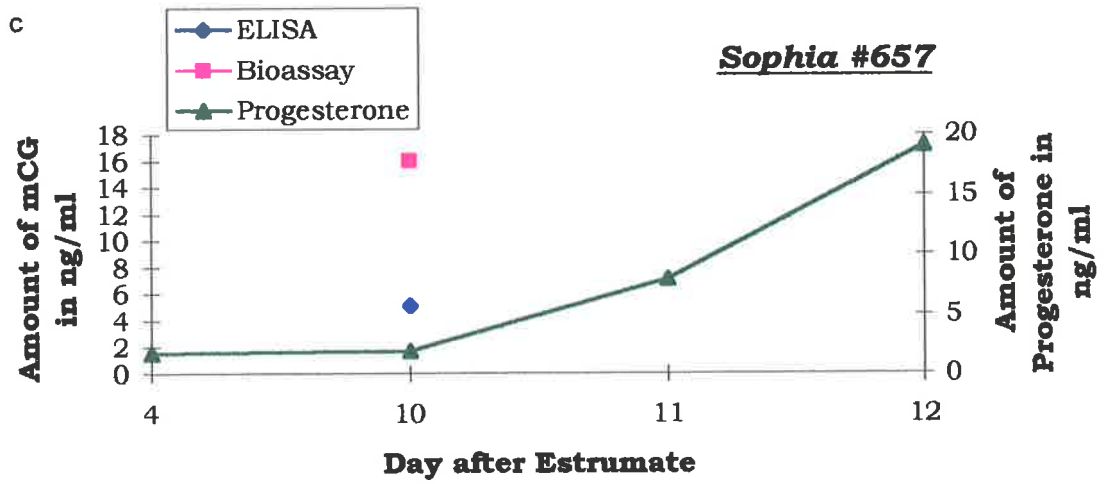
a



b



c

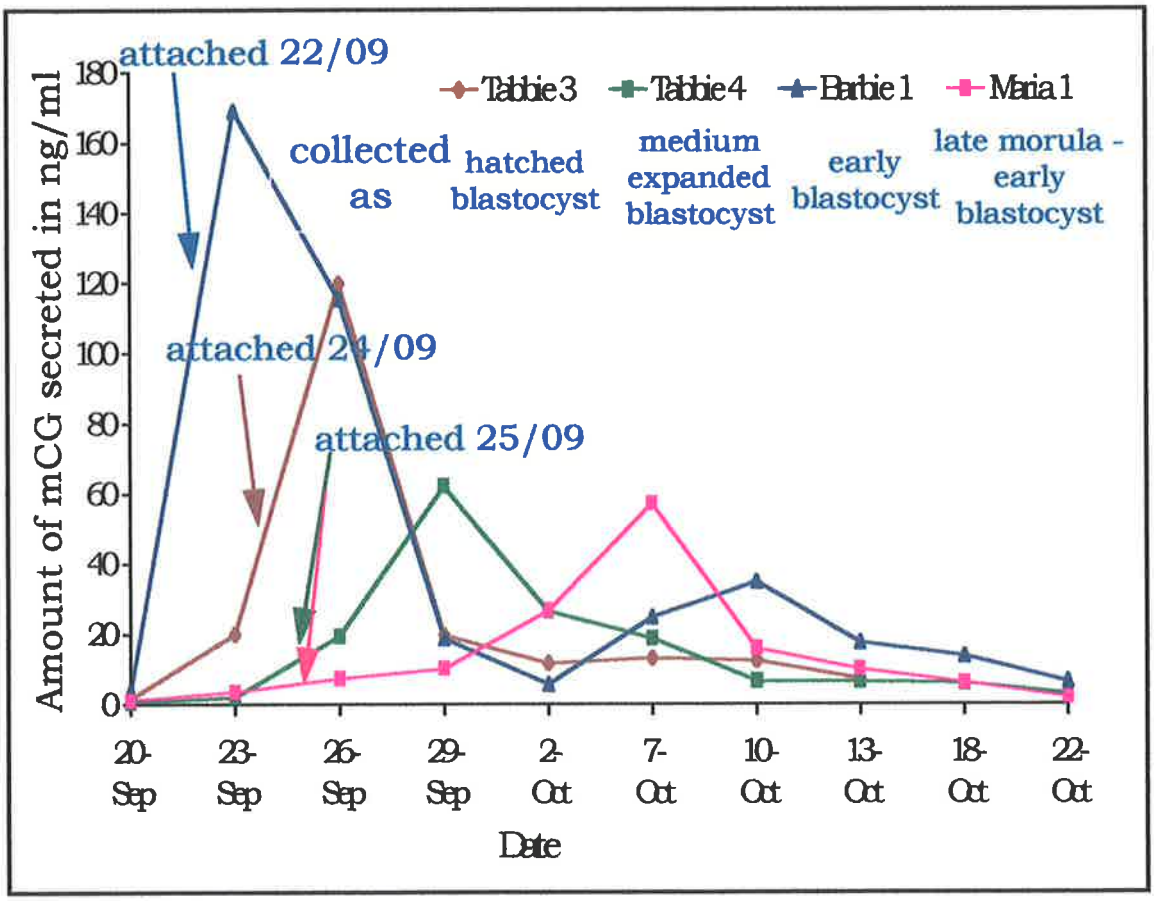


5.4.2 *mCG Protein in Culture*

5.4.2.1 mCG Protein Secretion by Embryos

Four embryos collected at various stages (from the late morula to the hatched blastocyst stage) were cultured through to attachment and formation of trophoblastic vesicles. The ELISA detected mCG protein prior to attachment and up to 48 days in culture. The maximum level was measured immediately after attachment (*see Figure 5.8*).

Figure 5.8 Secretion of mCG from four embryos cultured *in vitro* from the stage of collection shown until after trophoblastic vesicle formation.



5.4.2.2 mCG Protein Secretion by Trophoblastic Vesicles

Marmoset CG protein was measured in vesicle fluid (fluid within the vesicle) at much higher levels than the surrounding medium (87.6 ng/ml *c.f.* o.93 ng/ml; *see Figure 5.9*). Protein secretion of mCG in medium of trophoblastic vesicles was also determined by ELISA (*see Figure 5.10*). Most of the large vesicles (>1.5 mm) showed protein secretion.

Figure 5.9 Comparison of the level of mCG detected in vesicular fluid and the medium surrounding the vesicle.

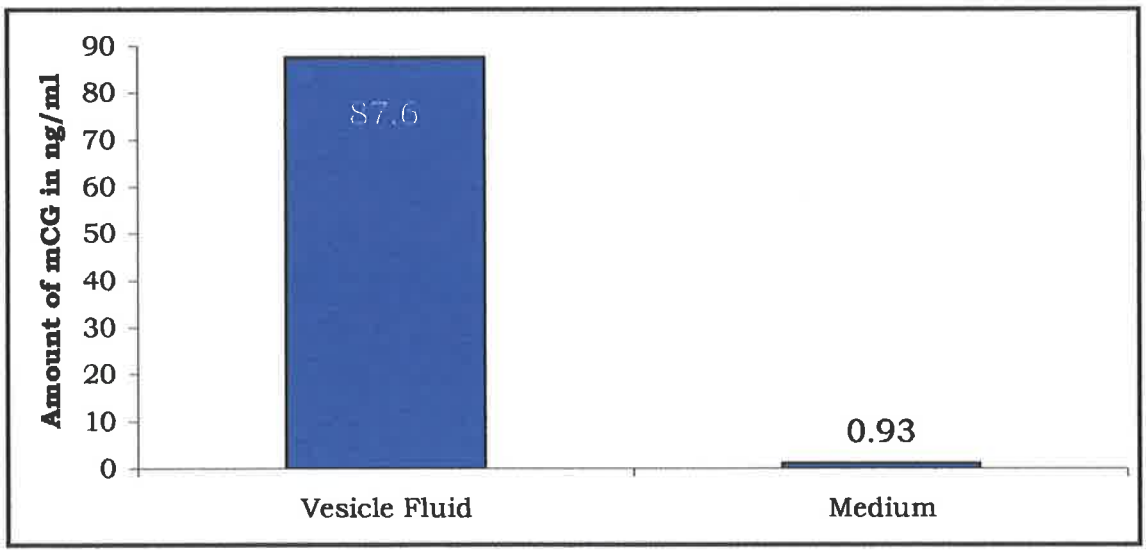
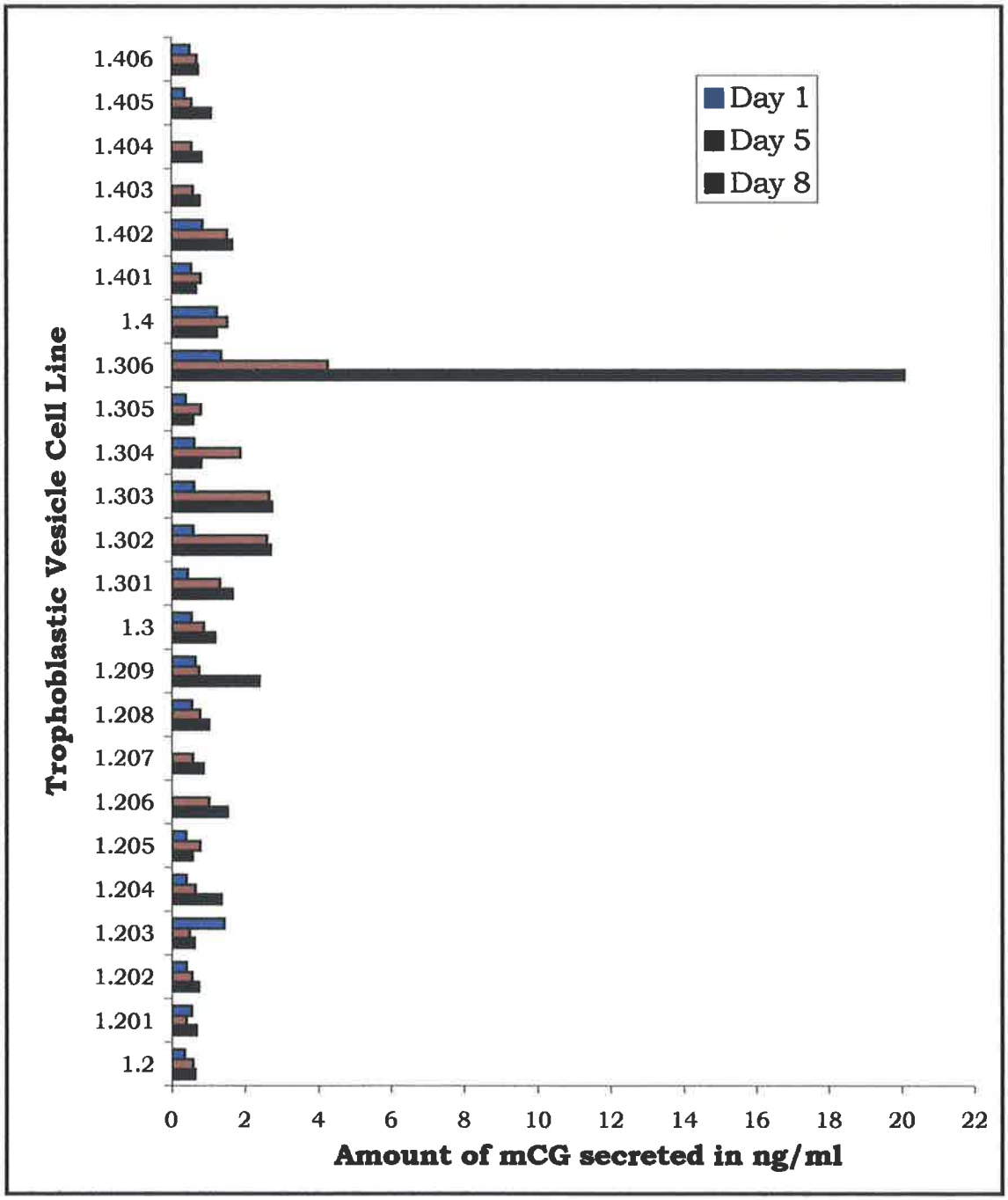


Figure 5.10 Secretion of mCG from a number of trophoblastic vesicle cell lines cultured in 4-well plates. The amount of mCG detected is shown from media collected three times over 8 days.



5.5 Discussion

This chapter has described some of the characteristics of the mCG ELISA, which is specific for mCG. The actual procedure has been published recently (Amato *et al.* 1998) and is described in *Chapter Two*.

The cross-reactivity of the ELISA to other gonadotrophins, including LH, LH-β and FSH from the baboon and cynomolgus monkey and hCG was tested. The low cross-reactivity of the assay with these gonadotrophins (*shown in Figure 5.1*) suggests a specific assay for mCG. In *Figure 5.2*, the dilutions of the extracts of placenta and pituitaries, pregnant marmoset urine and plasma, media collected from cultured trophoblastic vesicles and three different forms of recombinant mCG resulted in parallel regression lines. This confirms the fact that the assay is detecting the correct protein species.

Plasma was collected from cycling and pregnant marmoset monkeys. The ELISA measured mCG from Day 58 after ovulation in the plasma of one monkey (*see Figure 5.3*). Plasma was collected before Day 58, however, there was not enough plasma to measure mCG. Thus, two other monkeys were allowed to stay pregnant until the time that Estrumate is effective (approximately Day 44 of pregnancy; *see Figures 5.4 and 5.5*). Marmoset CG was not detected until Day 21 after ovulation in one monkey (*Figure 5.4*), whilst mCG was detected by Day 7 in the other monkey (*Figure 5.5*).

However, the levels of mCG detected was much lower in the early pregnancy bleeds than later in pregnancy (compare the scales on the right for mCG of *Figures 5.4 & 5.5 with Figure 5.3*). The results from later in pregnancy compare well with previous results (Chambers and Hearn 1979), however the early pregnancy results with the much lower levels are unexpected.

A bioassay to measure mCG, described by Ascoli in 1981 and used in our laboratory previously (Simula *et al.* 1995) was compared with the ELISA and found to correlate well (correlation coefficient 0.9672; reported in (Amato *et al.* 1998) and in *Section 5.3.1*). A marmoset LH-β cDNA has not beeen isolated to date. Some evidence in our laboratory (A.P. Simula, personal communication) suggests that the marmoset may only express a CG-like luteinising hormone in both the pituitary and placenta. Hence, plasma from four marmosets was tested around the time of ovulation to determine if any immunoactive and/or bioactive proteins could be detected at this time. Both the ELISA and bioassay measured mCG before the time of expected ovulation, suggesting a CG/LH peak. Given that 1) this ELISA is specific for mCG, 2) that there is immunoactivity in the marmoset pituitary (*Section 5.3.4, Figure 5.2*) and 3) that PCR results suggest a higher abundance in the pituitary than in the placenta (*Chapter Four*), it suggests that the marmoset has a gene for CG and not a separate gene for LH in the pituitary (Simula *et al.* 1995; Simula *et al.* 1996). In the human, hCG expression has been described in the pituitary (Chen *et al.* 1976).

Embryos collected from various stages were cultured through to trophoblastic vesicle stage and the medium collected to measure mCG. Secretion of mCG was measured prior to attachment as shown in *Figure 5.8*, which has been previously shown in cultured human embryos (Lopata and Hay 1989a). The differences in mCG levels secreted by individual embryos in culture may be an indicator of viability. The late morula-early blastocyst (Maria 1) secretes much lower levels of mCG and has a later peak than the other three embryos, thus the *in vitro* culture of embryos may not be ideal. If CG is secreted prior to implantation, then the monitoring of CG levels in *in vitro* fertilisation embryos prior to transfer to the uterus may be used as an indicator of viability. Hence only the highest expressing embryos could be implanted to maximise success.

