The Control of Placental Corticotrophin-Releasing Hormone and Adrenocorticotrophic Hormone Production

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Thesis submitted to the University of Edinburgh for the degree of Doctor of Medicine.

September 1995

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DECLARATION

Except where acknowledgement is made by reference, the experiments detailed in this thesis were the unaided work of the author. No part of this work has previously been accepted for any other degree nor is any part of it being concurrently submitted in canditure for any other degree. This thesis was written by myself.

Elizabeth Sarah Cooper

Acknowledgements

I am most grateful to Professor I.A. Greer and Dr A.N. Brooks for their supervision and guidance throughout this study, which was funded as a Medical Research Council Training Fellowship.

I am grateful to Mr M. Millar for his help in setting up the immunocytochemistry and in situ hybridisation methods.

I wish to thank the midwifery and medical staff of the Simpson Memorial Maternity Pavillion, particularly the staff of ward 54, for their help with the collection of tissues.

I also wish to thank Mr T McFetters for his patience and help with the photography and graphs.

I would like to thank Professor D.W. Lincoln for accepting me as an MRC training fellow in the MRC Reproductive Biology Unit, and Professor A.A. Calder for allowing me to use the facilities of the Department of Obstetrics and Gynaecology, University of Edinburgh.

Finally I wish to thank my parents for their constant encouragement.

ABSTRACT

The human placenta contains many hormones identical to those of the hypothalamo-pituitary axis. It was proposed, in the 1950's that these may influence the hypothalamo-pituitary-adrenal axis of both the fetus and the mother. More recently it has been suggested that placental corticotrophin-releasing hormone (CRH) and adrenocorticotrophic hormone (ACTH) may play an important role in the mechanism initiatiating parturition. The plasma concentration of CRH is known to increase with gestation rising to high levels prior to delivery. *In vitro* studies have investigated the modulation of its synthesis, and have led to suggestions that it is involved in a positive feedback mechanism leading to birth. The studies described in this thesis were undertaken to investigate the control of its production *in vivo.* Less is known about placental ACTH, and disagreement exists over whether its output increases during pregnancy. Studies to clarify this point, and to investigate the modulation of ACTH production were also performed.

The cellular localisation of placental CRH has previously been investigated using immunocytochemistry, but results are conflicting. The results discussed in this thesis are in agreement with the more recent of these studies. Immunoreactive CRH was localised to the syncytiotrophoblasts of the placenta and to the amnion, chorion and decidua throughout gestation. Since it has been hypothesised that CRH may play a role in the onset of parturition, the effects of labour on both immunostaining, and placental CRH content were investigated. There was no difference in placentas collected after spontaneous delivery compared with those from women who had not laboured. Previous *in vitro* studies have suggested that prostaglandins may stimulate, and progesterone may inhibit, placental CRH production. Placentas collected after prostaglandin induced delivery in the first and second trimesters, and at term were studied. There was found to be no difference in either immunostaining, or in placental CRH content in any of these tissues compared to tissues collected after suction curettage, or spontaneous

delivery. The effect of progesterone was studied using the antiprogestin mifepristone in the first trimester. Tissues collected after termination of pregnancy with mifepristone showed no differences to those collected after curettage.

These studies are the first to localise POMC gene expression within the placenta by in situ hybridisation. In combination with immunocytochemistry both mRNA transcribed from the gene, and the gene product translated from the mRNA can be identified. The results of these studies provide very strong evidence that POMC is synthesised in cytotrophoblasts in the first trimester, and syncytiotrophoblasts thereafter. In addition both POMC mRNA and immunopositive staining were found in amnion and chorion. The placental ACTH content increased with advancing gestation, and this finding was supported by both an increase in immunostaining and POMC gene expression over this period. Placental ACTH content, immunostaining and POMC gene expression were unaffected by labour, the administration of prostaglandins in the first trimester and at term, or by the administration of mifepristone in the first trimester.

Thus both CRH and ACTH appear to be constitutively present in the placenta throughout gestation. These results suggest that these peptides may have other roles during pregnancy rather than being simply related to parturition. They contribute to the normal endocrine environment of the placenta and may have a 'housekeeping' role throughout pregnancy. In addition, they may also enter the fetal circulation influencing its hypothalamo-pituitary-adrenal axis and may potentially affect the maturation of other important organs.

List of Abbreviations

ACTH	Adrenocorticotrophic hormone
α-MSH	alpha-Melanocyte Stimulating Hormone
β-EP	Beta-Endorphin
CLIP	Corticotrophin-like Intermediate Peptide
CRH	Corticotrophin-Releasing Hormone
CRH-BP	Corticotrphin-Releasing Hormone Binding Protein
DAB	3.3'-diaminobenzidine tetrahydrochloride
DHEAS	DihydroxyepiandrosteroneSulphate
GnRH	Gonadotrophin Releasing Hormone
HCG	Human Chorionic Gonadotrophin
HPA	Hypothalamo-pituitary-adrenal
HPL	Human Placental Lactogen
PBS	Phosphate buffered saline
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$

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CHAPTER 1

LITERATURE REVIEW

1.1. Introduction

The placenta contains many hormones that are identical or similar to those of the hypothalamo-pituitary axis. In particular it synthesises corticotrophin-releasing hormone (CRH), the plasma concentration of which rises with advancing gestation. CRH is thought to play an important role in the mechanism by which parturition is initiated. In addition, the presence of an adrenocorticotrophin-like hormone has been demonstrated in placenta. Both these hormones may influence the hypothalamo-pituitary-adrenal axis of the fetus and also possibly the mother, and, at the same time have a paracrine/autocrine role within the placenta and fetal membranes.

This review will deal with the anatomical and histological structure of the human placenta, the placenta as an endocrine organ and then give a brief overview of the physiology and biochemistry of normal labour. It will also review the historical background to the discovery of CRH, both hypothalamic and placental, including its hypothesised intracellular mode of action on the corticotroph, the changes in CRH during pregnancy in maternal plasma, fetal plasma and amniotic fluid and its interactions with CRH-binding protein. It will then discuss CRH in the placenta and fetal membranes including its tissue and cellular origins, the control of CRH production by the placenta and fetal membranes including its relevant molecular basis, and its interactions with steroid hormones, prostaglandins and oxytocin. Finally, it will discuss the suggested role that placental CRH may play in parturition. Background information will be provided of the discovery of ACTH in the placenta, the changes in plasma and placental levels during pregnancy and the control of its production. It will then suggest possible explanations for the role of ACTH in pregnancy.

1.2. The structure of the placenta and fetal membranes

1.2.1. The placenta

The human placenta consists of layers of trophoblast cells, connective tissue and branching blood vessels which are all of fetal origin. As early as the third week of gestation, there is a villous system capable of supplying the embryo with nutrients and oxygen. The primary stem villus, consisting of a cytotrophoblast core surrounded by a syncytial layer, is penetrated by mesoderm to form the secondary stem villus. These mesodermal cells differentiate to form the fetal capillaries of the tertiary stem villus, by the fourth week of gestation, connecting the placenta with the embryo. By the end of the eighth week, the trophoblast consists of a great number of secondary and tertiary villi the surface of which is formed by the syncytiotrophoblasts resting on a layer of cytotrophoblasts covering the fetal capillary system. In the mature placenta, villi of syncytiotrophoblast, containing mesodermal cores in which fetal blood vessels run, penetrate deeply into maternal tissue (the decidua). Each villus, formed by progressive branching of the main divisions of the umbilical vessels is separated from the surface by a thin syncytiotrophoblast layer. The branches of the villi are arranged in lobules with the terminal villi projecting inwards with a maternal spiral artery at the base of each lobule which injects blood into the space. There are approximately two hundred such lobular units and several are grouped together to form a cotyledon. Maternal blood circulates around the terminal villi containing the fetal capillaries and both active transport and passive diffusion of molecules occurs across this membrane depending on such factors as molecular weight, electrical charge and hydrophobicity. The chorionic villi are thus the functional unit of the placenta. Each is surrounded by an outer layer of syncytiotrophoblast and an inner layer of cytotrophoblast. The syncytiotrophoblast is a continuous layer covering the villi, and on the maternal side, is in direct contact with maternal blood. Morphologically the trophoblasts may be cellular (cytotrophoblasts) syncytial (syncytiotrophoblasts) or intermediate. Mitotic figures are

completely absent from the syncytium, being confined purely to the cytotrophoblasts. Midgely (1963) found that although Tritium-labelled thymidine appeared at first only in the nuclei of the cytotrophoblasts, it could be detected twenty-two hours later in the syncytiotrophoblast. It therefore seems likely that the syncytium is derived from cytotrophoblast and is a mitotic end stage. Thus, cytotrophoblasts are prominent in first trimester placenta, but, as they differentiate into syncytiotrophoblast, they are sparse at term. A basement membrane lies between the trophoblast and the stroma. The stroma contains loose mesenchymal tissue, macrophages (Hofbauer cells) and fetal capillaries (one to six per villus). The chorionic villus consists of thick areas containing the organelles for synthetic functions and thin areas overlying fetal capillaries (vasculosyncytial membranes) that are specialised for transfer between maternal and fetal circulations. Of all the placental components, the trophoblast is the most variable in terms of structure, function and development and has the most diverse roles. These include attachment and nutrition of the early conceptus, prevention of maternal rejection, transfer of substances between the maternal and fetal circulations, and as an endocrine organ secreting a large number of steroid and peptide hormones, enzymes and placental proteins. (For the structure of placental villi at term and in the first trimester, see figure 1.1)

1.2.2. The amnion

The amnion develops from fetal ectoderm and arises as a layer of epithelial cells between the ectodermal disc of the inner cell mass and the trophoblast (chorion). The amnion is 0.02-0.5mm thick and has five layers comprising cuboidal epithelium with a microvillus surface, prominent intracellular canals and numerous vacuoles, basement membrane, the compact layer, fibroblastic layer and the spongy layer of mucoid reticular tissue. It is avascular and aneuronal and has no lymphatics. It also has diverse roles including the production of amniotic fluid and prostaglandin synthesis. (For the structure of the amnion, see figure 1.2)

1.2.3. The chorion

Chorion has only four layers, comprising a cellular layer of fibroblasts, a reticular layer, a basement membrane and then trophoblast. The trophoblast layer is two to ten cells thick, and is continuous with placental trophoblast, lying adjacent to decidua. In placental areas chorion contains both a vascular and a nervous supply, but elsewhere is avascular and aneuronal. As well as its synthetic functions, it has substantial prostaglandin dehydrogenase activity.
(For a diagram of the structure of chorion, see figure 1.2).