Marmoset CG secretion was also measured in trophoblastic cell lines (*Figure 5.10*). Although the amount secreted was dependent on the size and number of vesicles present within the individual cultures, generally the amount of mCG increased as the vesicles proliferated. These trophoblastic vesicle cell lines were actually derived from the embryos cultured in *Figure 5.8*. The 1.2 - 1.209 cell line was derived from Tabbie 3, the 1.3 - 1.306 cell line from Tabbie 4 and the 1.4 - 1.406 cell lines were derived from Barbie 1. Maria 1 did not proliferate into a primary vesicle and so could not produce any vesicles, this embryo may have been a female embryo, or it could be that it was not healthy enough to

produce vesicles. There is evidence from Alex Lopata's laboratory in Melbourne that the Y chromosome is important for trophoblast growth, thus a female embryo with two X chromosomes will presumably not proliferate to a primary cytotrophoblastic vesicle very easily, whilst a male embryo will, if the culture conditions are correct.

As previously found, the amount of mCG within the vesicle is substantially more than in the surrounding medium (*Figure 5.9* and (Summers *et al.* 1987b). Possibly receptors for mCG are present on the inside of the vesicle, which correlates to the inside of the exocoelomic cavity (Moore and Persaud 1993) and the protein has a local autocrine/paracrine role. However, the localisation of mCG receptors needs to be investigated by *in situ* hybridisation and/or immunohistochemistry.

In summary, we have developed a specific and sensitive assay for the measurement of mCG in all biological fluids so far studied. In its current form it will prove useful for measuring mCG from marmoset embryos and trophoblastic vesicles cultured under various conditions, in order to examine the impact of cytokines and growth factors on embryonic expression of CG.

Chapter Six

Culture of Marmoset Trophoblastic Vesicles and Embryos with Cytokines

6.1 Introduction

Chorionic gonadotrophin's most widely recognised role is sustaining the life of the corpus luteum of pregnancy, however, it seems to also be involved in implantation, as well as trophoblastic differentiation (Sawai *et al.* 1995a; Shi *et al.* 1993). Although CG is essential for implantation and a viable pregnancy in primates, an understanding of the factors regulating expression is lacking.

A number of cytokines and growth factors have been shown to influence the expression and secretion of CG. Transforming Growth Factor-beta (TGF-β) has been shown to induce release of hCG from human trophoblasts (Matsuzaki *et al.* 1992). Whilst the addition of Colony Stimulating Factor-1 (CSF-1) to cultures of human term cytotrophoblast cells, resulted in the differentiation of the cells to syncytiotrophoblasts and an increase of hCG and human placental lactogen (hPL; Garcia-Lloret *et al.* 1989).

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) has been shown to induce production of ovine trophoblastic protein-1 (oTP-1), an analogue of CG in sheep, in ovine embryo cultures (Imakawa *et al.* 1993). Interleukin-6 (IL-6), as well as GM-CSF and CSF-1 is known to modify CG expression in human placental cell lines (Ben-Rafael and Orvieto 1992).

Finally, Leukaemia Inhibitory Factor (LIF) has been shown to be imperative for implantation in the mouse (Bhatt *et al.* 1991) and the addition of hLIF to cultures of murine eight-cell embryos resulted in the embryos developing to post-hatching stages (Lavranos

et al. 1995). Human LIF also has an effect on trophoblast outgrowth (Robertson *et al.* 1991). Human endometrium has been shown to express LIF mRNA at the time of implantation, moreover, the human blastocyst has been shown to express mRNA for the LIF receptor (Charnock-Jones *et al.* 1994b). Recently, Nachtigall and associates (1996) examined the effect of LIF on purified cytotrophoblast cultures. Knowing that hCG secretion is a marker of syncytiotrophoblast differentiation and oncofetal fibronectin secretion a marker of anchoring junctional trophoblast differentiation, they found that the addition of hLIF to their culture system markedly decreased hCG protein secretion and expression, as well as significantly increasing the expression and secretion of oncofetal fibronectin. Thus, they suggest that LIF appears to directly modulate trophoblast differentiation and hence is an important regulator of human embryonic implantation.

Trophoblastic vesicles derived from marmoset cytotrophoblast cells have been shown to retain their ability to synthesise and secrete CG during culture (Summers *et al.* 1987b). Thus, the aim of the experiments described in this chapter was to culture marmoset embryos and trophoblastic vesicle fragments, derived from *in vitro* implanted embryos, in the presence of candidate cytokines: LIF, GM-CSF, TGF-β and IL-6 and determine their influence on mCG gene expression and secretion. Marmoset CG expression will be monitored using RT-PCR and immunological techniques described in the previous chapters.

6.2 Collection of Embryos

Marmoset embryos of various stages (morula through to hatched blastocyst stage; *see Table 6.1*) were collected by the non-surgical flushing technique (*as described in Section 2.2.2; see Table 6.2*). The collected embryos were incubated at 37°C in α-MEM buffered with HEPES, before being washed and transferred into culture media (*as described in Section 2.3*). Some embryos were cultured to trophoblastic vesicles (*described in Section 6.3*) and then either dissected to trophoblastic vesicle fragments and cultured with cytokines or, left intact and cultured with cytokines (*both described in Section 6.4*). Other embryos were cultured in the presence or absence of cytokines (*described in Section 6.5*).

Table 6.1 Number and stage of development of embryos that were collected by non-surgical flushing, for culture experiments.

Embryo Stage	Number of Embryos
16-32 cell	2
morula	16
early blastocyst	4
blastocyst	6
expanded blastocyst	3
hatched blastocyst	1
total	**32**

Of the total, five were discounted from experiments: one blastocyst was heat-shocked, one morula and one expanded

blastocyst were lost in transfer and the two 16-32 cell embryos, collected in November 1995 were cultured in the wrong medium.

Table 6.2 Number of successful cannulations, flushes and number of embryos collected by the non-surgical flushing technique.

Year	1995†	1996	1997	1998†
Number of Cannulations (#*C*)	10	45	90	31
Number of Flushes (#*F*)	4	19	23	7
Number of Embryos Collected (#*E*)	6	23	30	8
#E/#C	0.60	0.51	0.33	0.26
#E/#F	1.50	1.21	1.30	1.14

†There was only three months of flushing in 1995 and four months in 1998.

6.3 Culture of Embryos through to Trophoblastic Vesicles

Embryos were cultured to form trophoblastic vesicles (*as described in Section 2.3.1*). A total of ten embryos (*see Table 6.3*) were cultured through to vesicles for subsequent trophoblastic vesicle fragment culture with cytokines and growth factors. The first two embryos (collected 14/04/97) were allowed to culture indefinitely as vesicles, changing and collecting media every three days, however, due to incubator problems the tissue was discarded. All collected media was assayed for mCG using the ELISA (*results in Section 5.4.2.1*). Of the others, two degenerated (Barbie 18/08 & Maria 19/08) before they hatched, possibly due to exposure to low

oxygen tension. The remaining six were propagated to form vesicles and used for the trophoblastic vesicle fragment experiments (*described in Section 6.4*).

Table 6.3 Number and stage of development of embryos that were cultured for trophoblastic vesicles.

Embryo Stage	Number of Embyos	Code
morula	4	Tabbie 1 29/07, Barbie 18/08, Tabbie 2 18/08, Maria 1 16/09*
early blastocyst	1	Barbie 1 16/09*
blastocyst	4	Tabbie 1 & 2 14/04, Maria 19/08, Tabbie 3 17/09*
hatched blastocyst	1	Tabbie 4 17/09*
total	**10**	

* Maria 1 16/09, Barbie 1 16/09, Tabbie 3 17/09 & Tabbie 4 17/09 are the embryos that had their media analysed for the ELISA in *Chapter Five*.

6.4 Culture of Trophoblastic Vesicles and Fragments with Cytokines

6.4.1 *Methods*

6.4.1.1 Culture

Four cytokines were cultured with trophoblastic vesicle fragments: Transforming Growth Factor-beta (TGF-β), Interleukin-6 (IL-6), Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and Leukaemia Inhibitory Factor (LIF). Two trophoblastic vesicle fragments per well were cultured, in duplicate, in serum-free medium overnight (Day 0), before the addition of various doses of cytokine the next day (Day 1). Concentrations of cytokine

ranged from 0 to 0.2 ng/ml for TGF-β and 0 to 2.0 ng/ml for IL-6, GM-CSF and LIF. Media was changed, collected and stored at -20°C every day, before the experiment was terminated on Day 6.

Intact trophoblastic vesicles were cultured only with GM-CSF. Two vesicles per well were cultured in serum-free medium overnight (Day 0), before the addition of three doses of GM-CSF, the next day (Day 1). Medium was changed, collected and stored on Days 2 and 3 and then every second day (Days 5, 7, 9), before the experiment was terminated on Day 11.

Two of the cytokines, TGF-β and IL-6 were cultured with fragments derived from frozen-thawed vesicles in Associate Professor Alex Lopata's laboratory in Melbourne. At the end of the experiment, one of the fragments was lysed for RNA and subsequent RT-PCR analysis (*as described below*). The other two cytokines, GM-CSF and LIF were cultured with trophoblastic vesicle fragments derived from fresh vesicles from embryos collected by the non-surgical flushing technique. Both fragments were lysed for RNA and subsequent RT-PCR analysis at the end of the culture period.

6.4.1.2 RNA Extraction

RNA was extracted from each vesicle fragment and intact vesicle at the end of the culture period by placing each individual sample into an RNase-free tube with 500 µl of Solution D and proceeding as described in *Section 2.4.3.*

6.4.1.3 Qualitative RT-PCR

Qualitative RT-PCR was performed to establish the pattern of expression of mCG-α, -β and mGAPDH, using placenta as a positive control and tRNA or water as negative controls. Complementary DNA was synthesised using Superscript™II and 10 µl of RNA (*as described in Section 2.5.1.2*). The cDNA was then amplified for 30 cycles using mCG-α, -β and mGAPDH oligonucleotide primers and *Taq* DNA polymerase (*as described in Sections 2.5.2.2 and 3.4.2*). The primary PCR product was then diluted 10-fold and a 2 µl aliquot was reamplified for 35 cycles in a fresh PCR reaction using the same assay primers to give a secondary PCR product that was clearly detectable on a 2% agarose gel, stained with ethidium bromide and visualised with a UV light source (*as described in Section 2.5.3.1*).

6.4.1.4 Competitive PCR

Samples that showed mCG-β and mGAPDH expression were subsequently analysed with the competitive PCR assay. Each cDNA sample was diluted 4-fold and amplified with a dilution series of cDNA internal standard, using *Taq* DNA polymerase (*as described in Section 4.4*). In some cases, a 10-fold dilution of the primary PCR products was then reamplified in a fresh PCR reaction with the same assay primers (*as described in Section 2.5.3.1*).

6.4.1.5 ELISA

All collected media samples were analysed for mCG protein by ELISA (*as described in Chapter Two, Section 2.7*).

6.4.2 *Results*

6.4.2.1 Culture

Trophoblastic vesicles were micro-dissected into similar-sized fragments (*as shown in Figure 6.1a*). In some cases, the fragments would reform odd shaped vesicles (*as shown in Figure 6.1b*). The fragments and vesicles sometimes reattached to the plastic, although this was not always observed. For TGF-β and IL-6 treated fragments, the following results are for two fragments in each group, whilst the GM-CSF and LIF treated fragments had four fragments in each group. The GM-CSF treated vesicles had two in each group.

6.4.2.2 Qualitative RT-PCR

The fragments and vesicles were all individually assessed for expression of mCG-α, -β and mGAPDH. Photographs of the gels of the secondary PCR are shown in *Figure 6.2* for TGF-b (a) and GM-CSF (b) treated trophoblastic vesicle fragments and in *Figure 6.3* for IL-6 (a) and LIF (b) treated trophoblastic vesicle fragments. In

Figure 6.1 Photographs of trophoblastic vesicle fragments and intact trophoblastic vesicles.

(a) shows two freshly micro-dissected trophoblastic vesicle fragments and (b) shows the same fragments reformed as vesicles. (c) and (d) show other examples of vesicles. The vesicle on the right of (d) is actually a small vesicle on the side of a larger one, seen on the left.

Figure 6.2

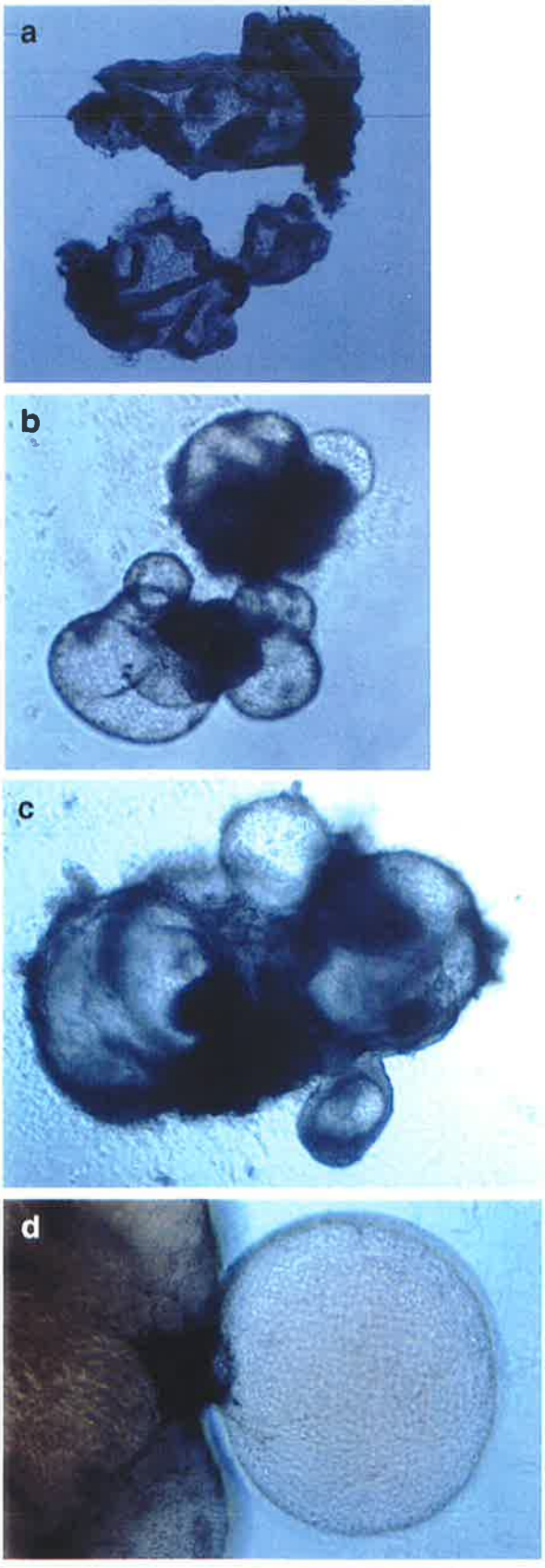


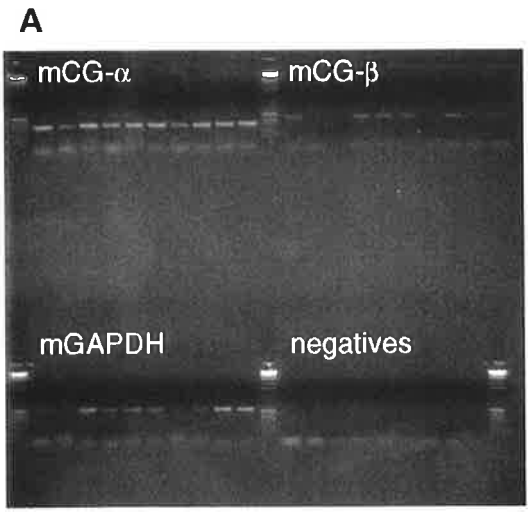
Figure 6.4, the secondary PCR of the qualitative RT-PCR for GM-CSF treated whole vesicles can be seen. In *Table 6.4*, the results obtained from the qualitative RT-PCR are summrised.The concentration of cytokine present in the culture is shown in the second column.