1.2.4. The decidua

Unlike the amnion, chorion and trophoblast, the decidua is maternally derived. It is the endometrium of the pregnant uterus, thickening throughout gestation, to become 10mm thick. The decidua adjacent to the site of implantation is the decidua basalis, that overlying the developing embryo is the decidua capsularis, and the rest of the uterus is lined by the decidua parietalis. After the fourth month of pregnancy, the decidua parietalis and capsularis fuse, and the uterine cavity is obliterated. Decidual stromal cells are large polyhedral epitheliod cells surrounded by a basement membrane. The decidua is thought to be concerned with the nutrition of the blastocyst (Kearns and Lala 1983), trophoblast invasion (Kishimoto et al 1987) and protection of the embryo from maternal immune rejection (Golander et al 1984).

1.3. The placenta as an endocrine organ

1.3.1. Placental steroid hormones

The placenta in many species is the source of many steroid and peptide hormones. The placental synthesis of steroid hormones is a vast subject, and since this thesis will focus on placental peptides, steroids will only be discussed here briefly. As mentioned above, during pregnancy the placenta undergoes a continuous differentiation process,

with cytotrophoblasts differentiating into syncytiotrophoblasts. Using immunohistochemical techniques investigators have localised steroid hormones to the syncytiotrophoblast. Large amounts of oestrogens, progesterone and pregnenolone are produced during pregnancy. By the eighth week, the placenta has started to produce progesterone (Tulchinsky and Hobel 1973), and as much as 600mg is produced per day by late pregnancy. Steroid precursors from the fetus such as dehydroepiandrosterone sulphate and 16α-hydroxydehydroepiandrosterone are converted by placental sulphatases and aromatases to oestradiol and oestriol respectively (Bolte et al, 1964; Sitteri and Macdonald1963; Klausner and Ryan 1964; Sitteri and Macdonald1966). (See figure 1.3 for a flow diagram of placental steroid production and metabolism)

1.3.2 Placental peptide and polypeptide hormones

Almost all the placental peptide or polypeptide hormones investigated are structurally and functionally either identical or very similar to their pituitary and hypothalamic counterparts. Thus placental lactogen shares properties with prolactin, chorionic gonadotrophin is similar to luteinising hormone, placental pro-opiomelanocortin (POMC) derived peptides are similar to those produced by the pituitary, and a number of peptides, including CRH, are produced that are very similar to hypothalamic hormones. (see figure 1.4)

1.3.2.1. Placental pituitary-type hormones

Pituitary-type hormones, HCG, HPL and POMC peptides have been primarily localised to the syncytiotrophoblast (Al-Timini and Fox 1987) and some studies have located hypothalamic-type peptides to the cytotrophoblast (Petraglia et al 1987; Saijonmaa et al 1988) or the syncytiotrophoblast (Riley et al 1991). In vitro studies have shown that GnRH stimulates the release of trophoblastic HCG production (Merz et al 1991; Kelly et al 1991), and CRH stimulates placental ACTH production (Margioris et al 1988a) leading to speculation that the cytotrophoblast may regulate syncytiotrophoblast function in a paracrine manner (Petraglia et al 1990b). In addition, these neuropeptides may enter

the maternal and fetal circulations and exert endocrine effects (Gennazzani et al 1975; Goland et al 1986; Economides et al 1987). Placental production of POMC peptides will be discussed in detail in later sections. In addition to POMC peptides, the placenta produces other pituitary-type hormones.

Human chorionic gonadotrophin (HCG) is one of the earliest hormones to be detected in pregnancy and is essential for the maintainence of the corpus luteum of pregnancy (Braunstein 1988). Its regulation is different to that of other placental proteins in that its levels peak in late first trimester and then fall whilst other placental hormones show a continual increase correlating with increasing placental mass (Braunstein et al 1976). The regulation of its synthesis is not well understood but may involve factors produced by decidua and trophoblast (Vicovac and Genbacev1988; Yagel et al 1989; Masuhiro et al 1991; Yuen et al 1986).

Placental lactogen can first be detected in plasma as early as 6 weeks gestation (Grumbach and Kaplan 1964), and its secretion increases linearly to
30 weeks (Grumbach et al 1968). It has been suggested that placental lactogen acts as an insulin antagonist in the mother, inducing lipolysis and proteolysis, thus facilitating the transfer of glucose and amino acids to the fetus (Grumbach et al 1968). More recently, it has been suggested that placental lactogen may affect fetal growth by a direct action on the fetus (Freemark and Handwerger 1989). Placental lactogen was, for many years, thought to be the only sommatomammotrophin produced by the placenta, however the syncytiotrophoblast also produces a peptide similar to pituitary growth hormone (Frankenne et al 1988), and the decidua produces prolactin (Golander et al 1978; Riddick et al 1978; Rosenberg et al 1980).

Placental growth hormone is thought to have similar action in the mother as placental lactogen, and it has been suggested that decidual prolactin may play a role in the regulation of amniotic fluid volume (Tyson et al 1984), the regulation of fetal lung surfactant synthesis

(Grosso et al 1980), and the modulation of prostaglandin synthesis in the fetal membranes (Tyson et al 1985).

1.3.2.2. Placental hypothalamic-type hormones

In addition to CRH (which will be discussed in detail in later sections) other hypothalamic releasing or inhibiting hormones are produced by the placenta. Placental gonadotrophin-releasing hormone (GnRH) has been localised to the cytotrophoblast and is immunologically and biologically indistinguishable from hypothalamic GnRH (Gibbons et al 1975; Khoder and Siler-Khoder 1980; Lee et al 1981). Its release is modulated by steroid hormones, opioids, prostaglandins and noradrenaline (Petraglia et al 1987a; Petraglia et al 1990b). GnRH has a paracrine role stimulating the release of HCG from cultured placental tissue throughout gestation (Siler-Khoder 1983; Khoder and Siler-Khoder 1978; Siler-Khoder and Khoder 1989).
A thyrotrophin-releasing hormone like substance has been found in human placenta (Gibbons et al 1975), and a growth hormone releasing hormone has been identified in rat placenta (Baird et al 1985). The function of both these peptides within the placenta is unknown.

For most placental peptides and proteins a definitive physiological role has not been established. Instead, it has been suggested, that many of the placental proteins and peptide hormones exist to maintain the trophoblast as a functional unit, providing information about the maternal environment, rather than having specific endocrine actions. This view is supported by the finding that normal pregnancy can occur when specific peptides, for example prolactin, (Simon et al 1986) are absent. The synthesis of these peptides would enable a fairly constant level of general activity throughout the trophoblast, maintaining constant transfer of waste and nutrients between fetal and maternal circulations (Chard 1993).

1.4. The physiology and biochemistry of labour

Effective labour involves both ripening and dilation of the cervix and the production of coordinated uterine contractions capable of expelling the conceptus. Uterine contractions occur throughout pregnancy (Caldeyro-Barcia 1959) and increase in strength over the last five weeks of pregnancy, reaching a maximum during labour. Over the same period a number of structural changes cause the cervix to soften, dilate, and become effaced (Anderson and Turnbull 1969). The cervix is composed of fibrous connective tissue, the main component of which is collagen fibrils bound together by the proteoglycan complexes of the ground substance (Van der Rest 1980). Proteoglycans are glycosaminoglycans, particularly dermatan sulphate in the human cervix, connected to a protein core (Uldbjerg et al 1983b). The sulphate group on this mucopolysaccharide makes it extremely hydrophilic and it is this modification of the cervical water content that alters the mechanical properties of the cervix (Uldjberg et al 1983c). The softening of the human cervix is due to marked changes in the connective tissue (Danforth 1983) with an increase in water content and a change in the content of glycosaminoglycans (Von Maillot et al 1979; Uldjberg et al 1983a). Compared with the non-pregnant cervix there is fall in collagen content of more than 50% (Uldjberg et al 1983c). In addition, the number of intermolecular cross-links is decreased causing the dispersal of collagen fibrils, with resulting softening of the cervix (Junqueira et al 1980). Prostaglandins are involved in the control of this process. The evidence for this comes from clinical as well as physiologcal and pharmacological studies. There is a correlation between concentrations of prostaglandins E_2 and $F_{2\alpha}$ in amniotic fluid, and the degree of cervical ripening (Calder 1980), and prostaglandin E_2 is used to ripen the cervix prior to the induction of labour (Calder 1980; Greer et al 1989). PGE_2 reduces the collagen content of the cervix (Uldjberg et al 1983c), although whether this is due to an increase in collagenase activity (Uldjberg et al 1983; Szalay et al 1981), or by altering the composition of glycosaminoglycans in the ground substance (Cabrol et al 1987) is unclear. The importance of a local factor in cervical ripening is supported by experiments on isolated sheep cervix, where, despite the

loss of vascular and mechanical connections, softening still occured in labour (Ledger et al 1985). This experiment showed that cervical ripening is an active rather than a passive process and is not secondary to uterine activity. In addition oestradiol (Gordon and Calder 1977) and relaxin (MacLennan et al 1986) have been implicated in cervical ripening. Steroid hormones such as oestradiol may have effects on cervical ripening by a direct action or via changes mediated by prostaglandins.

As discussed above, the uterus is never entirely quiescent, but the nature of this activity at term, is entirely different to that in the first trimester. Several factors play a role in the transition from the uterus as a 'capacitance vessel' in early pregnancy, to that capable of expelling the fetus at term. Like the cervix, the body of the uterus has a matrix of collagen fibres. In this are embedded smooth muscle cells containing random bundles of actin and myosin (Marsten and Smith 1985) and it is through the interaction of these two molecules that contractions are generated. The interaction of actin and myosin in the mometrial cells is under the control of intracellular calcium levels (Carsten and Miller 1987). Prostaglandins E_2 and $F_{2\alpha}$ and oxytocin increase the availability of intracellular calcium and therefore stimulate uterine activity (Carsten and Miller 1987). During the last few weeks of pregnancy, and during labour, intercellular connections (gap junctions) develop. These junctions allow the cells to communicate both metabolically and electrophysiologically thus converting disparate myometrial cells to a functional syncytium (Garfield et al 1977; Garfield et al 1980a; Garfield and Hyashi 1981). The formation of these gap junctions is also under the control of steroids and prostaglandins (Garfield et al 1980); prostaglandins $F_{2\alpha}$ and E_2 and oestrogens stimulate, and progesterone and prostacyclin inhibit, gap junction formation. In addition, the antiprogestin mifepristone will induce gap junction formation. Progesterone decreases electrical conductivity between cells and inhibits the formation of gap junctions (McKenzie and Garfield 1985). Conversely, oestrogens reduce the membrane resting potential of cells, increase intracellular calcium ion concentrations and increase gap junction formation and thus stimulate myometrial contractility.