Table 6.4 Summary of Qualitative RT-PCR results for trophoblastic vesicle fragments cultured with four different cytokines and for intact vesicles cultured with GM-CSF
('+' *indicates presence of expression*, '-' *indicates absence of expression.*)

Cytokine	Concentration in ng/ml	Expression of mCG-α	mCG-β	mGAPDH
	trophoblastic vesicle fragments			
TGF-β	**0**	+	+	+
	0.01	+	+	+
	0.02	+	-	-
	0.1	+	+/-	+
	0.2	+	+	+
IL-6	**0**	+	+	+
	0.1	+	+	+
	0.5	+	+/-	+
	1.0	+	+	+
	2.0	+	+/-	+
GM-CSF	**0**	+/-	+/-	+
	0.1	+/-	+/-	+
	0.5	+/-	+/-	+
	1.0	+/-	+	+
	2.0	+/-	+/-	+
LIF	**0**	+	+/-	+
	0.1	+/-	+/-	+
	0.5	+	+	+
	1.0	+	+	+
	2.0	+	+/-	+
	intact vesicles			
GM-CSF	**0**	+	+	+
	0.1	+/-	+	+
	1.0	-	-	+
	2.0	+	+	+

Figure 6.2 Qualitative RT-PCR for mCG-α, mCG-β and mGAPDH of trophoblastic vesicle fragments cultured with TGF-β (a) and GM-CSF (b). The expected mCG-α, -β and mGAPDH products were 325, 433 and 567 bp, respectively. Molecular weight markers (M) were 1Kb DNA ladder.

Figure 6.3
Figure 6.4
Figure 6.5
Figure 6.6



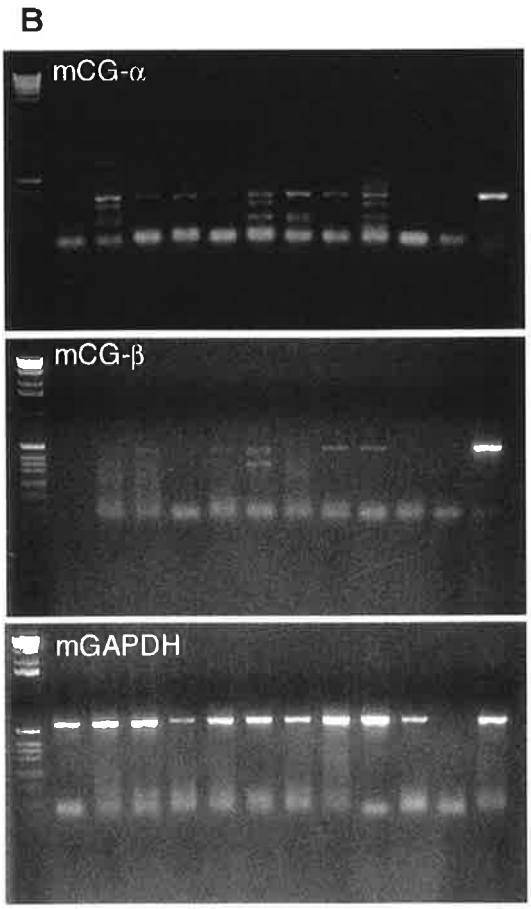
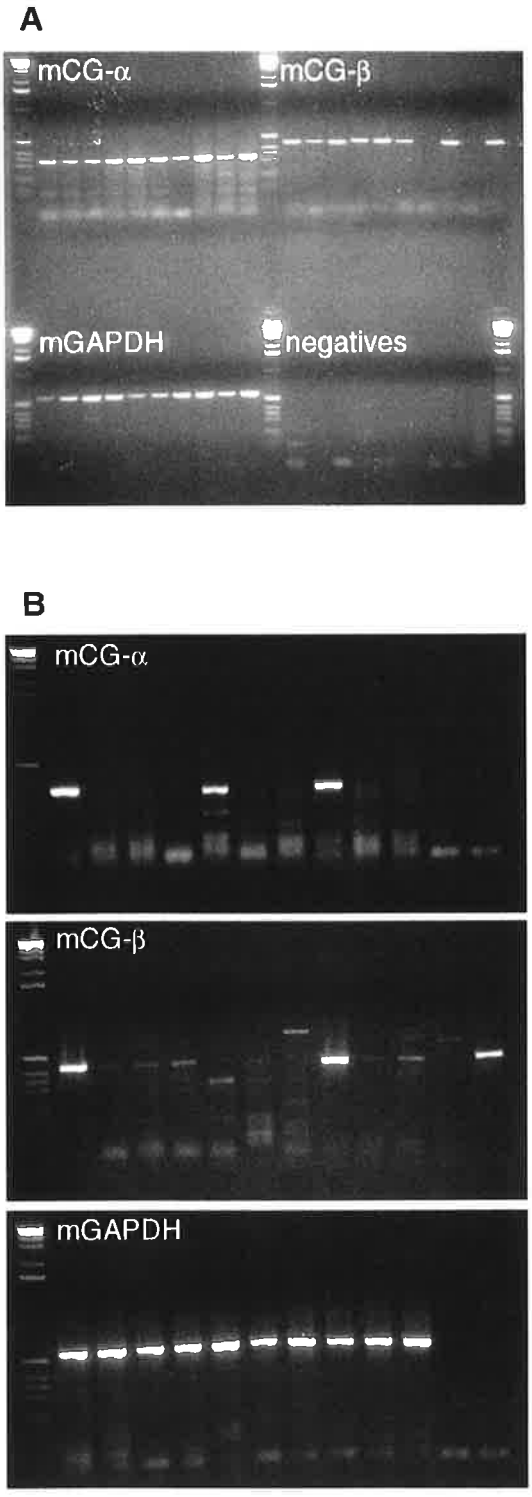


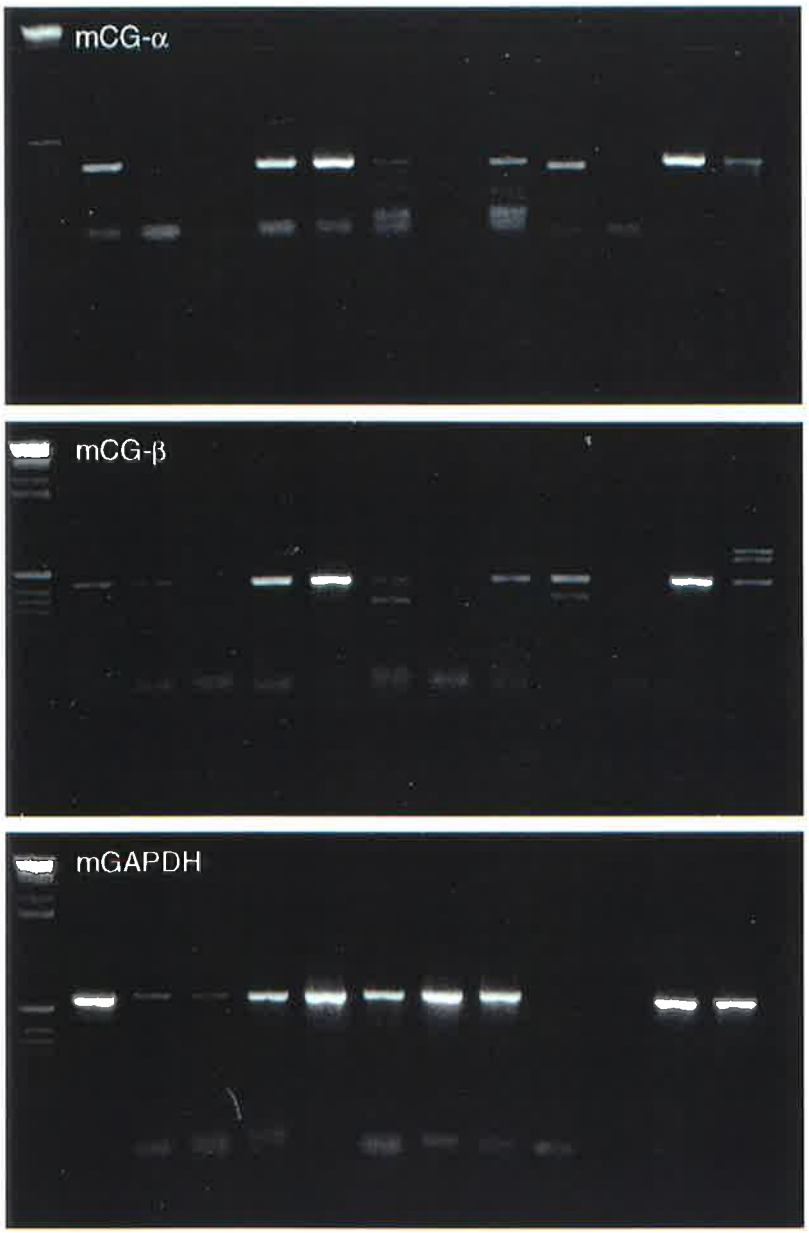
Figure 6.3 Qualitative RT-PCR for mCG-α, mCG-β and mGAPDH of trophoblastic vesicle fragments cultured with IL-6 (a) and LIF (b). The expected mCG-α, -β and mGAPDH products were 325, 433 and 567 bp, respectively. Molecular weight markers (M) were 1Kb DNA ladder.

Figure 6.3b



Figures 6.4 Qualitative RT-PCR for mCG-α, mCG-β and mGAPDH of intact trophoblastic vesicles treated with various doses of GM-CSF. The expected mCG-α, -β and mGAPDH products were 325, 433 and 567 base pairs, respectively. The molecular weight marker (M) was the 1Kb DNA ladder.

Figure 6.7



6.4.2.3 Competitive PCR

All samples that showed mCG-β and mGAPDH expression were analysed with the competitive PCR assay. The results are summarised in *Table 6.5* for both the trophoblastic vesicle fragments and intact vesicles. Representative scans of two trophoblastic vesicle fragments are shown in *Figure 6.5*.

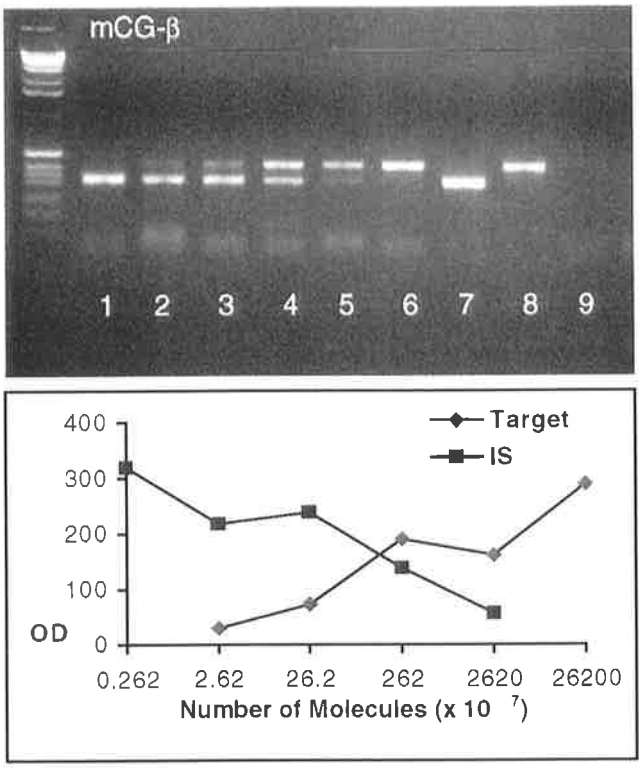
Table 6.5 Summary of Quantitative PCR results for trophoblastic vesicle fragments cultured with four different cytokines and intact vesicles cultured with GM-CSF.
('NA' = *expression too faint and variable to quantitate*)

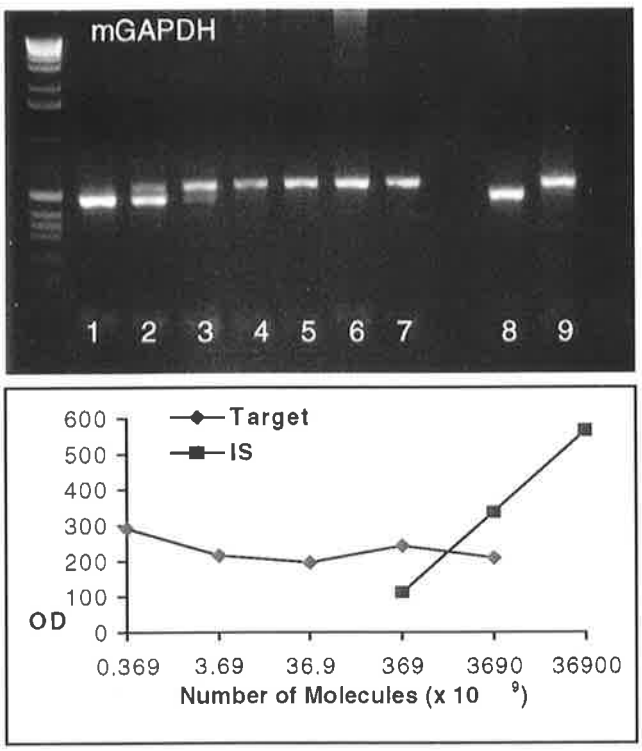
Cytokine	Concentration in ng/ml	Copies of mCG-β	Copies of mGAPDH
		trophoblastic vesicle fragments	
TGF-β	0	2.62×10^{7}	3.69×10^{12} - 3.69×10^{13}
	0.01	2.62×10^{7} - 2.62×10^{8}	3.69×10^{7} - 3.69×10^{8}
	0.02	no expression	no expression
	0.1	2.62×10^{9} - 2.62×10^{10}	3.69×10^{12} - 3.69×10^{13}
	0.2	2.62×10^{8}	3.69×10^{11}
IL-6	0	2.62×10^{10} - 2.62×10^{11}	3.69×10^{11} - 3.69×10^{13}
	0.1	2.62×10^{6} - 2.62×10^{9}	3.69×10^{10} - 3.69×10^{11}
	0.5	2.62×10^{6} - 2.62×10^{7}	3.69×10^{11}
	1.0	2.62×10^{8}	3.69×10^{11} - 3.69×10^{13}
	2.0	2.62×10^{9} - 2.62×10^{10}	3.69×10^{11} - 3.69×10^{12}
GM-CSF	0	NA	3.69×10^{12} - 3.69×10^{13}
	0.1	2.62×10^{10}	3.69×10^{12} - 3.69×10^{13}
	0.5	NA	3.69×10^{12} - 3.69×10^{13}
	1.0	NA	3.69×10^{12} - 3.69×10^{13}
	2.0	NA	3.69×10^{12} - 3.69×10^{13}
LIF	0	NA	3.69×10^{14} - 3.69×10^{15}
	0.1	NA	3.69×10^{11} - 3.69×10^{12}
	0.5	NA	3.69×10^{9} - 3.69×10^{10}
	1.0	NA	3.69×10^{12} - 3.69×10^{13}
	2.0	2.62×10^{12}	3.69×10^{14} - 3.69×10^{15}
		intact vesicles	
GM-CSF	**0**	2.62×10^{9} - 2.62×10^{10}	3.69×10^{11} - 3.69×10^{12}
	0.1	NA	3.69×10^{10} - 3.69×10^{11}
	1.0	no expression	3.69×10^{11} - 3.69×10^{12}
	2.0	2.62×10^{10} - 2.62×10^{11}	3.69×10^{11} - 3.69×10^{12}

Figure 6.5 Representative scans of mCG-β and mGAPDH competitive PCR assays of two trophoblastic vesicle fragments cultured with cytokine. The expected products were 433 and 371 bp for the mCG-β target and internal standard, respectively, whilst the products generated from the mGAPDH competitive PCR were 567 and 484 bp for target and internal standard, respectively. The molecular weight marker (M) was the 1Kb DNA ladder.

The graph below each photograph shows the number of molecules of internal standard against the optical density of the PCR bands of target and internal standard (as determined by NIH image) for both the target and internal standard.

Figure 6.8


mCG-β
1 2 3 4 5 6 7 8 9
400
300
200
100
0
OD
Target
IS
0.262 2.62 26.2 262 2620 26200
Number of Molecules (x 10 7)

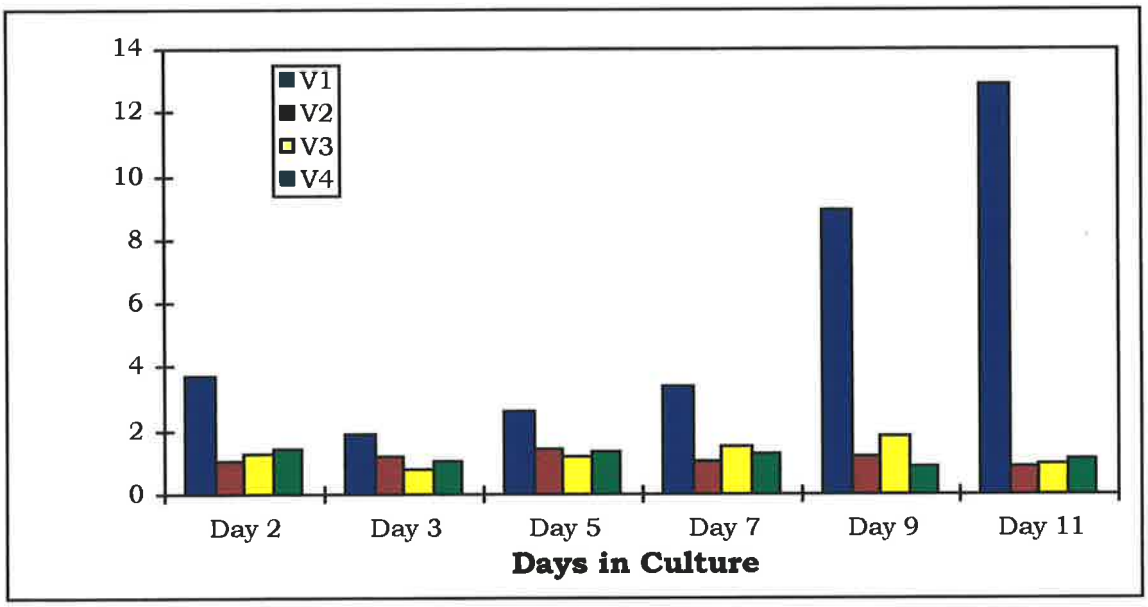

mGAPDH
1 2 3 4 5 6 7 8 9
600
500
400
300
200
100
0
OD
Target
IS
0.369 3.69 36.9 369 3690 36900
Number of Molecules (x 10 9)

As can be seen in *Table 6.5* expression is quite variable, which in some cases made it difficult to determine the range of internal standard for the competitive PCR assay. Hence some trophoblastic vesicle fragments could not be quantitated.

6.4.2.4 ELISA

Analysis by ELISA of most of the trophoblastic vesicle fragment medium showed no protein. So, the samples were concentrated, by lyophilising and the ELISA repeated. The ELISA still did not detect any protein secretion from most of the concentrated samples. The samples from the intact vesicles were analysed for mCG protein secretion by ELISA and these results are shown in *Figure 6.6*. The no cytokine vesicles showed an increase in CG secretion overall. The level of secretion of mCG from the treated vesicles was at the limits of detection of the ELISA and thus very low.

Figure 6.9 ELISA measurement of mCG protein secreted from the trophoblastic vesicles cultured with GM-CSF over 11 days. Media was collected Day 0, 1, 2, 3, 5, 7, 9 and 11, when the experiment was terminated. V1, V2, V3 and V4 correspond to vesicles cultured with 0, 0.1, 1.0 and 2.0 ng/ml of GM-CSF, respectively.



6.5 Culture of Embryos with Cytokines

6.5.1 *Methods*

6.5.1.1 Culture

Embryos were cultured overnight after collection in culture medium (Day 0), to recover, before being transferred to medium with 1,000 units of LIF (12 ng/ml; (Lavranos *et al.* 1995)), or 2 ng/ml of GM-CSF (personal communication, Sarah Robertson, 1997) or fresh culture medium (Day 1). The embryos were observed daily and the medium changed, collected and stored at -20°C every second day (Day 3, 5, 7, etc.). The day after attachment, the experiment was terminated. The embryo and any other cells present in the culture were lysed for RNA extraction and subsequent RT-PCR analysis.

6.5.1.2 RNA Extraction

RNA was extracted from the embryos and any cells found in the medium at the end of the culture period by placing each individual embryo and groups of cells into an RNase-free tube with 500 µl of Solution D and proceeding as described in *Section 2.4.3.*

6.6.3 *Qualitative RT-PCR*

Qualitative RT-PCR was performed to assess for expression of mCG-α, -β and mGAPDH. Reverse transcription was performed using Superscript™II and 10 µl of embryo RNA (*as described in Section 2.5.1.2*). The cDNA was amplified for 30 cycles with *Taq* DNA

polymerase and the mCG-α, -β and mGAPDH oligonucleotide primers (*as described in Sections 2.5.2.2 and 3.4.2*). In some cases, a 10-fold dilution of the primary PCR product was reamplified in a fresh PCR tube using the same assay primers to give a secondary PCR product that was clearly detectable on a 2% agarose gel (*as described in Section 2.5.3.1*).

6.5.1.4 Competitive PCR

Embryo samples that showed mCG-β and mGAPDH expression were subsequently analysed with the competitive PCR assay. Each cDNA was diluted 2-fold and amplified with a dilution series of cDNA IS, using *Taq* DNA polymerase (*as described in Section 4.4*). The primary PCR products were diluted 10-fold and reamplified in a fresh PCR reaction with the same assay primers, to give a secondary PCR product that was clearly detectable on a 2% agarose gel (*as described in Section 2.5.3.1*).

6.5.1.5 ELISA

All collected medium samples were analysed for mCG protein secretion by ELISA (*as described in Chapter Two, Section 2.7*).

6.5.1 *Results*

6.5.2.1 Culture

In total, 14 embryos were cultured with LIF, or GM-CSF or in culture medium with no cytokine, until hatching and attachment to the plastic (*see Table 6.6*). Another three embryos (Z1, Z2, M1) were cultured in serum-free culture medium from the time of collection, however, they did not develop and so were terminated, without analysis.

Table 6.6 Number and stage of development of embryos collected by the non-surgical flushing technique that were cultured with LIF or GM-CSF or without cytokine (control), until after attachment.

Embryo Stage	LIF	GM-CSF	Control	Total
morula	Z3, M3	T8, T9	T10, B2, B3, T5	8
early blastocyst	T6, T11	B4	S1	4
expanded blastocyst		M2	T7	2
Total	4	4	6	14

Of the total, three embryos (B2, B3, T5) which were collected as morulae, did not develop past the early blastocyst stage and so no cytokine was added to the system. Two of these embryos were still analysed (B2, B3), the other embryo (T5), however, was very compacted and degenerated and was not analysed. Another control embryo (S1) compacted before it hatched, however, the other two control embryos (T10, T7) hatched on Day 4 of culture and attached on Day 5. Both resembled primary vesicles (hollow ball of

cells) with a large inner cell mass (icm). Of the four LIF treated embryos, 3 of them (T6, T11, M3) hatched on Day 5 of culture and attached lightly the same day. At the end of the culture period, the three resembled primary vesicles with large icms, similar to control embryos. The fourth embryo (Z3) started hatching on Day 4 of culture, but late the next day, the herniated trophectoderm broke away and the remnants compacted in the zona. Of the GM-CSF treated embryos, one (T8) compacted after 3 days in culture, but the other 3 all hatched and attached. Two of them hatched on Day 5 of culture, T9 did not attach till late on Day 8 and was terminated Day 9 and M2 attached on Day 6 and was terminated on Day 7. Both resembled a tight bunch of cells, with no clear icm. B4 hatched and attached on Day 4 of culture, it also resembled a tight bunch of cells at termination on Day 6.

As all the embryos were observed daily, photographs were taken using slide film. Some of these slides, showing embryos of different stages have been scanned and are shown in *Figures 6.7 - 6.9*. LIF-treated embryos are shown in *Figure 6.7*. (c) show the embryos that compacted in the zona pellucida (Z3) and (d) shows a LIF-treated embryo just before termination. The trophoblast outgrowths are spindly. GM-CSF treated embryos are shown in *Figure 6.8*. The embryo in (c), just before termination has a few spindly outgrowths, but the majority of cells seen are rounded. Embryos cultured without cytokine, shown in *Figure 6.9*, resemble primary trophoblastic vesicles (d) at termination. The inner cell mass is very distinct.

6.5.2.2 Qualitative RT-PCR

The embryos and any other cells present in the culture were individually assessed for expression of mCG-α, -β and mGAPDH. The photographs of the gels of the secondary PCR are shown in *Figure 6.10* for LIF (a), GM-CSF (b) and no cytokine (c) embryos. The results obtained are summarised in *Table 6.7*.

The expected products for mCG-α, -β and mGAPDH are labelled α, β and g, respectively. The individual embryos are named at the base of each gel. Even though both Z3 and S1 compacted and did not attach, they both showed expression for mCG-α, mCG-β and mGAPDH. Every embryo exhibited mCG-α and mCG-β expression. A few embryos displayed only faint expression for mGAPDH (T6, B4 & T7).

Figure 6.7 Photographs of marmoset embryos cultured with LIF.

(a) morula

(b) blastocyst

(c) degenerate, compacted embryo still in zona pellucida

(d) end point of LIF treated embryos

Figure 6.10

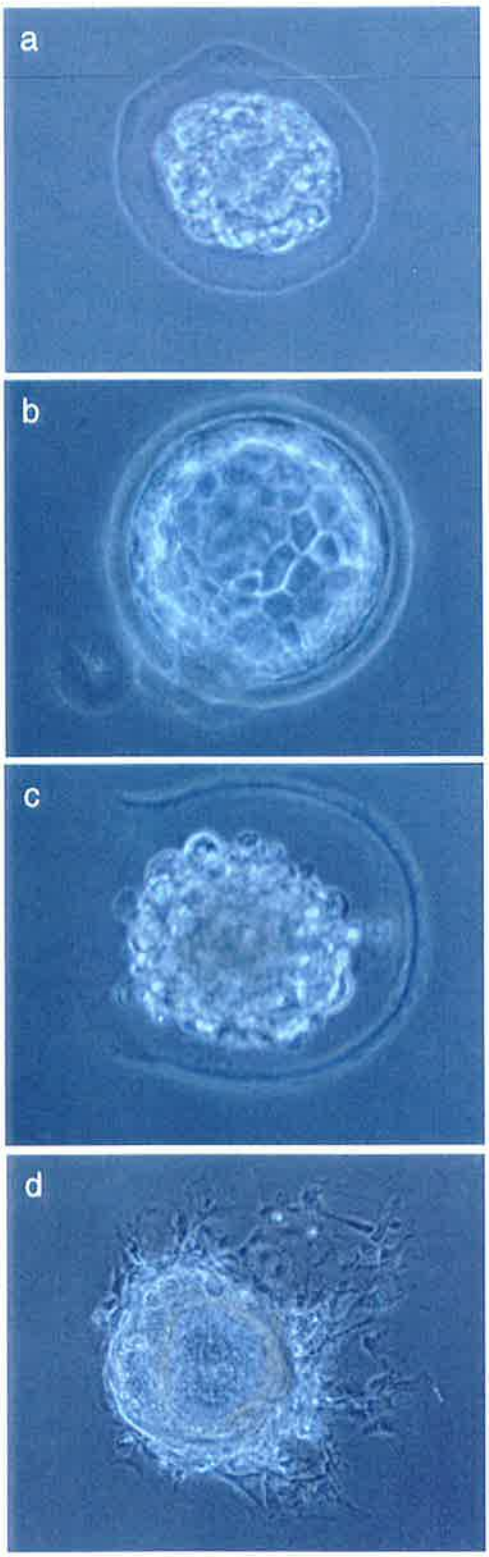


Figure 6.8 Photographs of marmoset embryos cultured with GM-CSF.

(a) expanding blastocyst, showing inner cell mass on the left

(b) hatching blastocyst

(c) end point of GM-CSF treated embryos

Figure 6.11

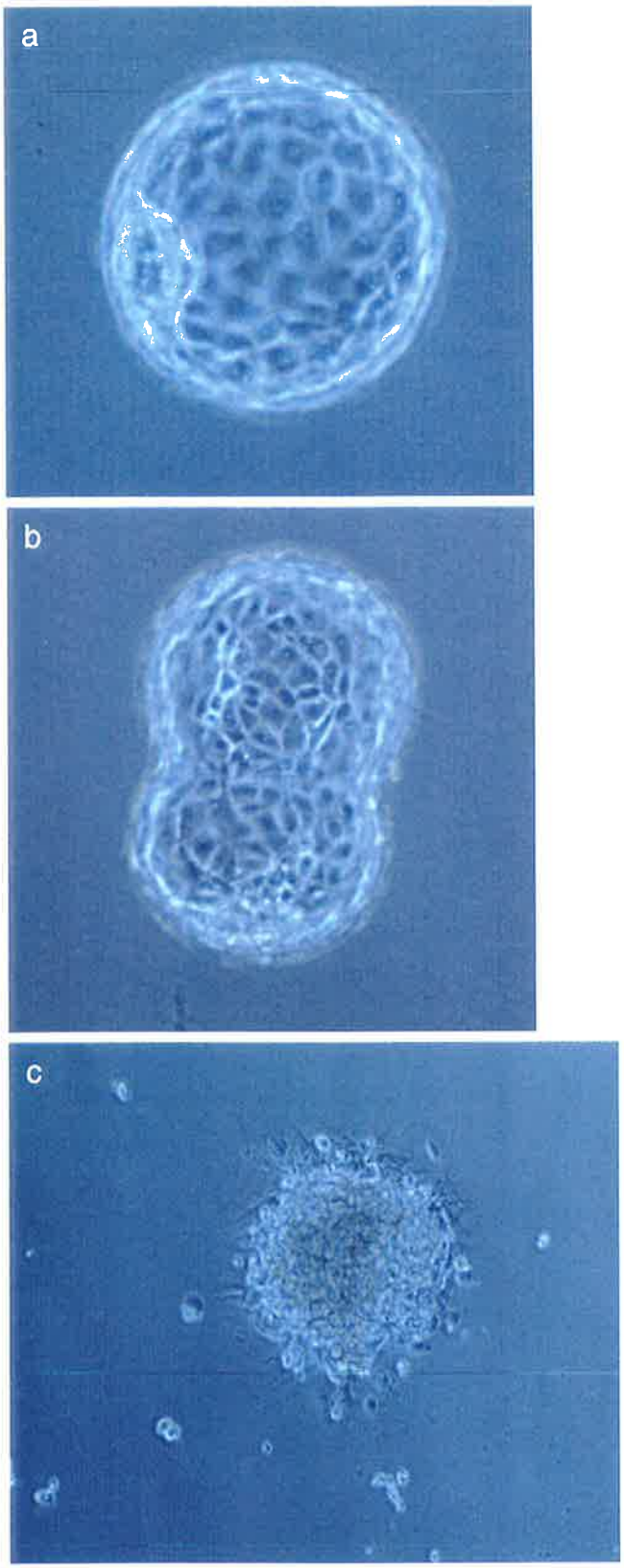


Figure 6.9 Photographs of marmoset embryos cultured without cytokine.