The activity of the uterus prior to labour thus depends on a balance of stimulatory and inhibitory factors, the appearance of gap junctions which allows synchrony between myometrial cells, and changes in membrane potentials. In sheep, parturition is preceded by a rise in the plasma concentrations of the myometrial stimulants oestrogens and prostaglandins combined with a fall in the plasma concentration of the myometrial relaxant progesterone (Csapo 1961). Such a fall does not occur in humans although the antiprogestin mifepristone increases the myometrial sensitivity to prostaglandins and is an effective abortifacent in humans and animals (Rodger and Baird 1987; Rodger et al 1988; Garfield and Baulieu 1987). Oestrogens may also stimulate prostaglandin production (Olsen et al 1983).

Prostaglandins play an important role in the generation of uterine contractility. Both PGE2 and PGF2α levls rise in amniotic fluid (Keirse 1979; Hillier et al 1974), and both metabolites rise in plasma during labour (Johnston et al 1990). The plasma concentrations of PGF2α during labour correlate directly with the duration and inversely with the need for augmentation, suggesting it is important for generating and maintaining uterine activity. The levels of PGE2 rise prior to the onset of established labour and it is the major prostaglandin produced by the amnion (Johnston et al 1990). It has high affinity receptors in the myometrium (Adelantado et al 1985) implicating it in the regulation of uterine contractility.

Over the last few weeks of pregnancy, and during the first stage of labour, the number of myometrial receptors for oxytocin increases (Alexandova and Soloff 1980; Fuchs et al 1982) with a resultant increase in sensitivity to oxytocin (Caldeyro-Barcia and Sereno 1961). At this time, the plasma level of oxytocin remains at a constant level (Thornton et al 1988). Oxytocin acts by increasing intracellular calcium availability and thus increases the contractions in a previously sensitised uterus (Fuchs 1983). Oestrogens increases (Alexandrova and Soloff 1980) and progesterone reduces (Soloff 1985) the concentration of oxytocin receptors and prostaglandins increase the uterine sensitivity to oxytocin.

Thus, steroid hormones and prostaglandins (regarded as the final common mediators of parturition) are involved in the regulation of both cervical ripening and myometrial contractility. Parturition in the sheep appears to be controlled by maturation of the fetal hypothalamo-pituitary-adrenal axis (Challis and Brooks 1989). The fetal hypothalamus secretes CRH which in turn stimulates the production of ACTH from the fetal pituitary over the last few weeks of gestation (Brooks and Challis 1988). The ACTH stimulates the production of cortisol from the fetal adrenal and this increases the activity of placental 17-hydroxylase, causing the conversion of progesterone to oestrogen (Flint et al 1975). Thus there is a change in the balance of myometrial stimulants and relaxants in favour of stimulants, thus resulting in increased gap junction formation production, myometrial excitability and prostaglandin production. In contrast, the human placenta is deficient in 17-hydroxylase. This means that there can be no placental conversion of progesterone to oestrogen. Indeed, there is no change in the plasma concentrations of these hormones at the onset of labour (Liggins 1983). However, a rise in fetal cortisol does occur, and there is further evidence of HPA axis activation in the rapidly rising levels of CRH prior to delivery (see sections 1.6-1.9). Maturation of the fetal HPA axis may affect the axis of hypothalamo-pituitary peptides produced by the placenta. Placental CRH and ACTH pass to the fetal circulation stimulating the fetal adrenal to produce cortisol and DHEA sulphate which in turn may be converted to oestradiol by placental aromatase. In addition, fetal cortisol may increase placental CRH production in a unique positive feedback mechanism (Jones et al 1989). As mentioned above, in common with the sheep, prostaglandins appear to be of central importance in the final pathway controlling human parturition. Placental CRH in turn, stimulates prostaglandin production by the fetal membranes, thus linking maturation of the HPA axis hormones with the onset of parturition.

1.5. Hypothalamic corticotrophin-releasing hormone

Corticotrophin-releasing hormone (CRH) is a 41 amino acid peptide first described, in the sheep, by Vale et al in 1981 and subsequently was isolated, characterised and synthesised (Vale et al 1983). It is produced in high concentrations by the parvocellular neurones of the paraventricular nucleus of the hypothalamus (Pelletier et al 1983). The axons of these neurones project to the median eminence where CRH is secreted (Takahashi et al 1989). It is then transported from the hypothalamus by the hypophyseal portal system and reaches the anterior pituitary where pro-opiomelanocortin products such as adrenocorticotrophic hormone (ACTH) and Beta-Endorphin (β-EP) are released (Grossman et al 1982). Ovine and human CRH both consist of 41 amino acids but seven of these, mainly found in the C-terminal region, differ (Vale et al 1983). Screening a human gene library with an ovine complementary DNA probe for CRH located the gene for human CRH precursor; the sequence of human CRH was then deduced (Shibihara et al 1983). Rat hypothalamic CRH was also found to differ from ovine CRH by seven amino acid residues (Rivier et al 1983), but rat and human CRH appear to be identical and their respective pre-pro CRH exhibit an 80% homology (Jingami et al 1985).

The CRH gene has been isolated from a variety of species and shows considerable interspecies homology. The rat and human sequences share 94% nucleotide homology, while the ovine sequence shares approximately 80% with each of these. In humans the gene is located on the long arm of chromosome 8 (Arbiser et al 1988).

The major role of hypothalamic CRH is to regulate the HPA axis via the stimulation of expression of the POMC gene (Affolter and Reisine1985) and ACTH release from the anterior pituitary gland. However, as well as the paraventricular CRH-containing neurones, multiple extahypothalamic sites of synthesis and action of CRH have been determined including the placenta.

CRH exerts its effects by binding to a high affinity membrane bound receptor. These binding sites have been so far identified in rat and human (DeSouza 1985) pituitary, and rat (DeSouza et al 1984) and human(DeSouza et al 1986) brain. The CRH receptor is coupled to adenylate cyclase (Bilezikjian and Vale 1982) through a GTP binding protein thus mediating an increase in intracellular cyclic AMP. Using a cDNA encoding a pituitary CRH receptor isolated from a Cushing's adenoma (Chen et al 1993) two forms of receptor were isolated, the alpha form consisting of a 415 amino acid protein in the form of seven putative membrane spanning domains. It is similar in structure to the family of G-protein coupled receptors which includes calcitonin, vasoactive intestinal peptide and growth hormone releasing hormone receptors. The second form of the receptor consists of a 415 amino acid protein with an insert of 29 amino acids in the first intracellular loop. Using the same probe, a rat brain CRH receptor was isolated (Perrin et al 1993), which differed by 12 amino acids, representing a species or a functional difference.

1.6. Maternal plasma CRH during pregnancy.

In 1982 a substance of similar size to hypothalamic CRH was extracted from human term placentae and it was found that this extract could stimulate the production of ACTH from rat anterior pituitary cell cultures; thus it showed similar bioactivity to hypothalamic CRH (Shibasaki et al 1982). CRH was then demonstated, by radioimmunoassay, in the plasma of women during the third trimester of pregnancy, levels being undetectable in normal, non-pregnant women. Chromatography of this plasma showed that the CRH was indistinguishable from that of placental extracts (Sasaki et al 1984). It was therefore concluded that the placenta may be the source of the high levels of plasma CRH in late pregnancy. In non-pregnant women, the concentration of plasma CRH is low < 10pg/ml (Sasaki et al 1984, Suda et al 1985), it then slowly rises during pregnancy until approximately thirty-five weeks gestation after which it rises exponentially until term reaching nanomolar concentrations (Goland et al 1986, Campbell et al 1987 , Cunnah et al 1986, Sasaki et al 1987, Laatikainen et al 1987b, Wolfe et al 1988a).

Furthermore, it was seen that plasma levels were further elevated during early labour, peaked at delivery and declined rapidly, halving within 20-30 minutes of delivery (Schulte and Healy 1987; Laatikainen et al 1987b), and reaching non-pregnant levels by 1-5 days post-partum (Sasaki et al 1987; Okamoto et al 1989). In addition, maternal plasma CRH is raised in preterm labour (Kurki et al 1991), but unaffected by the administration of tocolytics. Longitudinal studies of plasma CRH levels throughout pregnancy have shown that women who subsequently went into premature labour had abnormally high levels of CRH several weeks before delivery (Campbell et al 1987). High levels are also seen in women with pregnancy induced hypertension (but not chronic hypertension), twin pregnancy (although this is likely to be due to increased placental mass) intrauterine growth retardation (possibly secondary to placental compromise rather than just a mass effect) and antepartum haemorrhage at twenty-eight weeks gestation (Wolfe et al 1988b, Campbell et al 1987, Okamoto et al 1989, Warren et al 1990, Laatikainen et al 1991). Since raised CRH levels in plasma are seen several weeks prior to premature delivery, it has been suggested that measurement of CRH could form an important predictive test for preterm delivery (Campbell et al 1987; Wolfe et al 1988b).

1.7. Amniotic fluid CRH

Amniotic fluid, placental, and maternal and fetal plasma CRH levels are highly correlated (Sasaki et al 1990; Maser Gluth et al 1987; Economides et al 1987) but maternal plasma concentrations tend to be twice as high as amniotic fluid levels. As is the case in maternal and fetal plasma, a significant rise in amniotic fluid CRH concentration is seen between mid-trimester (Economides et al 1987) and the third trimester (Maser-Gluth et al 1987; Laatikainen et al 1988; Sasaki et al 1989). This is paralleled by an increase in the CRH gene expression in the amnion reflected by a rising level of CRHmRNA (Okamoto et al 1990). Concentrations of CRH in amniotic fluid were increased in patients with diabetes and in pregnancies complicated by pre-eclampsia and intra-uterine growth retardation. In cases of intrauterine stress such as in pre-eclampsia with intrauterine growth retardation, fetal maturation

is promoted by an unknown mechanism. One of the most important features, in terms of extrauterine survival, of fetal maturation is the production of surfactant by the fetal lungs. This occurs after twenty-four weeks gestation during a period when the CRH levels in maternal plasma are increasing (Sasaki et al 1984, Goland et al 1986, Campbell et al 1987, Laatikainen et al 1987b). Laatikainen et al (1988) studied amniotic fluid CRH concentrations in normal and in complicated pregnancies and also related levels to known parameters of fetal lung maturation. A lecithin/Sphingomyelin ratio >2 or a positive phosphatidylglycerol test in amniotic fluid indicates mature fetal lungs and these coincided with a rise in amniotic fluid CRH and cortisol (Laatikainen et al 1987b) suggesting that CRH may be involved, probably via cortisol, in the process of fetal lung maturation.