(a) expanding blastocyst, showing inner cell mass on the top right

(b) hatched blastocyst with empty zona on the left

(c) hatched blastocyst, with a distinct inner cell mass

(d) end point of embryos cultured without cytokine

Figure 6.12

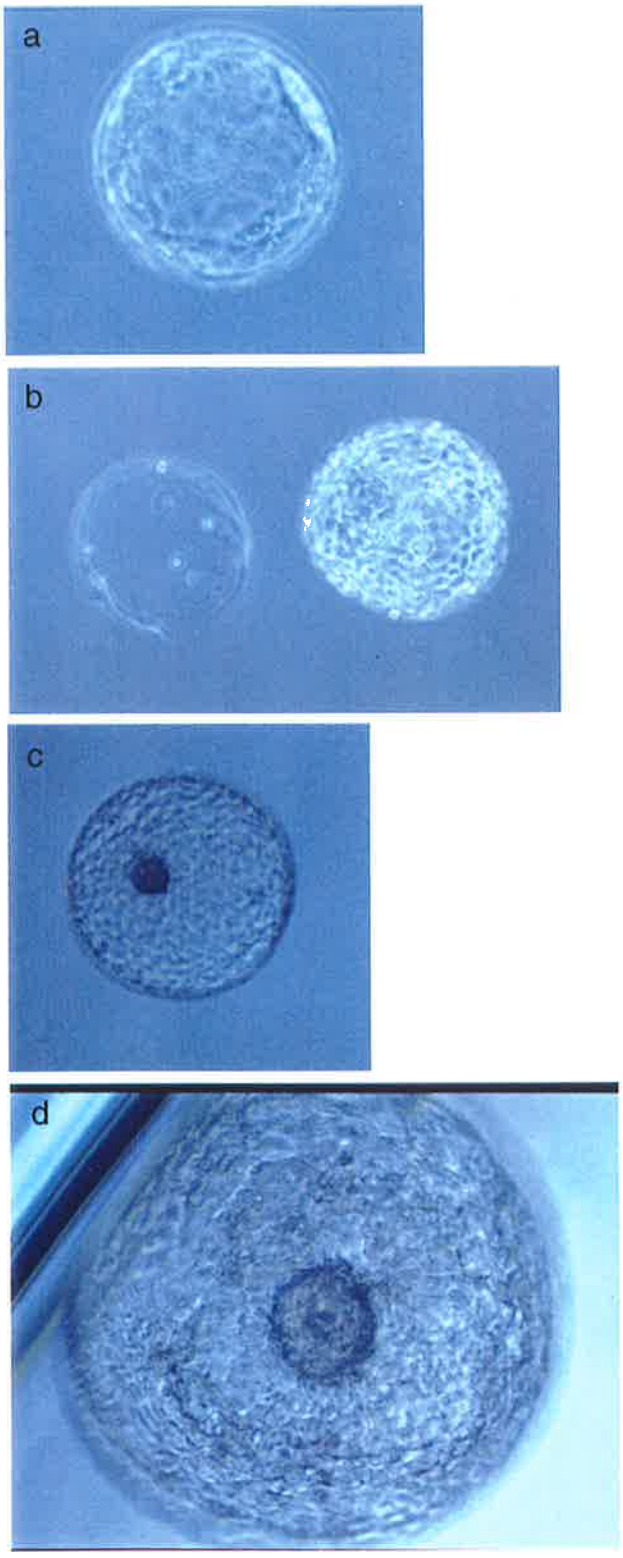


Figure 6.10 Qualitative RT-PCR of embryos cultured with LIF, or GM-CSF or no cytokine until the day after attachment. The first lane in each panel (α) shows the result of RT-PCR using the mCG-α primers, the centre lane of each panel (β) shows the product generated from the mCG-β primers and the right lane of each panel (g) shows the result from the RT-PCR using the mGAPDH primers. The expected mCG-α, -β and mGAPDH products were 325, 433 and 567 bp, respectively. The molecular weight markers (M) were the 1Kb DNA ladder.

Figure 6.13

Figure 6.14

Figure 6.15

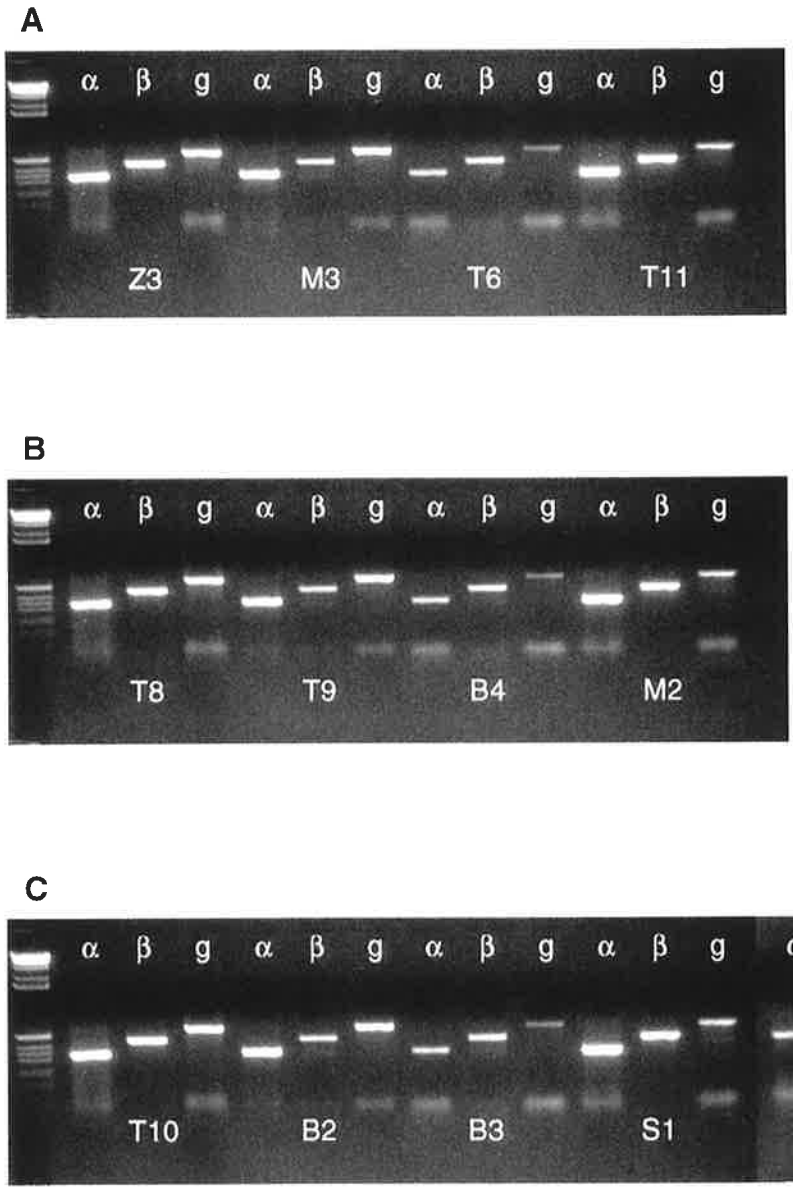


Table 6.7 Summary of Qualitative RT-PCR results for embryos cultured with LIF, or GM-CSF or no cytokine (control).
('+' *indicates presence of expression, '-' indicates absence of expression.*)

Treatment	Number of Embryos	Expression of mCG-α	mCG-β	mGAPDH
LIF	4	+	+	+
GM-CSF	4	+	+ (3/4)	+ (3/4)
Control	5	+	+	+

6.5.2.3 Competitive PCR

All samples showed expression for mCG-β and mGAPDH, so they were analysed with the competitive PCR assay. Representative scans of two embryos are shown in *Figure 6.11*. The equivalency points of each sample that was able to be quantitated is shown in *Table 6.8*. The expression of mCG-β, or mGAPDH or both was not quantitated in 4, 2 and 3 embryos, respectively. A plot for each of the equivalency point is shown in the Appendix.

Table 6.8 Summary of Quantitative PCR results for embryos cultured with LIF, or GM-CSF, or no cytokine (control).

		Copies of		mCG-β
Cytokine	**Embryo**	**mCG-β**	**mGAPDH**	**/mGAPDH**
LIF	Z3	1.65×10^{11}	3.69×10^{12}	0.0447
	M3			
	T6	2.25×10^{11}	2.58×10^{12}	0.087
	T11		3.28×10^{13}	
GM-CSF	T8			
	T9	2.27×10^{12}	2.92×10^{13}	0.0777
	B4		3.60×10^{12}	
	M2	2.02×10^{10}		
control	T10		3.30×10^{13}	
	B2			
	B3	2.34×10^{10}		
	S1	2.07×10^{12}	3.17×10^{13}	0.065
	T7		1.96×10^{13}	

6.5.2.4 ELISA

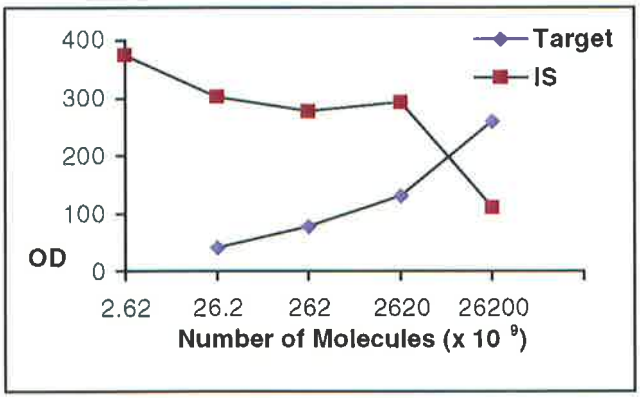
Analysis by ELISA of most of the medium samples showed variable mCG protein secretion, most being below our lowest standard of 0.5 ng. The results have been plotted on a histogram and are shown in *Figure 6.12.*

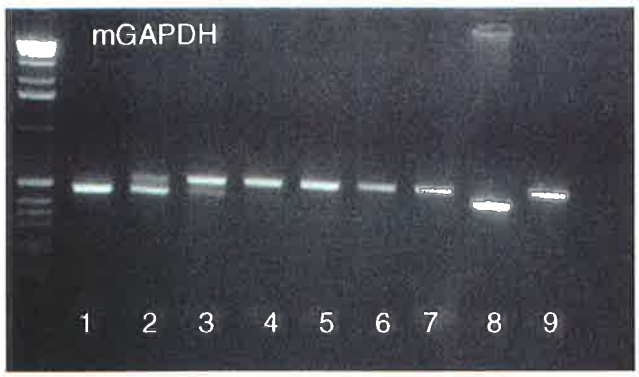
Figure 6.11 Representative scans of mCG-β and mGAPDH competitive PCR of two embryos cultured with cytokine. The expected mCG-β competitive PCR products were 433 and 317 bp for the target and IS, respectively, whilst the products generated from the mGAPDH competitive PCR were 567 and 484 bp for the target and IS, respectively. The molecular weight marker (M) was the 1Kb DNA ladder.

The graph below each photograph shows the number of molecules of internal standard against the optical density of the PCR bands of target and internal standard (as determined by NIH image) for both the target and internal standard.

Figure 6.16


mCG-β
1 2 3 4 5 6 7 8


400
300
200
100
0
OD
Target
IS
2.62
26.2
262
2620
26200
Number of Molecules (x 10 9)


mGAPDH
1 2 3 4 5 6 7 8 9

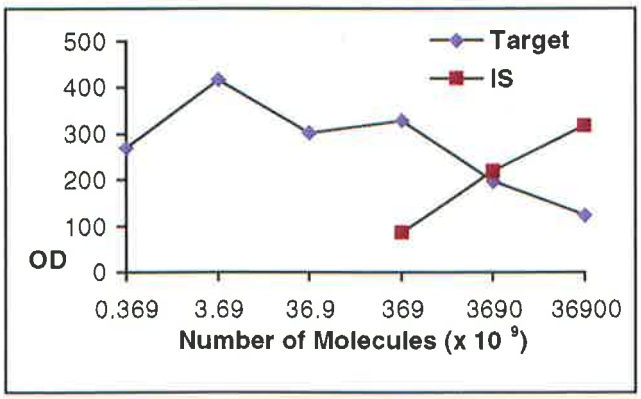
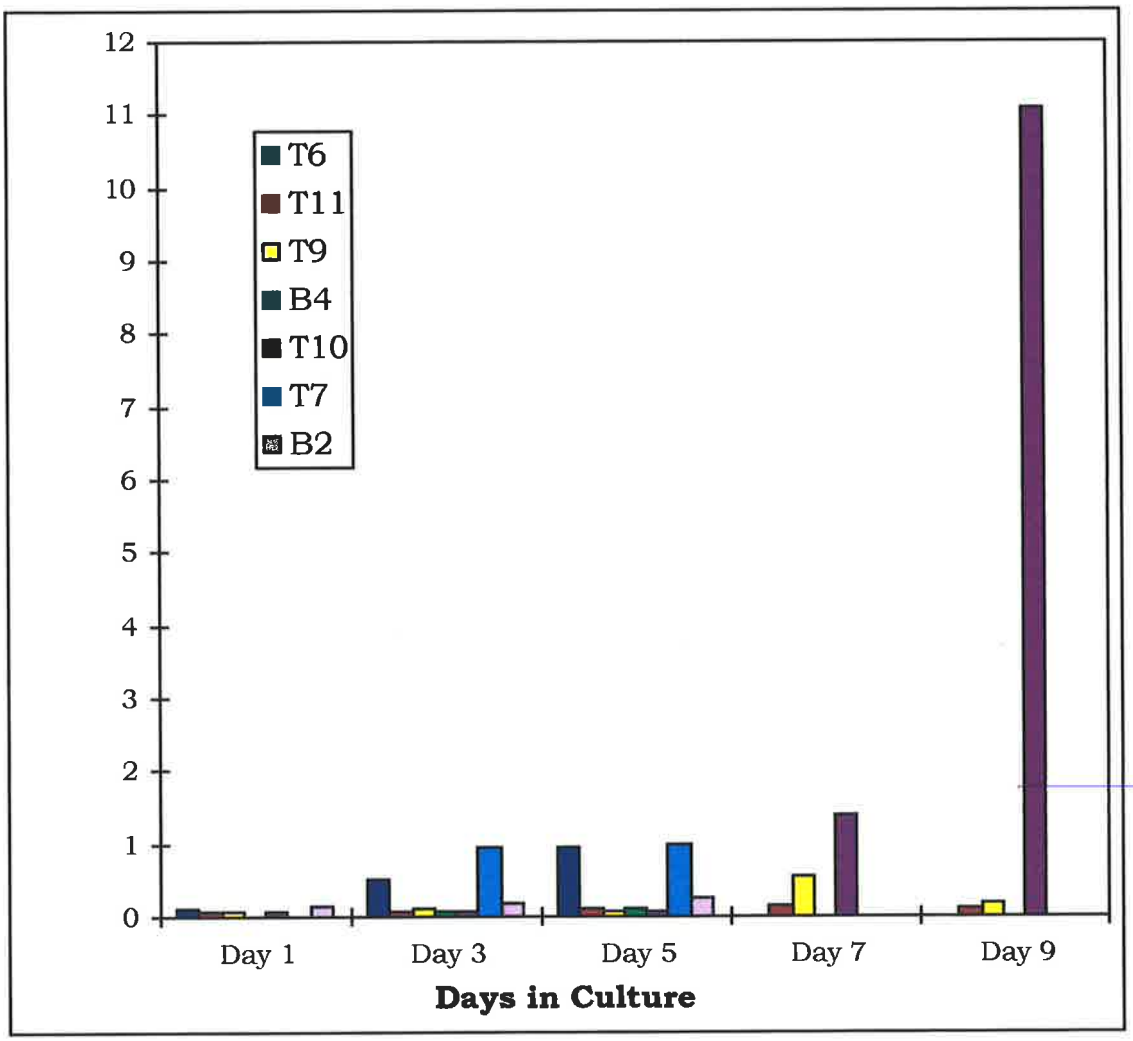

500
400
300
200
100
0
OD
Target
IS
0.369
3.69
36.9
369
3690
36900
Number of Molecules (x 10 9)

Figure 6.12 Measurement of mCG protein secretion over 9 days in culture from embryos cultured with LIF (T6, T11), or GM-CSF (T9, B4), or no cytokine (T10, T7, B2), using the ELISA.



6.7 Discussion

This chapter has described the culture of trophoblastic vesicle fragments (TVFs), whole trophoblastic vesicles (TVes) and intact marmoset embryos with or without various cytokines. Four cytokines were cultured with TVFs: Transforming Growth Factor-Beta (TGF-β), Interleukin-6 (IL-6), Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and Leukaemia Inhibitory Factor (LIF) for a period of 6 days. Only GM-CSF was cultured with whole vesicles for a period of 11 days. Both GM-CSF and LIF were cultured with intact embryos until they hatched and attached.

The aim of this chapter was to investigate the influence of various cytokines on mCG gene expression *in vitro*.

The non-surgical flushing technique (*developed by Thomson et al. 1994* and described in Section 2.2.3) is an excellent technique for obtaining embryos from the morula stage through to the hatched blastocyst stage of development. The monkeys were able to be flushed every month (c.f. every 3-4 months for the mini-laparotomy technique, described in Section 2.2.2) and generally, one (1) morphologically normal embryo was obtained every successful flush (see Table 6.2). During 1997 and the early few months of 1998, the monkeys were upset with frequent movement from one room to another. This seemed to have an impact on the number of embryos recovered by the flushing technique. A system of hierachy is present within a primate colony, the dominant

female exerts her pheromonal influence on other younger, less dominant monkeys and prevents them from ovulating. Between the cages in our colony, there are barriers to prevent this occurring, however, monkeys can still see other monkeys on the opposite side of the room. Environmental factors also come into play, for example stress of being moved around, who is next door and what is happening next door. The introduction of new monkeys and the removal of established monkeys also affect other members of the colony. The low embryo number to cannulation ratio (Table 6.2) also demonstrates the necessity to not disturb the 'flushing' group of monkeys. Of the 67 embryos collected in total by the non-surgical flushing technique (see Table 6.2), 32 were used for culture experiments (see Table 6.1): five were discounted from experiments (as described below Table 6.1), ten were cultured for trophoblastic vesicles (see Table 6.3), fourteen were cultured with or without cytokine (see Table 6.6) and another three were cultured in serum-free medium (described above Table 6.6). The other 35 embryos were either frozen for subsequent RT-PCR analysis (30 embryos of various stages: see Chapters Three and Four) or placed into formalin (one morula, two expanded and two hatched blastocysts: these have not been analysed as yet). Due to the scarcity of marmoset embryos and the success of trophoblastic vesicle culture (Summers *et al.* 1987), we decided to utilise the vesicle culture system. Trophoblastic vesicle fragments were used because the number of cells within a fragment is probably closer to the

number of cells within a blastocyst. Trophoblastic vesicle fragments were cultured with various doses of cytokine for a period of 6 days. From the qualitative RT-PCR results (*Figures 6.2 and 6.3 and Table 6.4*), it can be concluded that there is a great deal of variability of expression of mCG between the fragments, especially the LIF- and GM-CSF-treated fragments. This could be due to the difference in sizes of the fragments and therefore the variability in cell number of the fragments. Any fragment that showed mCG-β and mGAPDH expression was subsequently analysed with the competitive PCR assay (*see Table 6.5*). There was a greater variability in mCG-β than mGAPDH expression, but then there were quite a few fragments that had very faint expression for mCG-β from the qualitative RT-PCR results and could not be quantitated with the competitive PCR. This could be due once again to the variability in size of the fragments. IL-6 appeared to decrease the amount of mCG-β expression, compared to control fragments, whilst TGF-β appeared to increase mCG-β expression, compared to control fragments. This is the opposite to the literature. The amount of mCG protein secreted from the fragments could not be determined even after concentrating, so we assumed that the amount of protein being secreted by the fragments was very small and not detectable by our ELISA and that the volume of media (~1 ml) was diluting any protein that was being secreted, or perhaps the daily changing of media was diluting out other factors that the fragment needed to secrete

mCG. So, it was decided to culture whole vesicles with cytokine for a longer time period and not change the medium as much.

Intact vesicles were cultured with various doses of GM-CSF. The qualitative RT-PCR results (*see Table 6.4*) show that mGAPDH is expressed in all the vesicles, which indicates that the RNA extraction and subsequent qualitative RT-PCR were a success. However, mCG-β was not expressed in all the vesicles, particularly the vesicles treated with 1.0 ng of GM-CSF. The vesicles treated with 0.1 ng of GM-CSF showed very faint expression for mCG-β and so all the vesicles treated with 0.1 and 1.0 ng of GM-CSF could not have their mCG gene expression estimated. The amount of mCG protein secreted was also investigated using the ELISA (*see Figure 6.6*), however, most of the samples were at the lower limits of the ELISA (below 0.5 ng/ml). Nevertheless, the control vesicles (no GM-CSF) showed more mCG protein secretion than the vesicles treated with GM-CSF, which was a very unexpected result. But, perhaps GM-CSF enhances expression of mCG, not translation of the message into the protein, or perhaps the mCG was being secreted into the vesicle fluid and not into the media surrounding the vesicle. After this experiment was completed, we discovered that the vesicle fluid (fluid within the vesicle) contained more mCG protein than the surrounding medium (*see Chapter Five, Figure 5.9*).

Thus, we concluded that trophoblastic vesicles were a better culture system for investigating mCG genes mRNA expression than the fragment culture system. However, due to the fact that vesicles

and fragments mainly consist of trophoblast cells (either syncytiotrophoblast or cytotrophoblast cells) there is a lot of other factors that must come into play for regulating mCG expression and secretion in the intact embryo (inner cell mass). In fact it has been shown that the inner cell mass is important for the efficient secretion of CG by marmoset blastocysts (Summers *et al.* 1993). Moreover, the study of the effect of cytokines on isolated, cultured trophoblast cell lines may not give a true indication of the role of such factors in regulating CG expression in early embryos. The population of receptors present on *in vitro* cultured trophoblast cell lines may be different to the receptors on the cells of the early embryo and the cells expressing CG of the early embryo may be influenced by neighbouring embryonic cells. So it was decided to move on to culturing intact embryos.

Marmoset embryos were cultured in serum-free culture medium with 12 ng of LIF (Lavranos *et al.* 1995), or with 2 ng of GM-CSF (personal communication, Dr.S.A. Robertson, 1997) or in culture medium without cytokine. Three embryos were cultured in serum-free medium without cytokine, however, they did not develop further than the stage that they were collected and so it was decided to ensure hatching and attachment of the control embryos by culturing in 'normal' culture medium (α-MEM with 10% FCS, L-glutamine, antibiotics, insulin and transferrin, *as described in Section 2.3*). Even still, embryos cultured in 'normal' culture medium did not always hatch and attach, as shown by B2, B3 and T5. Moreover, embryos cultured with LIF and GM-CSF did not

always hatch and attach, *as described in Section 6.5.2.1 and shown in Figures 6.7 - 6.9.* This perhaps is due to those embryos not being viable from the start and so adding cytokine and culturing in serum-free medium is not always going to help matters!

Nevertheless, nearly every embryo cultured with or without cytokine showed expression for the mCG genes and mGAPDH, as shown by the results in *Table 6.7 and Figure 6.10.* The amount of mCG-β and mGAPDH could be quantitated by the competitive PCR assay, shown by *Table 6.8 and Figure 6.11.* However, the ELISA showed variable secretion and most of the samples were below our lowest standard of 0.5 ng (*see Figure 6.12*). This could be that the message is not being translated into protein, thus any increase in expression is not being seen as an increase in secretion.

Expression of mGAPDH was pretty constant between all groups (between 2.58×10^{12} - 3.30×10^{13} copies). Expression of mCG-β varied between the groups: there was great variability between embryos from the control group (range: 2.07×10^{10} - 2.34×10^{12}) and between embryos from the GM-CSF treated group (range: 2.02×10^{10} - 2.27×10^{12}). Only the LIF treated embryos showed a constant value of copy number of mCG (1.65×10^{11} or 2.25×10^{11}). Thus not many conclusions can be drawn from these results other than to repeat the experiments with more embryos. There does however, appear to be a morphological difference between the three groups. The LIF treated group (*see Figure 6.7d*), shows spindly cells at attachment, compared to the rounded cells seen in the GM-CSF treated culture (*see Figure 6.8c*). The no

cytokine treated embryos resembled primary cytotrophoblastic vesicles (*see Figure 6.9d*).

Both LIF and GM-CSF have been shown to modulate CG expression and secretion, or it's ruminant analogue, trophoblastic protein (TP; see Roberts *et al.* 1992a for a review). The addition of hLIF to eight-cell mouse embryos, resulted in the embryos developing past the hatching stage (Lavranos *et al.* 1995) and hLIF has also been shown to affect trophoblast outgrowth (Robertson *et al.* 1991). Recently, LIF has been shown to markedly decrease mCG protein secretion and expression in purified cytotrophoblast cultures (Nachtigall *et al.* 1996). Moreover, in the mouse, maternal LIF has been found to be essential for implantation (Bhatt *et al.* 1991; Shen and Leder 1992; Stewart *et al.* 1992). More recently, a study investigating infertility in Interleukin-11 receptor (IL-11R) deficient mice found that the IL-11R complex which involves gp130, also part of the LIFR complex, is absolutely required for female fertility. They suggest that IL-11 is required one day later than LIF, in the mouse and is important for the normal decidual response to the implanting blastocyst, however, the IL-11 ligand deficient mouse needs to be investigated (Robb *et al.* 1998). In the human, the endometrium at the time of implantation has been shown to express LIF mRNA and the human blastocyst to express mRNA for the LIFR (Charnock-Jones *et al.* 1994b).

The addition of GM-CSF to ovine embryo cultures resulted in the production of ovine trophoblastic protein (oTP), the analogue of CG in sheep (Imakawa *et al.* 1993). GM-CSF has been shown to induce

the proliferation of pure ectoplacental cone trophoblast from mouse embryos (Armstrong and Chaouat 1989), as well as stimulate both differentiation of human cytotrophoblast cells from full term pregnancies to syncytiotrophoblast and release of hCG and human placental lactogen (hPL; Garcia-Lloret *et al.* 1989). Wegmann's group has also shown that GM-CSF can promote implantation of mouse embryos using an *in vitro* model of implantation (Wegmann 1990b).

The marmoset embryo is an individual and its viability is dependent on many things, one of them being the expression of genes, including mCG and mGAPDH. The expression of genes can be modulated by factors present within the culture system, however, whether this change in expression is actually translated into protein is another consideration. This strategy of culturing marmoset embryos with or without cytokine and then analysing the mCG gene expression by qualitative and quantitative PCR technology and determining the protein secretion by ELISA is a useful method to investigate the effect of cytokines on CG expression and secretion. However, more embryos are required to investigate the full effects of these two cytokines.

Chapter Seven

Final Discussion

Final Discussion

The major aim of this study was to use the marmoset monkey as a model primate to determine the regulation of expression and secretion of chorionic gonadotrophin (CG) from the pre-implantation embryo. In particular, we wished to investigate the factors regulating expression and secretion of CG, thereby determining the viability of the embryo. This knowledge will aid us in improving culture conditions and viability of human *in* vitro fertilisation (IVF) embryos, as well as enhancing our understanding of primate reproductive physiology.

Due to the ethical constraints governing the use of human embryos and because CG is a primate specific hormone, we have used the common marmoset, *Callithrix jacchus*, as a model primate. The marmoset is small, easily handled and trained and has a hormonal profile for CG similar to that of the human, of 28 days. Their ovarian cycle can be regulated by the administration of prostaglandin F_{2_α} and they routinely ovulate two or more oocytes per cycle. With the advent of the non-surgical flushing technique, embryos can be obtained from each marmoset every month, although pheromonal and environmental factors still have an impact on the number of embryos collected. Of the 67 embryos that were collected, 30 were frozen for RT-PCR analysis, as described in *Chapters Three and Four* and 32 were cultured, as described in *Chapters Five and Six.*

Initially the ontogeny of expression of the two subunits of mCG, alpha and beta in the pre-implantation embryo was investigated. Expression of mCG-α was detected from the four cell stage, whilst mCG-β expression was detected from the morula stage. Expression of the house-keeping gene glyceraldehyde-phosphate dehydrogenase (mGAPDH) was also identified from the morula stage, and was used as a reference mRNA species for the competitive PCR. The identity of the PCR products for each target sequence was confirmed by sequencing and restriction enzyme digestion. The absence or inability to amplify a PCR product for mGAPDH in the four- and eight-cell stage embryos may be due to the recovery of low levels of RNA from these early embryos, thus it may also be possible that mCG-β is expressed at this stage. The expression of these three genes was also investigated in degenerate blastocysts, showing the absence of mCG-β expression. Hence, suggesting that CG-β may be an indicator of viability of the embryo. This is the first time that mCG expression by the embryo has been shown to occur prior to implantation and thus prior to the establishment of a link between the mother and the embryo. The presence of mCG at these early pre-implantation stages may indicate a possible local function for CG at peri-implantation.

Having established the expression of both mCG subunits, assays were developed to determine the relative abundance of mCG-β in trophoblastic tissue under various culture conditions. It

was not possible to develop a similar assay for mCG-α, and so this still needs to be further investigated. Due to the potential problems with the amount of RNA recoverable from individual embryos, the competitive RT-PCR assay developed was only used on tissue samples from marmosets. For all the embryo and trophoblastic tissue experiments a competitive PCR assay was developed and used.

The competitive RT-PCR assay was originally tested on RNA isolated from marmoset pituitaries and placenta. This revealed a higher abundance of mCG-β-like species in the pituitary than in the placenta. Related work in the laboratory, using various combinations of primer pairs was unable to isolate an LH-like cDNA. This may suggest that the marmoset has a gene(s) for LH-β which produce a protein product structurally similar to CG, with an extended C-terminus (Personal communication, Dr. A. P. Simula). The relative abundance of mCG-β was investigated in trophoblastic vesicles and pre-implantation embryos, however, under the current conditions, the competitive RT-PCR assay was not sensitive enough to quantitate the limited amount of mCG-β mRNA, even though mGAPDH was detected.

To ensure the competitive PCR assay was consistent with the competitive RT-PCR assay, the results for the levels of mCG-β and mGAPDH in placental and pituitary tissues was compared. This was confirmed to be the case. The competitive PCR assay was first tested using placental and trophoblastic vesicle cDNA. Subsequently, mCG-β expression was quantitated using marmoset

embryo cDNA from a morula and expanded blastocyst. This showed that embryos have a variable level of gene expression, which may be dependent on the viability of the embryo.

A specific and sensitive assay for the detection of mCG protein has recently been developed (Amato*et al.* 1998). This assay was used to measure mCG secreted from pre-implantation marmoset embryos, revealing the secretion of mCG prior to attachment. Differences were seen in CG output from the individual embryos, thus the presence of high levels of CG may be an indicator of viability.

Plasma mCG was also investigated in cycling and pregnant marmoset monkeys. The profile of CG in pregnancy of the marmoset was similar to that already published (Chambers and Hearn 1979). As mentioned above, there is some evidence suggesting that the marmoset may only express a CG-like luteinising hormone in both the pituitary and placenta. Thus, the presence of a CG/LH peak was investigated in the plasma of four marmosets around the time of ovulation. Both the ELISA and bioassay detected an immunoactive species prior to the time of expected ovulation, hence, suggesting a CG/LH peak.

Given that 1) the ELISA is specific for mCG, 2) the ELISA is able to detect an immunoactive species in the marmoset pituitary, 3) the PCR results suggest a higher abundance of mCG in the pituitary than in the placenta and 4) previous reports suggest that mCG may serve as mLH when expressed in the pituitary gland

(Simula *et al.* 1996), it suggests that the marmoset may have a gene(s) for CG-β expressed in the pituitary and placenta, and not a separate gene for LH-β. This is the case with the equine LHβ which is a single gene expressed in both the pituitary and the placenta (Murphy and Martinuk 1991).