1.8. Fetal plasma CRH concentration

CRH is detectable in umbilical arterial cord plasma in both term and preterm (between 26 and 37 weeks gestation) infants (Nagashima et al 1987). Furthermore umbilical venous CRH concentrations are higher than umbilical arterial plasma concentrations when the samples are taken simultaneously suggesting that the placenta is a major site of CRH production (Goland et al 1986, Sasaki et al 1987, Goland et al 1988) and that it secretes large amounts of biologically active CRH into the maternal and fetal circulations during pregnancy. The umbilical arterial plasma CRH level is extremely elevated in growth retarded compared with normal fetuses (Goland et al 1993). This suggests that placental CRH may be stimulated in a similar way to that from the hypothalamus in conditions of chronic stress.

Using fetoscopy, fetal blood samples were obtained at gestations of between sixteen and twenty-four weeks (Economides et al 1987). There was a positive correlation between maternal and fetal plasma CRH agreeing with the hypothesis that the placenta was the common source of CRH for both compartments, it being unlikely that significant quantities of CRH cross the placenta on account of its size and

hydrophobicity. The placental CRH is secreted from the trophoblast into the maternal villous blood and thence to the fetal circulation. However, other groups have suggested that CRH concentrations do not correlate well with those of ACTH or β-endorphin in either fetal or maternal plasma, indicating that placental CRH is not the major determinant of either fetal or maternal ACTH secretion (Goland et al 1986). However, work done prior to the studies of Economides in 1987, did not use fetoscopy to obtain samples, instead using hysterotomy or abortion specimens, possibly allowing more time for degradation of peptides.

1.9. CRH in placenta, fetal membranes and myometrium

Extracts of human placenta contain corticotrophin releasing hormone-like activity similar in activity and size to hypothalamic CRH (Shibasaki et al 1982). When added to cultures of anterior pituitary cells the extracts showed bioactivity with the release of ACTH and β-EP. Furthermore when placental tissue from different stages of pregnancy was investigated it was noted that levels of immunoreactive CRH were much lower at ten weeks gestation than at term (Schulte et al 1987, Frim et al 1988, Saijonmaa et al 1988) and this correlated with maternal plasma CRH levels (Sasaki et al 1987). The effects of labour on placental CRH have also been studied, and the mean concentration of CRH in placental tissue was found to be significantly larger after spontaneous vaginal delivery than at elective caesarean section performed before the onset of labour (Saijonmaa et al 1988).

In addition to measuring CRH in placental extracts, it has also been possible to measure CRH output from primary cultures of placenta and fetal membranes (Jones et al 1989), and from superfused placental fragments (Thomson et al 1988). There is an increased output of CRH from short term cultures of cells obtained from spontaneous deliveries compared with those from elective caesarean section.

Thus, the increasing concentrations of CRH in placental extracts as pregnancy progresses and during labour (Schulte and Healy 1987, Saijonmaa et al 1988), the positive correlation between fetal and maternal plasma CRH (Economides et al 1987) and the difference in umbilical arterial and venous levels of CRH (Goland et al1986, Sasaki et al 1983, Goland et al 1988) all provide indirect evidence that the placenta produces the increasing amounts of CRH seen in maternal plasma. Further evidence to support this has been provided by the study of CRH gene expression in the placenta. Using oligonucleotide probes and northern blot hybridisation, CRHmRNA was detected in the human placenta (Grino et al 1987) supporting the idea that the placenta is the site of CRH biosynthesis. That the rising levels of CRH in pregnancy are of placental origin was further supported by the finding that placental CRHmRNA increases more than twenty-fold in the five weeks preceding parturition and this parallels the placental CRH peptide content (Frim et al 1988). Furthermore the CRHmRNA in human placental tissue is similar in size to hypothalamic CRHmRNA and the transcription initiation site for placental CRHmRNA is identical to that previously predicted for hypothalamic CRHmRNA, 23-26 nucleotides downstream from a promoter element (Frim et al 1988). CRHmRNA has also been demonstratated in the amnion (Okamoto et al 1990), in increasing amounts through gestation. It may be responsible for the increasing concentrations of CRH in the amniotic fluid during gestation (Laatikainen et al 1988). In the decidua, like placenta and amnion, the expression of CRHmRNA was higher at term than during the first and second trimester (Petraglia et al 1992).

1.10. CRH localization at a cellular level

In the late 1980s two studies used immunohistochemical techniques to localize CRH to the cytotrophoblast cells of the placenta (Petraglia et al 1987, Saijonmaa et al 1988) but their results were conflicting in two respects. Saijonmaa et al found positive immunoreactive CRH staining in the cytotrophoblast cells of first trimester placenta, but were unable

to show staining in term placenta. However Petraglia et al, using indirect immunofluorescence suggested that immunoreactive CRH was present in only term placenta. As gestation proceeds the number of cytotrophoblasts decreases to a relatively small number at term. If these were the CRH producing cells this would not be consistent with the rising CRH content of placental extracts and CRHmRNA content with increasing gestation.

More recent work by Riley et al (1991) used an ovine polyclonal CRH antibody and placentas of different gestations from normal and pathological pregnancies. They found that positive CRH staining was localized to the syncytiotrophoblast and the intermediate trophoblast but not the cytotrophoblast. From week twenty of gestation onwards the distibution of CRH staining was the same in placentas obtained from normal births, preterm births, pregnancies complicated by pre-eclampsia and post term births. CRH was also localized in the epithelium and subepithelial layer of the amnion, the reticular layer of chorion, the invasive trophoblast and the stromal cells of the decidua. In diabetic pregnancies, where there are an increased number of cytotrophoblast cells at term, the placentas showed no positive staining of cytotrophoblast at this time. The presence of CRH immunoreactivity in amnion, chorion and decidua is consistent with the release of CRH by these tissues in primary culture. The number of syncytiotrophoblast cells at term is relatively high and, considering the large output of CRH at this time, this is maybe more likely to be the site of secretion. In addition, the syncytiotrophoblast has been shown to be the site of synthesis for other placental peptide and glycoprotein hormones (see sections 1.3.2.1-1.3.2.2).

1.11. CRH - binding protein

As described above plasma levels of CRH are markedly raised by the third trimester of pregnancy. Despite this, plasma levels of ACTH remain within the normal range at this time. In addition, direct radioimmunoassay of human CRH in unextracted plasma show spurious

results suggestive of some interfering factor in the plasma (Ellis et al 1988). Several groups have reported the existence of a binding protein with a large capacity to bind CRH which may explain these anomalies (Linton and Lowry 1986; Suda et al 1988). Sephadex G50 chromatography of unextracted human pregnant plasma at a neutral pH shows two peaks, the largest eluting in the void volume. When the plasma is pretreated with urea all the CRH elutes in the expected position suggesting that the peptide is bound to a large molecular weight carrier substance (Linton et al 1988). This carrier substance was later identified by electophoresis and autoradiography as a 37kDa protein of 322 amino acids (Orth and Mount 1987; Behan, Linton and Lowry 1989; Potter et al 1991). It is unlikely that the binding protein is a form of CRH receptor as it is considerably smaller and does not bind to ovine CRH (unlike the human CRH receptor) (Suda et al 1988), and CRH BP has no sequence homology with the cloned receptor (Chen et al 1993). Some regions of the brain express both the CRH receptor and the CRHBP (Potter et al 1992), but the functional relationship between the two remains to be determined.

CRHBP is expressed in human liver, placenta and brain, and in rat brain (Potter et al 1991; Potter et al 1992). CRHBP gene expression and peptide have been colocalized using human CRHBP cDNA riboprobes and immunocytochemistry to the syncytiotrophoblasts of human term placenta (Petraglia et al 1993). The local production of CRHBP by the trophoblast may be a mechanism by which the placenta may control CRH activity during pregnancy. As mentioned above, primate CRH binding protein mRNA is expressed in liver, placenta and brain, whereas in the rat CRHBP mRNA has only been observed in the brain (Potter et al 1991). The difference in tissue expression could reflect an adaptation in primates which prevents inappropriate pituitary effects of placental CRH during gestation. In contrast the rat placenta does not produce significant amounts of CRH and therefore a binding protein would not be needed to protect the pituitary.

The existence of this binding protein at concentrations sufficiently high to bind nanograms of CRH (such levels are seen in late pregnancy, as

previously discussed) probably explains the problems encountered in the development of radioimmunoassays for the direct measurement of hCRH in human plasma. It seems that the binding protein interferes with CRH antisera (Orth and Mount 1987). No such interference is seen when measuring ovine CRH in human plasma by radioimmunoassay (Nicolson et al 1983) suggesting that the binding protein does not bind to ovine CRH (Linton et al 1987). Specific immunoradiometric assays have been devoloped that are not affected by the presence of CRH-binding protein in plasma. These report a considerably shorter half life for ovine CRH in man (42 mins)(Saphier et al 1992) compared with that measured by radioimmunoassay (55-73 mins) (Nicolson et al 1983, Schulte et al 1984, Schurmeyer et al 1984). Radioimmunoassay may detect fragments of peptide giving a spuriously high result and therefore a falsely extended half life.

Plasma ACTH and cortisol responses to exogenous CRH are similar in pregnant and non-pregnant women, suggesting that plasma CRHBP has the capacity to bind additional CRH (Suda et al 1989). Although plasma ACTH is not raised above the normal range in pregnant women, they are still able to show activation of the HPA axis during stress. The CRH binding protein must therefore be able to inhibit the action of placental CRH but not hypothalamic CRH on the pituitary (Linton et al 1990). Purified CRH-binding protein, in concentrations similar to those found in pregnancy, was used in binding studies with CRH. It was found that equilibrium was reached after 15 mins incubation and it was hypothesised that acutely released hypothalamic CRH, which is in relatively high local concentrations (1μg/l in the rat) in the hypothalamopituitary portal blood, exerts its effect before being bound to CRH binding protein.

One of the major differences between man and sheep with respect to the HPA axis is the presence of CRH BP in human plasma (but not in sheep plasma) which binds to hCRH not oCRH (Linton et al 1988). The half life of hCRH in man is 30 mins, which is significantly shorter than the half life of ovine CRH in man and the half life of both human and ovine CRH in the sheep suggesting that CRH BP

enhances the clearance of human CRH in man. However then mechanism of this remains unclear.