The qualitative RT-PCR and competitive PCR assays and ELISA were then applied to trophoblastic vesicles and intact embryos cultured with or without various cytokines, in an attempt to investigate the influence of these factors on mCG expression and secretion *in vitro*. The two main cytokines chosen: LIF and GM-CSF have been shown to down-regulate and up-regulate CG expression and secretion, respectively, in human cytotrophoblast cultures (Nachtigall *et al.* 1996; Garcia-Lloret *et al.* 1989). Four embryos at either the morula or blastocyst stage of development were cultured with LIF or GM-CSF and five embryos were cultured in control culture medium. Embryos were cultured from collection, through blastocyst formation and hatching, and terminated on the day after attachment. The expression of mCG-α, -β and mGAPDH was detected in all embyos, regardless of the presence or absence of cytokine. Possibly due to the small sample size (4, 4 and 5 embryos in LIF-, GM-CSF-treated and control groups, respectively) the competitive PCR assay did not reveal any major differences in the relative amounts of mCG-β relative to mGAPDH expression. This may suggest that the regulation of mCG by these cytokines is not at the mRNA level, or that the time in culture was not

sufficiently long to detect any changes in expression. The ELISA showed variable secretion of mCG and most of the samples were below the limit of sensitivity for the assay.

Trophoblastic vesicle fragments were cultured with TGF-b, IL-6, LIF and GM-CSF, however, the results were very inconsistent, in all the assays, which could possibly be due to the differences in cell number in the fragments. Whole trophoblastic vesicles were cultured with GM-CSF only. These results were more consistent, surprisingly, the no cytokine vesicles showed increased mCG secretion, compared to the treated vesicles.

This strategy of culturing marmoset embryos with or without cytokine and then analysing the mCG gene expression and secretion, which is an indicator of viability is a useful method to investigate the effect of cytokines on CG expression and secretion. However, for this study, more embryos are required to determine the full effects of these two cytokines.

The restricted use of human embryos for research purposes has limited the investigation into the regulation of CG expression. Given the importance of this protein in early embryonic development much effort has been placed into developing alternative animal models to study its regulation. The marmoset monkey is one such species, which has been used over the last 20 years to investigate primate reproductive physiology. Until recently appropriate DNA and antibody probes have not been

available to study CG expression in this species (Simula *et al.* 1995, Amato *et al.* 1998).

In this sudy I have used a non-invasive technique to recover pre-implantation embryos from mated marmosets. While the technique has its advantages in respect to the welfare of the animal, the number of embryos recoverable limited the number of experiments that were carried out. I therefore, turned to trophoblastic tissue derived from cultured embryos after their attachment. This enabled experiments to be carried out to investigate the influence of cytokines on CG expression.

In order to monitor CG expression and secretion in the experiments PCR and immunological assays were developed and characterised in this study. These assays were used to measure CG in the experiments described earlier. While the depth of the investigation in this study was not very extensive, there was sufficient evidence to suggest that the marmoset could be a very useful model to study primate pre-implantation development. Currently there is no proven method for the induction of superovulation in the marmoset, although work is ongoing in this regard. The development of such a method would allow for the recovery of a greater number of embryos from a in a single flushing procedure. This should allow for the design of better experiments to study CG regulation. In turn this will bring reproductive physiologists closer to developing improved culture methods for

IVF-derived embryos prior to transfer to a recipient. Alternatively, the IVF advances could be used in programmes to preserve the existance of endangered species held in captivity.

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Appendix

Appendix

This list of recipes is in order of appearance from Chapter Two Materials and Methods.

Estrumate

To 20 mls of sterile PBS, 20 µl of estrumate (Estrumate: synthetic prostaglandin for cattle and horses, 250 µg/ml cloprostenol as sodium salt; Jurox Pty. Ltd.) was added. The solution was kept in a sterile bottle at 4°C. A 200 - 400 µl (0.5 - 1.0 µg) intra-muscular injection was administered to each marmoset every three or four weeks.

Saffan

Saffan (anaesthetic injection for cats; Pitman-Moore Australia Limited) was aseptically transferred by needle and syringe (Terumo) into a sterile bottle (Department of Pharmacy, QEH). Depending on the weight of the monkey, 0.5 - 0.7 ml of anaesthetic was injected into their inner thigh (half into the left and half into the right).

α-MEM, buffered with HEPES

To 100 mls of MQ water, 1.008 g of α-MEM (powdered media, with Earles salts and L-glutamine and without nucleosides and sodium bicarbonate; Trace Biosciences), 298 mg of HEPES acid salt (Sigma) and 325 mg of HEPES sodium salt (Sigma) were dissolved. The pH

of the solution was adjusted to below 7.4 before filter sterilising through a 0.22 μM disposable filter (Millipore). Two millilitres of fetal calf serum (FCS; Trace Biosciences), 2 mls of antibiotic solution (penicillin G 5,000 U/ml and streptomycin sulphate 5,000 μg/ml; CSL Biosciences) and 1 ml of heparin (Sigma) were added and the flushing media was filter sterilised again into 10 ml aliquots in Falcon tubes (Becton-Dickinson). This media was air equilibrated.

α-MEM culture medium

To 90 mls of α-MEM (liquid media, with Earles salts and sodium bicarbonate and without L-glutamine and nucleosides; Trace Biosciences), 10 mls of FCS, 2 mls of antibiotic solution (as above), 100 μl of transferrin (see below), 100 μl of insulin (see below) and 1 ml of L-glutamine (Gibco) were added. The media was filter sterilised into 10 ml aliquots in Falcon tubes. This media was CO_2 equilibrated.

Transferrin

To 100 mg of transferrin (human; Boehringer-Mannheim), 4 mls of sterile MQ water was added to prepare a 25 mg/ml solution. Aliquots were stored at -20°C. Ten microlitres per 10 mls of media were added.

Insulin

To 100 mg of insulin (from bovine pancreas; Boehringer-Mannheim), 4 mls of sterile MQ water was added to prepare a 25 mg/ml solution. Aliquots were stored at -20°C. Ten microlitres per 10 mls of media were added.

Serum-free α-MEM

To 100 mls of α-MEM (liquid media, as above), 500 mg of bovine serum albumin (BSA; Sigma) was dissolved. Two millilitres of antibiotic solution and 1 ml of L-glutamine were added and the solution filter sterilised into 10 ml aliquots. This solution was CO_2 equilibrated.

Diethyl pyro-carbonate (DEPC)-treated Water

To 500 ml of MQ water, 500 µl of DEPC (Sigma) was added. The solution was shaken vigorously, before autoclaving the next day.

Solution D

For each sample, 733 µl of 4 M guanidinium thiocyanate (ICN), 44 µl of 0.75 M sodium citrate, pH 7.0 (Sigma), 65 µl of 10% N-lauroyl sarcosin (Sigma) and 9 µl of 2-mercaptoethanol (Sigma) were pipetted into an RNAse-free eppendorf tube. The tube was vortexed and used immediately.

2 M Sodium Acetate, pH 4

To 40 ml of MQ water, was added 16.42 g of sodium acetate (anhydrous; BDH) and dissolved. The pH was adjusted to 4 with glacial acetic acid (BDH) and the volume made up to 100 ml with MQ water.

Water-saturated Phenol

Phenol (ICN) was melted at 65°C and approximately 25 mls was aliquoted to 50 ml Falcon tubes. DEPC-treated water was added to the 50 ml mark of the Falcon tubes, the lids sealed tightly and the tubes shaken vigorously. The two phases were allowed to separate and the aqueous phase was removed by aspiration and the process repeated at least 5 times. Finally, fresh DEPC-treated water was added to the phenol, the tubes were shaken and stored at -20°C.

Chloroform-isoamyl Alcohol (49:1)

The solution of chloroform (Ajax) and isoamyl alcohol (Sigma) was prepared as a 49:1 ratio and stored in a light excluding bottle at room temperature.

50 x TAE Buffer

To 400 mls of DEPC-treated water, 121 g of Tris.-HCl (ICN), 28.55 ml of glacial acetic acid (BDH) and 50 ml of 500 mM ethylene diamine tetra acetic acid (EDTA), pH 8.0 (Sigma) were added and stirred until all solutes had dissolved. The pH was adjusted to 8

with 1 M NaOH and the volume made up to 500 ml and stored at room temperature.

Ethidium Bromide

A 10 mg/ml solution of ethidium bromide was made. The bottle was wrapped in aluminium foil and stored at room temperature.

RNA Loading Buffer

To 30 ml of glycerol (ICN), 0.25 g of bromophenol blue (Sigma) was added. The solution was made up to 100 ml with DEPC-treated water and shaken vigorously. The solution was autoclaved and stored at 4°C, with aliquots ready for use at room temperature.

DEPC-treated Phosphate-buffered Saline

One tablet of Dulbecco's PBS (ICN) was dissolved in 100 mls of MQ water. One hundred microlitres of DEPC was added and the solution shaken vigorously, before autoclaving the nest day.

tRNA

To 100 mg of baker's yeast tRNA (Boehringer-Mannheim), 10 mls of DEPC-treated water was added. Aliquots were made and stored at –20°C.

dNTP mix

Ten µl of each dNTP (Promega Corporation) was added to 60 µl of DEPC-treated water. Aliquots were stored at -20°C.

oligo $(dT)_{15}$ primer

The tube containing the lyophilised oligonucleotide (Boehringer-Mannheim) was centrifuged to ensure collection at the base of the tube. To make a 500 µg/ml concentration, 80 µl of DEPC-treated water was added to the tube containing 40 µg. The tube was allowed to sit at room temperature for about 5 minutes and then the total volume required was pipetted to another tube to be aliquoted. The solution was stored at -20°C.

6x DNA Loading Buffer

To 30 ml of glycerol (ICN), 0.25 g of bromophenol blue (Sigma) and 0.25 g of xylene cyanol FF (Sigma) were added. The solution was made up to 100 ml with MQ water and shaken vigorously. The solution was stored at 4°C, with aliquots ready for use at room temperature.

TE (10 mM tris, 1 mM EDTA, pH 8)

This solution was prepared by adding 0.5 mls of 2 M Tris.-HCl, pH 8 (ICN) and 0.2 mls of 500 mM EDTA, pH 8 (Sigma) to 99.3 mls of DEPC-treated water. The solution was autoclaved and stored at room temperature.

Phenol:Chloroform:Isoamyl Alcohol

The solution of phenol, chloroform and isoamyl alcohol was prepared as a 25:24:1 ratio and stored in a light excluding bottle at 4°C.

3 M Sodium Acetate

To 800 ml of MQ water, 408.1 g of sodium acetate.$3H_2O$ was dissolved. The pH was adjusted to pH 6 (or 4.8) with dilute acetic acid and the volume made up to 1 litre. This solution was sterilised by autoclaving.

10% APS

This solution was prepared by dissolving 1 g of ammonium persulphate (Merck) in 10 ml of MQ water. The solution was filter sterilised through a 0.2 μM filter and stored at 4°C.

Gel Fixing Solution

To 2,400 ml of MQ water, 300 ml of methanol (BDH) and 300 ml of glacial acetic acid (BDH) was added. This solution was stored at room temperature.

5x Tris-Borate-EDTA (TBE) Buffer

To 900 ml of MQ water, 54 g of Tris base (Sigma), 27.5 g of boric acid (Sigma) and 20 ml of 500 mM EDTA, pH 8.0 was added and stirred until all solutes dissolved. The volume was adjusted to 1 litre and stored at room temperature in glass bottles.

2x TY Broth

To 900 ml of MQ water, 16 g of bacto-tryptone (Difco), 10 g of bacto-yeast extract (Difco) and 5 g of sodium chloride (Sigma) was dissolved by shaking. The pH was adjusted to 7 with 5 N sodium hydroxide and the volume made up to 1 litre with MQ water. This solution was sterilised by autoclaving and stored at room temperature.

1 M Calcium Chloride

Calcium chloride (29.4 g $CaCl_2.2H_2O$; Sigma) was dissolved in 200 ml of MQ water, filter sterilised and stored at 4°C.

L-broth

To 950 ml of MQ water, 10 g of bacto-tryptone (Difco), 5 g of bacto-yeast extract (Difco) and 10 g of NaCl (Sigma) were dissolved by shaking. The pH was adjusted to 7.0 and the volume made up to 1 litre. The solution was sterilised by autoclaving and stored at room temperature.

L-Agar

To 1 litre of L-broth, 15 g of bacto-agar (Difco) was added just before autoclaving. After autoclaving, the bottle was carefully swirled to distribute the melted agar. The medium/agar was allowed to cool to 50°C before adding ampicillin.

L-Agar Plates

30-35 ml of medium/agar per 90 mm plate was poured directly from the bottle. The surface of the medium in the plates was flamed to eliminate air bubbles on the surface. Plates were inverted and stored at 4°C after setting of the agar. 1-2 hours before use, the plates were placed at 37°C, inverted.

L-Agar Plates with X-gal and IPTG

To a pre-made L-broth agar plate (with or without ampicillin) 40 µl of X-gal (20 mg/ml; 5-bromo-4-chlor-3-indolyl-β-D-galacto-pyranoside, Boehringer-Mannheim) and 4 µl of IPTG (200 mg/ml; isopropyl-β-D-thiogalactopyranoside, Boehringer-Mannheim) were spread individually over the entire surface of the plate with a sterile glass spreader. The plate was incubated at 37°C until the fluid was absorbed.

Ampicillin

A 100 mg/ml stock solution of ampicillin (Boehringer-Mannheim) was filter sterilised through a 0.22 μM disposable filter (Millipore) and stored at -20°C.

T-broth

To 900 mls of MQ water, 12 g of bacto-tryptone, 24 g of bacto-yeast extract and 4 ml of glycerol was dissolved by shaking. The solution was sterilised by autoclaving and allowed to cool to 60°C before adding 100 ml of an autoclaved solution of 170 mM potassium phosphate (monobasic, KH_2PO_4; Sigma) and 720 mM potassium dihydrogen orthophosphate (K_2HPO_4; Ajax). This solution was stored at room temperature.

10x PBS pH 7.4

Sodium phosphate (21.4 g $Na_2HPO_4.2H_2O$, 0.012 M; Ajax), sodium hydrogen phosphate (4.1 g $Na_2H_2PO_4.H_2O$, 0.003 M; BDH) and sodium chloride (87.7 g NaCl, 0.15 M; BDH) were dissolved in MQ water. The pH was adjusted to 7.4 and the volume made up to 1 litre. This was a 10x concentrate solution of PBS and was stored at room temperature.

Assay Buffer

To 1 litre of 1x PBS, 0.5% bovine serum albumin (BSA; Boehringer-Mannheim) and 0.01 % thimersol (Sigma) was added. The solution was stored at 4°C.

Coating Buffer

Sodium carbonate (11.45 g $Na_2CO_3.10H_20$, 0.04 M; Ajax), sodium bicarbonate (5.04 g $NaHCO_3$ {MWt = 84.01}, 0.06 M; Ajax) and 0.01% thimersol (Sigma) were dissolved in 900 ml of distilled water. The pH was adjusted with HCl to 9.6 and the volume made up to 1 litre. The solution was stored at 4°C.

Coating Buffer with Anti-IgG

To 200 ml of coating buffer, 160 µl of anti-rabbit IgG (0.9 µg; Silenus Laboratories) was added. The solution was stored at 4°C.

Blocking Buffer

To 500 ml of coating buffer, 1% BSA (Boehringer-Mannheim) was added. The solution was stored at 4°C.

Wash Buffer

To 1 litre of 1x PBS, 0.05% Tween 20 (Ajax) was added. The solution was stored at room temperature.

Primary Antibody

To 199.9 ml of assay buffer, 100 µl of primary antibody (R64) was added. The solution was stored at 4°C.

Secondary Antibody

To 199.9 ml assay buffer, 100 ml of biotinylated secondary antibody was added. The solution was stored at 4°C.

Standards

Partially purified recombinant dimer was diluted in assay buffer. The range of standards was from 0.3 ng/ml to 24 ng/ml.

Streptavidin/Horse Radish Peroxidase Enzyme Conjugate

To 49.995 ml of peroxidase protective buffer (Silenus Laboratories), 5 µl of streptavidin/horse radish peroxidase (Boehringer-Mannheim) was added. This solution was stored at 4°C.

Substrate

To 9.725 ml of substrate buffer (below), 250 µl of TMB-DMSO (below) and 25 µl of 3% H_2O_2 (below) was added. This solution was freshly prepared and used immediately.