Thus, CRH BP may protect the body from the effects of the high CRH level seen in pregnancy. The slow time course of the association of CRH with CRH BP will still allow rapid hypothalamic pulses of CRH to stimulate pituitary ACTH release. Recent data suggests that there may be a marked fall in CRH BP levels in the final weeks of pregnancy (Linton et al 1993) and this may be part of the mechanism that initiates parturition, by allowing more free, biologically available CRH. There is a wide normal range for plasma CRHBP, and in the first and second trimesters this does not differ from non-pregnant levels. In the third trimester levels are lower than in non-pregnant women, but return to normal within 48 hours of delivery (Linton et al 1993) suggesting that the fetoplacental unit may have an influence. In addition, pregnancies complicated by pre-eclampsia and preterm labour show reduced levels of CRHBP which would allow for higher plasma levels of free CRH (Perkins et al 1992); however it is not known whether this may be only a consequence of the disease and particularly of the associated deranged liver function.

1.12. The control of CRH secretion by the fetal membranes and placenta.

1.12.1 Prostaglandins

Prostaglandins E2 and F2α increase in maternal and fetal plasma in late pregnancy and during labour, and have been implicated as mediators of the stimulation and maintenance of myometrial contractility and cervical ripening at term. Prostaglandins stimulate the output of CRH and ACTH in cultures of term placental cells (Petraglia et al 1987b). The output of ACTH is itself inhibited by CRH inhibitors suggesting that CRH may mediate this. Conversely, CRH has been seen, in short term cultures (48hours) to stimulate prostaglandin output from amnion, chorion, placental and decidual cell cultures obtained at term elective caesarean section and in amnion and placental cultures of tissue from

second trimester termination of pregnancy (Jones and Challis 1989, Jones and Challis 1990b). The output of prostaglandins was inhibited by ACTH antisera (Jones and Challis 1990a), thus, ACTH may have a modulatory role in both the stimulation of prostaglandins by CRH and the stimulation of CRH by prostaglandins. Furthermore, in early pregnancy tissues, and in term placenta and decidua, ACTH itself stimulates prostaglandin output (Jones and Challis 1989). In cultured amnion cells, glucocorticoids stimulate PGE_2 output in a dose dependant manner (Potestio et al 1988), and potentiates the effect of adding the PG precursor, arachidonic acid. It has therefore been suggested, that this effect is secondary to increased synthesis of prostaglandin synthase. This may be mediated through CRH since glucocorticoids stimulate CRH and CRH stimulates prostaglandin output.

1.12.2. Steroids

Steroid hormones modulate the placental production of CRH. In short term cultures (48 hours) of amnion, chorion and decidua, cortisol and dexamethasone stimulated CRH output in a dose dependent manner (Jones et al 1989). Furthermore, in 3 day old cultures of placental cells, dexamethasone increased CRHmRNA levels after incubation for 48 hours (Robinson et al 1988). However, when betamethasone, a long acting steroid, was given to women in preterm labour up to 21 hours prior to delivery no change in plasma CRH was seen although ACTH and cortisol levels were suppressed (Tropper et al 1987). It seems that glucocorticoids stimulate CRH secretion by the placenta but inhibit the HPA axis. Robinson has proposed that CRH from the placenta passes via the umbilical vein to the fetus and stimulates ACTH secretion by the fetal anterior pituitary. This stimulates the secretion of cortisol by the fetal adrenal cortex which passes back to the placenta and may act there as a positive feedback stimulator of CRH synthesis and release. Because glucocorticoids have an important role in the maturation of fetal organs the CRH/ glucocorticoid positive feedback loop holds advantages for fetal wellbeing, as well as being central to a glucocorticoid-ACTH-CRH cascade postulated as a mechanism which

occurs at the onset of parturition (Challis and Hooper 1989) CRH stimulates the output of POMC peptides, ACTH (Petraglia et al 1987), α-melanocyte-stimulating hormone and β-endorphin, in a dose dependant fashion from placental explants in culture (Margioris et al 1988).

In contrast, in short term cultures of amnion, chorion, placenta and decidua, progesterone inhibited CRH output (Jones et al 1989). The high levels of progesterone in maternal plasma during pregnancy may thus inhibit CRH output until some as yet unknown mechanism overrides this prior to birth when the CRH level is allowed to rise. In man, unlike the situation in the sheep, there is no fall in plasma progesterone prior to parturition suggesting that this change may occur at a local level.

1.12.3. Oxytocin

Oxytocin is a positive inotrope which stimulates myometrial contractility and CRH potentiates its effects (Quatero and Fry 1989). Preincubation of strips of myometrium with CRH primed the muscle for the inotropic effect of oxytocin. Furthermore, CRH and oxytocin together had a greater effect on myometrial contractility than oxytocin alone (CRH alone has no inotropic effect) (Quatero and Fry 1989), and this CRH potentiation of the action of oxytocin appears to be mediated by prostaglandins, probably $PGF_{2\alpha}$ (Quatero et al 1991). Recent work has shown that the myometrium has binding sites for CRH that increase in affinity during pregnancy and may influence parturition by sensitizing the myometrium to oxytocin (Quatero et al 1992, Hillhouse et al 1993). Furthermore oxytocin stimulates the release of CRH from placental cells (Petraglia et al 1989b). It has been hypothesised therefore that during labour release of oxytocin stimulates further production of CRH which in turn potentiates the effects of oxytocin on myometrial contractility.

1.12.4. Other factors

Acetylcholine, noradrenaline, oxytocin, vasopressin and angiotensin II have also been shown to stimulate CRH production from cultured placental cells in a dose related manner (Petraglia et al 1989b), although the significance of this is unclear. Acetylcholine and noradrenaline also stimulate CRH release from rat hypothalamic tissue in vitro (Rivier and Plotsky 1986), and the presence of adrenergic and cholinergic receptors in placental tissue (Olubadewo and Rama Sastri 1977; Divers et al 1981) suggests these neurotransmitters may have a role in placental endocrine regulation. Similarly, oxytocin, vasopressin, angiotensin II and interleukin-1 increase the release of CRH from the hypothalamus (Sapolsky et al 1987).
These findings suggest that there may be a similar mechanism for control for placental and hypothalamic CRH release.

The cytokines interleukin 1 alpha and 1 beta also stimulate the production of CRH from placental cells cultured for one week (Petraglia et al 1989b, Petraglia et al 1990b). These substances are released in response to infection and the link between infection and preterm labour is well known. However, studies correlating CRH levels, cytokines and preterm labour are yet to be done.

1.13. Hypothesis for the role of CRH in parturition

Thus the placenta (probably the syncytiotrophoblast) and fetal membranes produce increasing amounts of CRH with advancing gestation as a result of increasing CRH gene expression. CRH increases placental ACTH production and both may affect prostaglandin output by the fetal membranes. The maternal HPA axis is protected from high circulating levels by CRH BP, it is not yet known if the fetal HPA axis is similarly protected. Placental CRH may pass into the fetal circulation in sufficient concentrations to release ACTH from the fetal pituitary, resulting in increased plasma cortisol and organ maturation. Fetal cortisol may also act to positively feedback on placental CRH gene expression and thus CRH production, thus forming a positive

feedback loop. In addition placental CRH may have a paracrine role in stimulating local prostaglandin production which itself stimulates the release of CRH forming another positive feedback loop. Finally CRH may sensitize the myometrium to the inotropic effects of oxytocin and potentiate its effects once labour has started. A fall in the level of CRH BP just prior to parturition will make more bioactive CRH available for these pathways. The ultimate result of this series of positive feedback loops is birth (Challis and Hooper 1989).

1.14. Placental ACTH

1.14.1. ACTH background

ACTH is a 39 amino-acid peptide which is synthesised as part of a precursor molecule POMC. Following the signal peptide, there is an N-Terminal fragment followed by ACTH(1-39) which is followed by the β-lipotropin sequence. ACTH(1-39) contains within it the sequences of alpha-melanocyte-stimulating hormone (α-MSH) (1-13) and corticotropin-like intermediate lobe peptide (CLIP) (18-39). β-lipotropin contains within it the sequence for γ-lipotropin, β-MSH and β-endorphin (Nakanishi et al 1979) (see figure1.6).

1.14.2. Previous studies of placental ACTH secretion.

The presence and release of an ACTH like substance in the placenta was first suggested in the 1950's with an observation that pregnancy stimulated adrenocortical activity in a patient with Addisons disease (Jailer and Knowlton, 1950). This was supported by the detection of ACTH-like activity in human placental extracts (Silman and Bergmann, 1953; Assali and Hamermesz, 1954). In the rat (Knobil and Briggs 1959), and in the rhesus monkey (Hodgen et al 1975), pregnancy reduces the extent of adrenal atrophy that occurs in response to maternal hypophysectomy. More importantly, even after subsequent fetectomy (removal of the fetus but not the placenta), cortisol levels remained relatively high, but then fell markedly after placental delivery,

suggesting that the placenta provides direct support for the maternal adrenal cortex. In human pregnancy, most, though not all, studies have found a rise in free, biologically active cortisol as pregnancy advances (Nolten and Rueckert, 1981; Demey-Ponsart et al 1982). Plasma ACTH concentrations are not depressed, in fact some studies have shown a rise (Rees et al 1975; Genazzani et al 1975; Okamoto et al 1989; Smith et al 1990), suggesting that the increase in cortisol is secondary to relatively increased ACTH secretion. Rees et al (1975) also noted that the potent synthetic glucocorticoid, dexamethasone, which readily supresses cortisol in non-pregnant subjects by turning off the production of ACTH, had little effect on circulating cortisol levels in pregnant women. Two theories have been suggested to account for this finding; the first postulates an ectopic source for the ACTH (such as the placenta) the second, a resetting of pituitary and/or hypothalamic sensitivity to negative feedback, perhaps mediated by other hormonal changes which occur during gestation.

1.14.3. Placental ACTH concentrations

The placenta contains immunoreactive and bioactive ACTH- like activity (Genazzani et al., 1975, Rees et al 1975: Liotta et al 1977), immunoreactive β- lipotropin and β-endorphin (Nakai et al, 1984; Odagiri et al 1979), a small β-endorphin-like molecule (placental b-endorphin) (Chan and Smith 1992), and immunoreactive α-MSH (Clark et al 1978). The placenta is not merely a storage site for pituitary POMC-derived peptides, but can actively synthesise them (Liotta and Krieger 1980). There is some disagreement as to whether ACTH concentrations change with gestation. Concentrations reported by Liotta et al (1977), Rees et al (1975) and Odagiri et al (1979) are essentially similar and vary from 1-4ng/g. Lower values were reported by Genazzani et al (1975); but there was lack of parallelism in the immunoassay of placental extract with standard ACTH. Placental ACTH concentrations are several orders of magnitude less than that present in the anterior pituitary, in which the ACTH concentrations are of the order of 1μg/mg tissue. Total human pituitary content of ACTH is approximately 500μg; with a placental weight of 500g, placental content would be

approximately 1/1000 that of the pituitary. Peptides within the pituitary are sequestered within granules, whereas in the placenta they are not, implying alternative secretory mechanisms other than exocytosis.

Reported placental β-endorphin-like concentrations range from 1-2ng/g (Nakai et al 1978; Krieger et al 1980), β-lipotropin-like concentrations from 3-9 ng/g (Nakai et al 1978; Krieger et al 1980) and α-MSH- like concentrations from 0.2-0.6 ng/g (Krieger et al 1980).

Immunocytochemistry has localised ACTH (Al-Timini and Fox 1986), β-lipotrophin and β-endorphin (Laatikainen et al 1987a) to the syncytiotrophoblasts of the placenta; and in vitro studies have shown that these cells are capable of secreting ACTH (Liotta et al 1977), and β-endorphin-like immunoreactivity (Liotta and Krieger 1980).

POMC mRNA has been identified in human, rat and mouse placenta using rat pituitary cDNA probes (Cheng et al 1986). This POMC mRNA is shorter than its pituitary counterpart due to shortening of the 5' end. In rat placenta, POMC gene expression seems to be constitutive as the concentration of POMC mRNA does not change during pregnancy.

1.15. Control and effects of placental POMC secretion

Both vasopressin and CRH are potent releasers of ACTH and other POMC peptides from the anterior pituitary. CRH also stimulates the release of ACTH from cultured placental cells in a dose dependant manner (Petraglia et al 1987b). In addition, dibutyryl cyclic AMP (dcAMP) produces a similar effect, but does not potentiate the action of CRH. It has been suggested therefore, that placental CRH may regulate placental POMC fragment release by the cAMP-dependant intracellular messenger system and may indirectly affect placental steroidogenesis (Petraglia et al 1990c). ACTH in turn stimulates the output of placental CRH as well as oestradiol and progesterone secretion from placental explant cultures (Barnea et al 1986). It may also have a role in placental

glucose metabolism, as it increases aerobic glycolysis and oxygen consumption.

1.16. Plasma concentrations of POMC-derived peptides and cortisol

Both free cortisol (Nolten and Rueckert 1981; Demey-Ponsart et al 1982) and urinary excretion of cortisol increases during pregnancy. Concentrations still, however, show diurnal variation, and increase in response to stressors such as labour (Namba et al 1980; Nolten et al 1980; Patrick et al 1980). High plasma cortisol concentrations are thought to result from placental ACTH.

There is considerable debate over the concentration of pro-opiomelanocortin derived peptides in the maternal blood. ACTH plasma concentrations show their normal circadian variation in pregnant subjects (Carr et al 1981). There is some question as to whether plasma ACTH concentrations in pregnant women are appropriately low for the somewhat elevated free cortisol concentrations present. Genazzani et al (1975) reported elevated plasma ACTH concentrations throughout pregnancy. However these results are dubious since parallelism with standard was not obtained in the radioimmunoassay used. Rees et al (1975) demonstrated a progressive rise in plasma ACTH concentrations during pregnancy, although most values were in the normal range. A similar rise was noted by Carr et al (1981), but these values were less than those seen in normal ovulatory women. Their interpretation of this was that maternal plasma concentrations are initially suppressed but rise progressively throughout pregnancy. If the placenta is the source of these pepides, it may not be subject to negative feedback by cortisol or progesterone.

Reports of plasma levels of β-endorphin during pregnancy also vary between increased (Smith et al 1990; Newnham et al 1983, Chan and Smith 1992), decreased (Aboud 1988; Raisanen 1987) and similar (Goland et al 1981; Stark and Frantz 1983), compared to non-pregnant women. The placenta extensively acetylates β-endorphin and since most of the β-endorphin immunoreactivity is in a non-acetylated form it has been

suggested that this is of pituitary origin (Chan and Smith 1992). The high levels seen during pregnancy may be in response to the elevated placental CRH concentration in plasma (Chan et al 1993). Acetylated β-endorphin is devoid of opiate activity and placental β-endorphin is therefore unlikely to be involved in stress-induced analgesia during parturition. It has been suggested, that β-endorphin has an immunological role, affecting monocyte chemotaxis (Sacerdote and Panieri 1989), a cardiovascular effect (Furuhashi et al 1986) and a neuroleptic effect (DeWied et al 1980) during pregnancy.

The level of α-MSH during pregnancy is also the subject of some dispute with some studies showing a rise in the third trimester (Clark et al 1978) and some not (Silman et al 1975).

1.17. Fetal and placental ACTH interactions.

Early reports suggested that fetal plasma ACTH and amniotic ACTH levels throughout gestation were markedly greater than those in maternal plasma (Winters et al 1974; Tuimala et al 1976) throughout gestation. However, more recently, fetal blood sampling by direct fetoscopy has enabled more accurate measurements to be made. These have demonstrated that the levels do not differ significantly (Economides et al 1987). The evidence for a placental source of ACTH in the fetal circulation is only circumstantial. Exogenous ACTH does not cross the placenta (Miyakava et al 1974; Dupouy et al 1980) but, growth of the adrenal cortex appears to be normal in anencephalic fetuses up to mid gestation, (Bernischke 1956) suggesting that a factor other than fetal or maternal pituitary ACTH is responsible for support of the fetal adrenal. It is likely that at least some of this support may be provided by placental ACTH, as well as hCG and prolactin (Jaffe et al 1981; Pepe et al 1988). Late in pregnancy, the placenta may also provides trophic support for the fetal adrenal cortex. Until birth the rate of steroidogenesis is very high (Jaffe et al 1981; Pepe and Albrecht 1990), but after delivery its size (Schulz et al 1962) and secretory activity (Jaffe et al 1981; Pepe and Albrecht 1990) rapidly decline possibly as a result of removal of placental support.

1.18. Summary of placental ACTH

The human placenta can produce and release bioactive and immunoreactive ACTH and other POMC-derived peptides throughout gestation. There is some debate as to whether the levels of these peptides increase or remain constant in placenta and in plasma during pregnancy. The production of placental ACTH is under the control of placental CRH in a paracrine manner via cAMP-dependant intracellular messenger system. In turn placental ACTH may influence placental steroidogenesis via the secretion of CRH, oestradiol and progesterone. There is indirect evidence that placental ACTH may be secreted into both the maternal and fetal circulations. There is thus clearly a potential role for placental ACTH in directly influencing the maternal and/or fetal hypothalamo-pituitary-adrenal axes during human pregnancy.

1.19. The aims of this thesis

The aims of this thesis are to investigate, in detail, the ontogeny of placental CRH production, linking placental peptide content with its precise cellular localisation throughout gestation and then to investigate the effects of labour, exogenous prostaglandin and antiprogestin administration at both early and late gestation. It will then investigate placental ACTH in the same groups of patients in terms of placental ACTH content, exact cellular localization and POMC gene expression.

Figure 1.1 The structure of the placental villi in (a) first trimester and (b) term placenta.