Substrate Buffer

Sodium acetate (8.2 g; Ajax) and EDTA (0.37 g; BDH) was dissolved in MQ water. The pH was adjusted to 5.0 with glacial acetic acid and the volume made up to 1 litre. The solution was stored at room temperature.

Hydrogen Peroxide

To 0.1 ml of 30% hydrogen peroxide (H_2O_2; Sigma), 0.9 ml of MQ water was added. This solution was stored at 4°C in 1 ml aliquots in aluminium foil.

TMB/DMSO

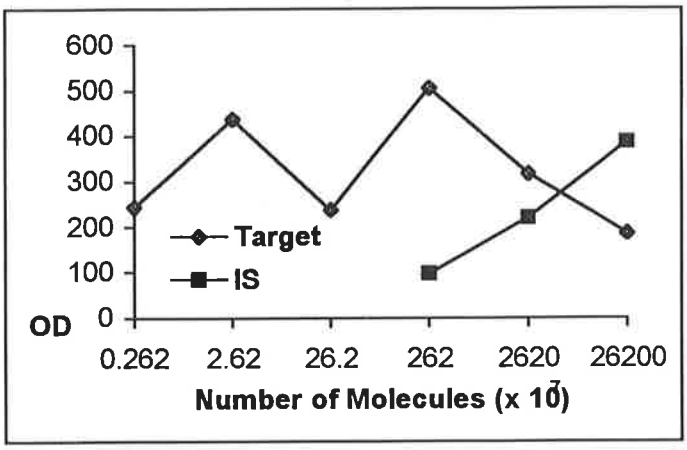
To prepare a stock solution, 100 mg/ml 3,3'-5,5' tetramethylbenzidine (TMB; Boehringer-Mannheim) was dissolved in dimethyl sulphoxide (DMSO; Sigma). This stock was then diluted further with DMSO to make a 10 mg/ml solution. Both solutions were stored at 4°C.

Stop Solution

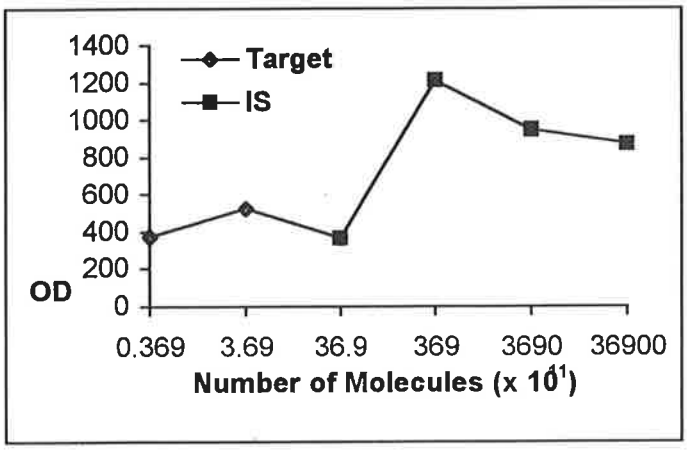
To prepare a 500 mM sulphuric acid solution, 22.5 ml of concentrated H_2SO_4 (BDH) was added carefully to 975.5 ml MQ water. The solution was stored at room temperature.

These graphs show the equivalency point for the cultured embryos that were able to be quantitated. On the left of each graph is the embryo code from *Table 6.6* and a b for mCG-β or a g for mGAPDH.

Z3 b



Z3 g



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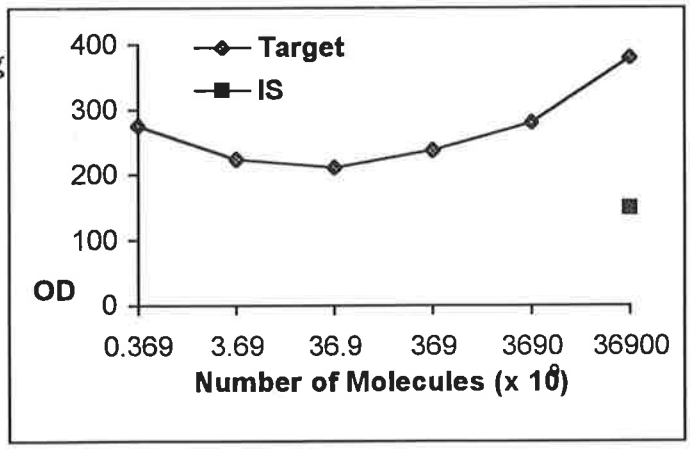

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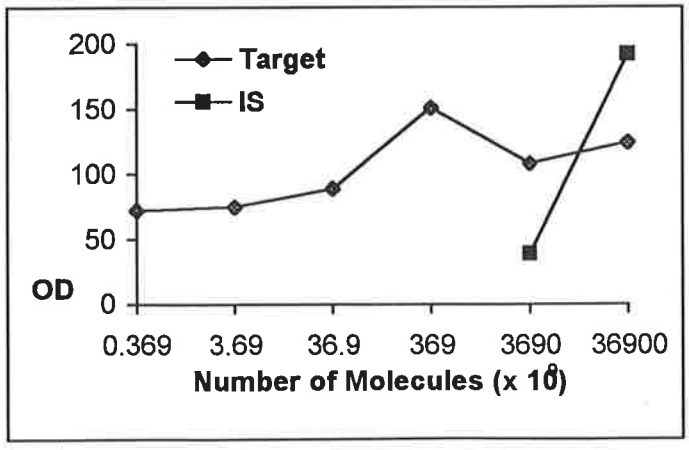

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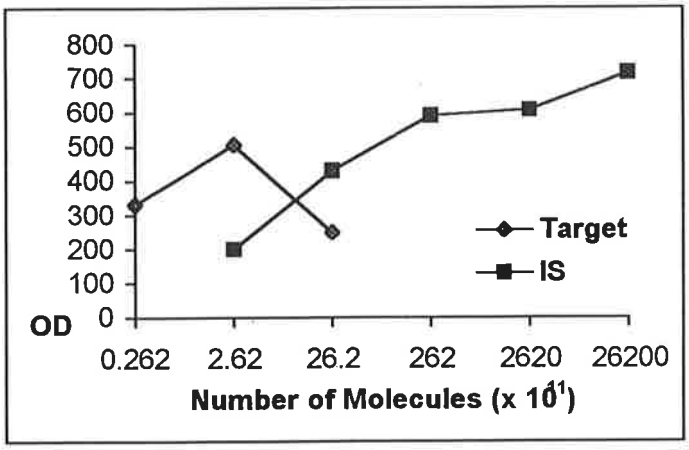

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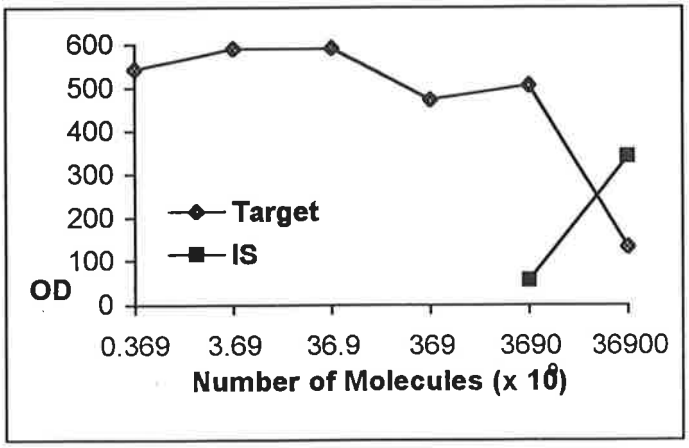
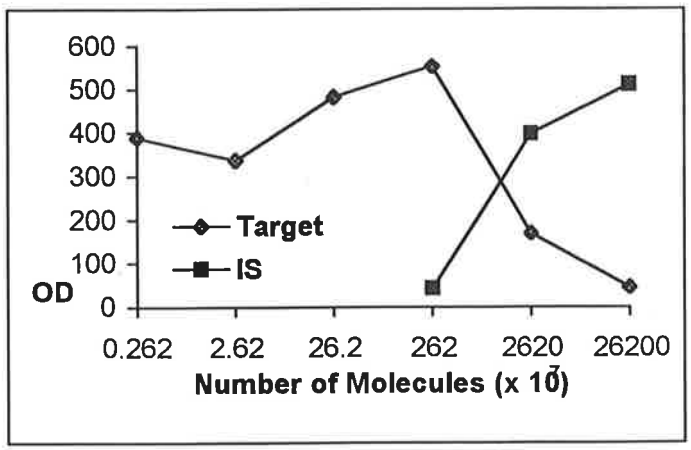


B3 b



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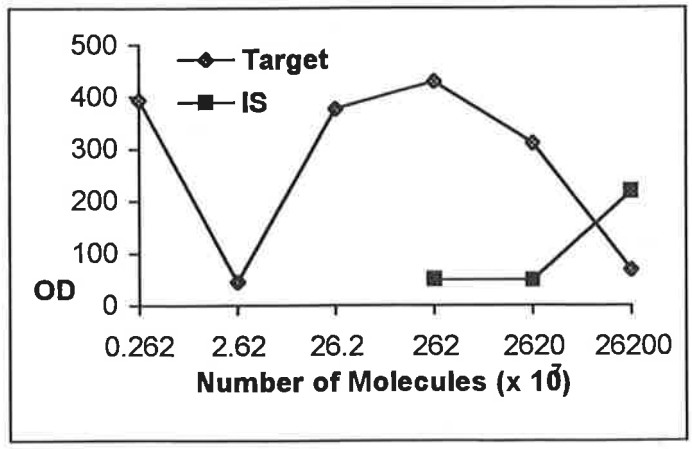

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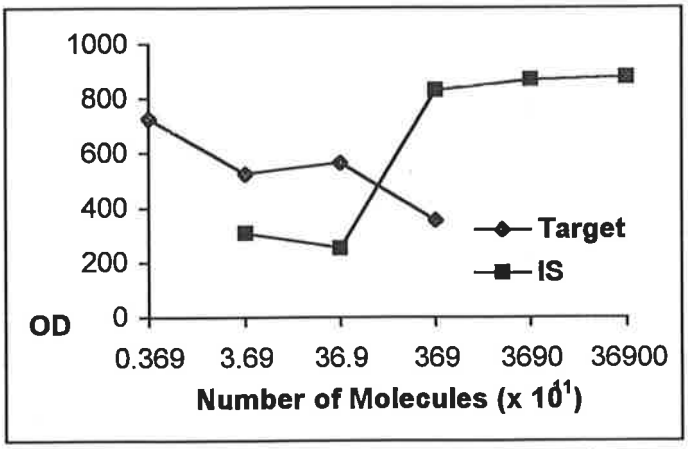

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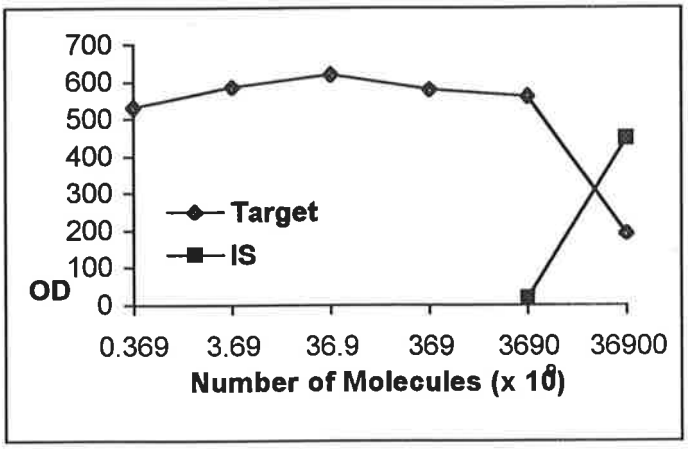

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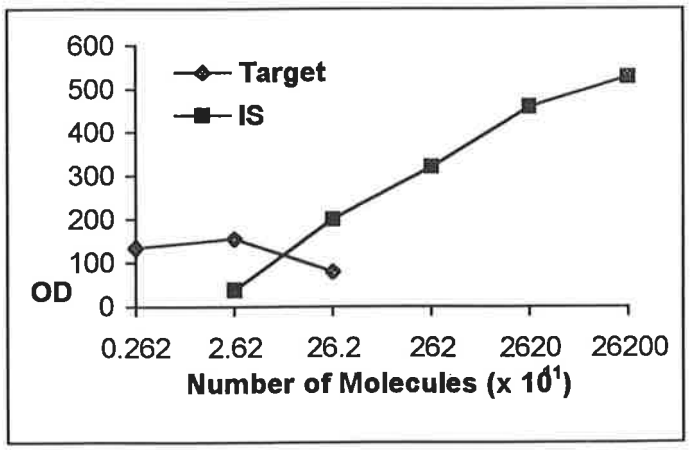

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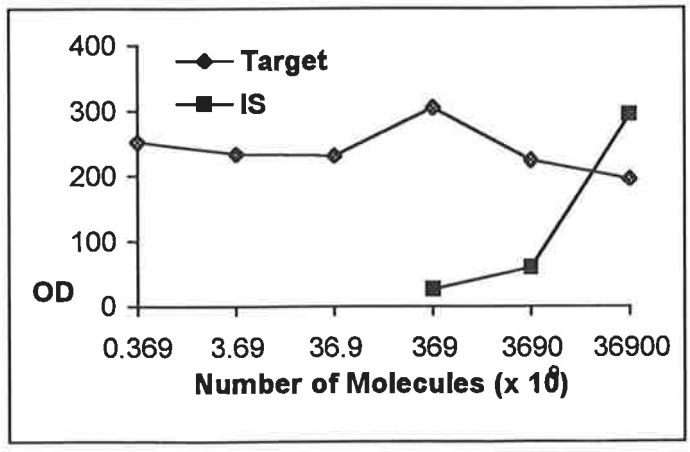

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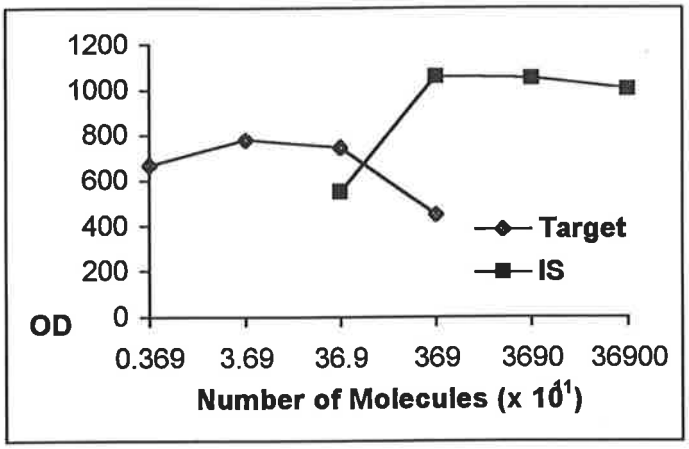
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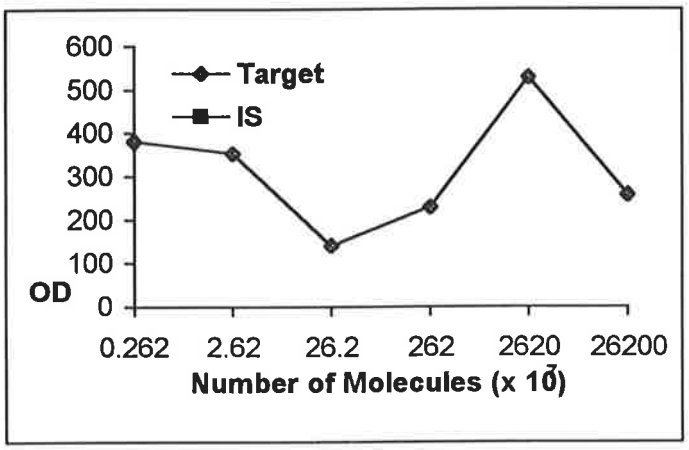
36900
Number of Molecules (x 10¹)

T7 b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26200
Number of Molecules (x 10⁷)