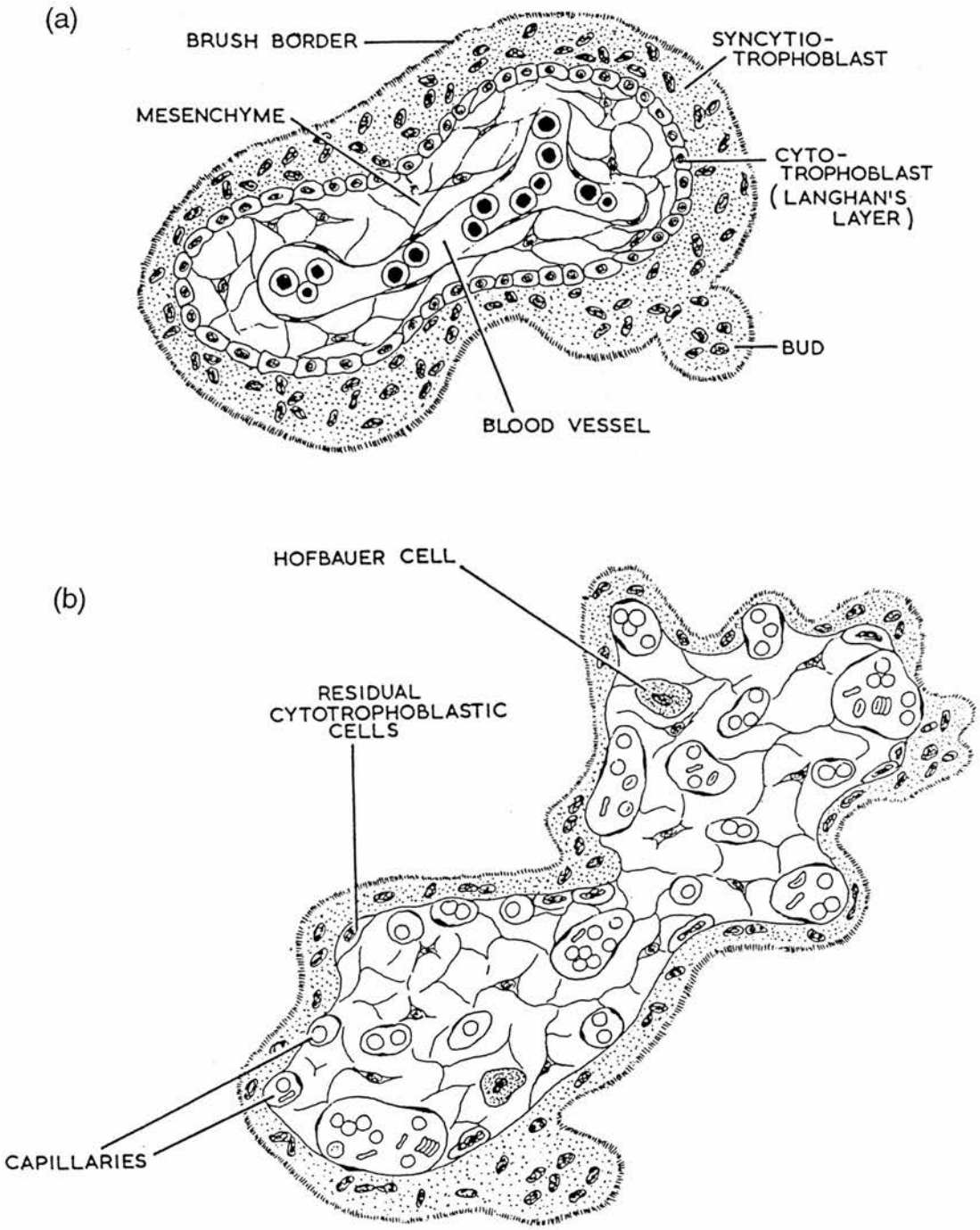


Figure 1.2 The structure of the fetal membranes

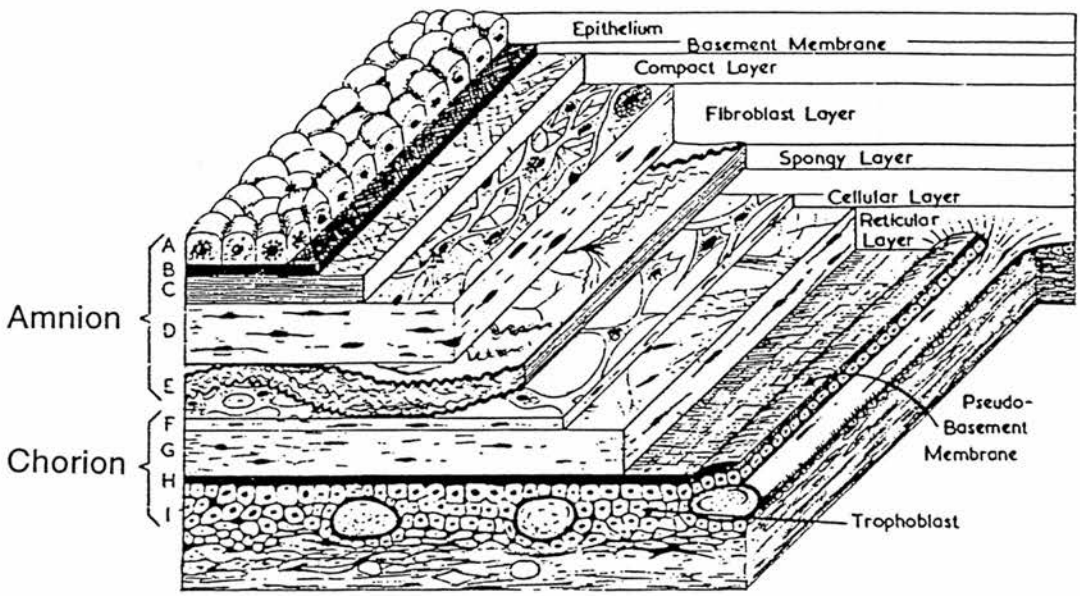


Figure 1.3 Steroid biosynthesis in the feto-placental unit

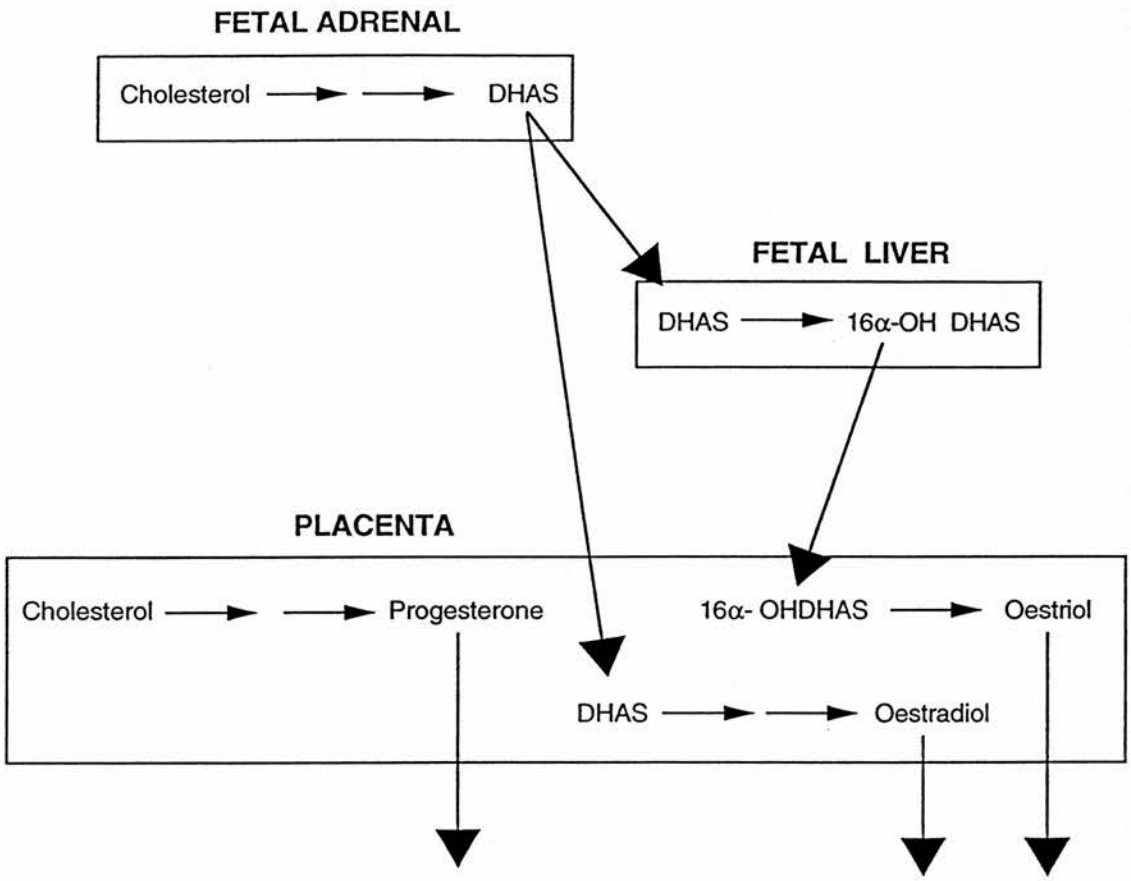
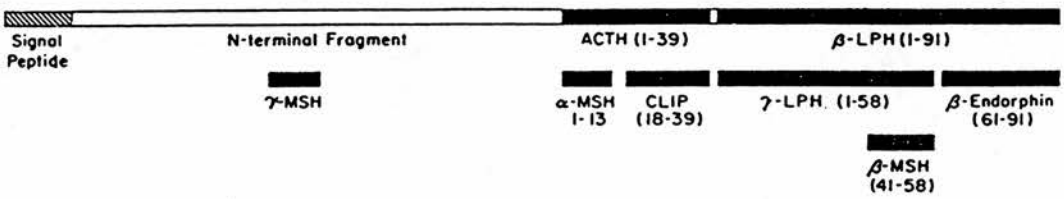


Figure 1.4 Placental peptide hormones

Pituitary-type hormones	Hypothalamic-type hormones	Others
Human chorionic gonadotrophin[c]	LHRH[a]	Inhibin[d]
Human placental lactogen[c]	TRH[b]	Somatostatin[e]
Human chorionic thyrotrophin[c]	CRH	
Pro-opiomelanocortin peptides		

a) Gibbons et al (1975)
b) Khoder and Siler-Khoder (1980)
c) Osathanondh and Tulchinsky (1980)
d) McLachlan et al (1986)
e) Lee et al (1982)

Figure 1.5 Structure of Pro-opiomelanocortin (Nakanishi et al 1979)



CHAPTER 2

GENERAL MATERIALS AND METHODS

This chapter describes basic materials and methods which are common to all experiments performed. The development and validation of specific immunocytochemical and in situ hybridisation methods are described in detail in chapters 3 and 4.

2.1.SEPHADEX CHROMATOGRAPHY

Chromatography comparing placental extracts with both standard human ACTH and standard human CRH was performed using a sephadex column. A Sephadex G-50 Fine (Pharmacia Fine Chemicals Uppsala Sweden) column was carefully poured to a length of 50x1cm in a glass column and equilibrated with ACTH assay buffer (phosphate-EDTA buffer; Na_2HPO_4 (63 mmol/l), EDTA (13mmol/l), pH7.4, containing 0.02% (w/v) sodium azide, 0.1% (v/v) Triton-X-100 and 250 Kallikrein inhibitor units of aprotinin/ml (Sigma). 10 mls of 10% bovine serum albumin solution were added to reduce non-specific binding, and the column re-eluted with ACTH assay buffer. Blue dextran was used to determine the void volume of the column. Standard human ACTH was applied to the column and sixty 1ml fractions were collected at a flow rate of 4 ml/hr. The column was flushed with ACTH assay buffer and placental extract which had been reconstituted in ACTH assay buffer was applied to the column in the same way, and the fractions collected at identical flow rate. All the fractions were collected and stored at -20^{o}C before radioimmunoassay for ACTH.

An identical Sephadex G-50 column was used for chromatography of CRH except that the column was equilibrated with CRH assay buffer (0.05mol phosphate buffer /l containing 0.1mol NaCL/l. 25mmol EDTA/l, 0.01% thiomersal, 0.1% Triton X-100 and 0.1% bovine serum albumin pH7.4). Standard human CRH (Bachem lot no. WG750) was applied to the column and sixty 1 ml fractions collected at a flow rate of 4 ml/hr.

The column was flushed with CRH assay buffer and placental extract which had been reconstituted in CRH assay buffer was applied and fractions collected at an identical rate. All the fractions were collected and stored at -20°C before radioimmunoassay for CRH.

2.2. PEPTIDE EXTRACTION AND RADIOIMMUNOASSAY

Intensity of staining in immunocytochemistry is valuable in localising the cellular site of the antigen. It is, however, only semiquantitative for the amount of antigen present in the cells. In order to more accurately measure peptide content in the different groups of tissues studied and support the findings of the immunostaining, samples of the same tissues collected for immunostaining and in situ hybridisation were collected, the peptide extracted and the content of both ACTH and CRH measured by radioimmunoassay. The peptide content with increasing gestational age, after the administration of exogenous prostaglandins and mifepristone, and after the effects of labour was investigated. These results were then compared with the immunostaining and in situ hybridisation results so that information would be available about both gene expression and the translated gene product in terms of both locality and quantity.

A human ACTH radioimmunoassay available within the unit was used to measure ACTH in both extracted placenta and membranes and in the column fractions produced after chromatography of placental extracts and standard human ACTH (see section 4.1). Because of problems encountered in setting up a human CRH radioimmunoassay, a hCRH RIA kit was purchased and used to quantify CRH in the placental extracts. To reduce expense, an ovine CRH radioimmunoassay was used to detect CRH in column fractions of placental extracts and hCRH standard. There is sufficient cross-reactivity between the two species to find the retention time of hCRH standard of the column,

and it was not necessary to accurately measure the absolute amount of peptide in each fraction.

2.2.1. Peptide extraction

The tissues collected were those as detailed in section 2.2.1. Immunocytochemistry. All tissues were placed, within 30 minutes of delivery into liquid nitrogen to preserve peptide content and were stored in liquid nitrogen until needed.

Frozen pieces of tissue were quickly weighed and then added to a known volume of hot acid (0.1M HCl, 1.0M Acetic acid 1:1 vol/vol) and boiled for 5 minutes. The samples were cooled on ice and homogenised. At this point, an aliquot of each was removed and stored at 4°C for later protein estimation. The samples were then centrifuged at 10,000G for 30 minutes, the supernatant removed, and lyophilised in aliquots for radioimmunoassay and chromatography.

2.2.2. Protein estimation

Protein content of the homogenised placentas was assayed using a Bio-rad protein assay. This is a colorimetric assay for measuring total protein concentration and is performed as follows;

The samples were treated overnight with 1M NaOH. The next day, a 2mg/ml solution of bovine serum albumin was made as a standard and dilutions between 15 and 2000µg/ml made by diluting this in 1M NaOH. 20µl of each solution was pipetted into a 96 well flat bottomed plate giving dilutions ranging from 0 to 40µg per well. Samples were added to the plate in duplicate. 100µl of Bio-rad protein reagent was added to the standard curve and samples and the plate was read at O.D 260nm. The standard curve was plotted on an Apple Macintosh computer using Cricketgraph and the straight part of the curve used to calculate the protein concentration of the samples.

2.3. Radioimmunoassay procedures

1. Ovine CRH radioimmunoassay
2. Human CRH radioimmunoassay kit (Penninsula laboratories)
3. Human ACTH(1-39) radioimmunoassay

2.3.1. Ovine CRH radioimmunoassay

Immunoreactive CRH from the sephadex chromatography fractions was determined by an ovine CRH radioimmunoassay (Brooks and Challis 1988) as follows;

The radioimmunoassay was performed in plastic tubes using CRH assay buffer (0.05 mol phosphate/l containing 0.1 mol NaCl/l, 25mmol EDTA/l 0.01% thiomersal, 0.1% triton-X-100 and 0.1% bovine serum albumin (Sigma chemical company) at pH 7.4). All standards, antibody and tracer were made up in this buffer. Rabbit-ovine CRH antiserum was used at a dilution of 1:100,000. Synthetic ovine CRH (tyr- CRH Bachem inc) was iodinated by the chloramine-T method as follows. 4µg of Tyr-0CRH in 10µl 0.1 mol acetic acid/l, 10µl 0.5mol phosphate buffer/l (pH7.4) and 10µl (1mCi) Na ^{125}I were mixed in a plastic eppendorf tube. 10µg of chloramine-T (1g/l) in 0.5 mol/l phosphate buffer was added and after 15 seconds the reaction was stopped by the addition of 25µl of sodium metabisulphite (1g/l) in phosphate buffer. The mixture was then applied to a Sep-Pak C_{18} cartridge (Waters Associates, Milford M.A. U.S.A) which had been pre-wetted with 5ml methanol followed by 10mls of distilled water. Free iodine was eluted in 6mls 0.05mol phosphate buffer/l containing 0.1% trifluoroacetic acid (TFA) and ^{125}I-labelled Tyr-oCRH was then eluted in 3mls of a mixture of acetonitrile (80%) and phosphate buffer (0.05 mol/l) containing 0.1% TFA (20%). Tracer was stored at 4°C. Before use in a radioimmunoassay, the binding of tracer to CRH2G antiserum was checked.

The assay standards (0.1ml) (in triplicate) and the unknown samples (0.2ml) (in duplicate) were incubated overnight at 4°C, with CRH2G

antibody(0.1ml) at a dilution of 1:100,000. Then approximately 10,000 cpm of tracer (0.1ml) (^{125}I-oCRH) was added and the mixture incubated for a further 24 hours. Free and bound steroid was separated by incubation with Magsep particles (donkey-anti rabbit seond antibody attached to magnetic particles kindly supplied by Dr R Kelly, MRC Reproductive Biology Unit Edinburgh). The detection limit of the assay is 10-15 pg/tube.

The CRH2G antibody shows minimal cross-reactivity (<0.001%) with a number of other hypothalamo-pituitary peptides including ACTH (1-39), ACTH (1-24), β-endorphin, α-melanocyte-stimulating hormone, met-enkephalin, leu-enkephalin, arginine vasopressin, oxytocin, thyrotrophin-releasing hormone and luteinizing hormone-releasing hormone.

Bound radioactivity was measured using a multigamma counter (1261 Multigamma, LKB Wallac OY, Turku, Finland), from which the output of the raw counts was recorded by a digital data logger (Data grabber, Mutek, Box, Wiltshire) onto a floppy disc. The data was then transferred from the data grabber disc onto an Apple Macintosh computer where raw counts were assayed by the AssayZap universal assay calculator program (Elsevier, Biosoft, UK). A four parameter weighted-regression model of a displacement curve was used for this program. This begins with the two-parameter logit-log model as a first estimate, but then adjusts the asymptotes, slopes and points of inflection (the four parameters) of model curve reiteratively, in order to give more weight to the standard points with smaller variance, until optimum squares fit is obtained. Assayzap also preserves previous information of assay history in order to compare with previous standard curves, quality control and binding level.

2.3.2. Human CRH radioimmunoassay kit

Because of the low titres of human CRH antibody raised in house, it was decided to purchase a human CRH radioimmunoassay kit (Peninsula laboratories, inc Belmont California USA). The asaay was performed following the manufacturers instructions. Briefly, all reagents were prepared in assay buffer supplied with the kit. The lyophilised placental extracts were reconstituted in assay buffer, and 0.1ml volumes were incubated overnight at 4°C with antiserum (rabbit anti human CRH). The next day, 0.1ml of tracer were added (10,000 cpm) and the mixture incubated for a further 24 hours at 4°C. Separation of bound and free CRH was achieved by addition of 0.1ml of goat antirabbit IgG serum, and 0.1ml of normal rabbit serum. The tubes were mixed and the contents incubated for 90 minutes. The samples were centrifuged at 3,000r.p.m for 20 minutes at 4°C and the supernatant aspirated. A standard curve was set up and run in parallel with the samples. Bound radioactivity was measured using a multigamma counter as for oCRH assay. The standard curve and unknown samples were calculated using AssayZap as for oCRH assay. The limit of sensitivity of this assay is 1 pg/tube.

2.3.3. Human ACTH radioimmunoassay

The concentration of ACTH in both placental extracts and in column fractions from chromatography comparing placental extract with standard ACTH was determined by double antibody radioimmunoassay using a human ACTH antibody elaborated by Dr A.F. Parlow (AFP6328031) (Director of The Pituitary hormones and Antisera centre Torrance , CA, USA) and supplied by the National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK). Synthetic ACTH (1-39) for use as a standard and for iodination was purchased from Cambridge Research Biochemicals. Iodinated ACTH was prepared using the chloramine-T method and separated using Sephadex G-50 Fine chromatography. Lyophilised placental extracts were reconstituted in ACTH assay buffer (Na_2HPO_4(63mmol/l), EDTA (13mmol/l), pH7.4) containing 0.02% (w/v) sodium azide, 0.1% (v/v) Triton-X-100 and 250 Kallikrein inhibitor units

of aprotinin/ml (Sigma, Dorset UK). Column fractions were assayed directly. Samples (0.1ml), in duplicate, were incubated overnight at 4°C with ACTH antiserum (at a final dilution of 1:12,500). Iodinated ACTH (approximately 15,000 cpm) (0.1ml) was added to all tubes and incubated for a further 24 hours. Standards, antibody and tracer were all diluted in ACTH assay buffer. Free and bound ACTH was separated by incubation with Magsep particles (donkey anti rabbit second antibody attached to magnetic particles, kindly supplied by Dr R Kelly MRC Reproductive Biology Unit Edinburgh). Bound radioactivity was measured using a Multigamma 1261 counter (see oCRH RIA). Standard curve samples were prepared in ACTH assay buffer and assayed simultaneously and used to calculate the concentration of ACTH in the unknown samples. The limit of sensitivity of this assay is 1.0pg/tube. The antibody used for this assay is highly specific for ACTH (1-39) having minimal cross-reactivity for ACTH(1-10), ACTH(11-24), ACTH (1-24), and α-MSH.

All the results of ACTH and CRH content of placental extracts were expressed in terms of the protein content of the tissues.

2.4. Statistical Analysis

All statistical analysis was performed on an Apple Macintosh computer using the Statworks program (Data Metrics Inc., U.S.A). Two way analysis of variance comparing placental ACTH or CRH content with gestational age, and comparing different tissue types and different modes of delivery was assessed by analysis of variance using a statistics program written for the Apple Macintosh Computer (CLR Anova, Clear Lake Research Houston Texas USA).

CHAPTER 3

DEVELOPMENT OF IMMUNOCYTOCHEMICAL METHODS TO LOCALISE CRH AND ACTH WITHIN THE PLACENTA AND FETAL MEMBRANES.

IMMUNOCYTOCHEMISTRY

3.1 Introduction

Immunocytochemistry is a very sensitive and specific method, using antibody-antigen complexes tagged by a visible label, to allow visualisation, in situ, of a wide variety of cell products. In early immunocytochemical methods, the specific antibody itself was labelled (the direct method) but indirect methods tend to be more sensitive and are now commonly used. In the development of immunolocalisation methods for this thesis both avidin-biotin and antibody-enzyme bridge techniques were used. Indirect methods involve the application of the primary, unlabelled, antibody to the section. A second, labelled, antibody from another species, raised to the IgG of the animal donating the first antibody, is then applied, the first antibody acting as an immunoglobulin antigen. Label intensity is significantly enhanced with this method compared with the direct method. The reasons for this are not clear. The primary antibody molecule may bind several molecules of the second, labelled, antibody, or the hyperimmunity and therefore higher titre of the second antibody may be responsible. Avidin has a very high affinity for biotin, one molecule being able to bind four molecules of biotin. Biotin will also bind the Fc portion of immunoglobulins, each also able to bind several molecules of biotin. Both avidin and biotin may be labelled and with the right combination of avidin, biotin and antibody it is possible to build up very high concentrations of label thus increasing the sensitivity. The avidin-biotin method is known to

produce a high level of sensitivity with paraffin embedded sections. Both the methods described in this thesis involve the use of antibodies and the enzyme peroxidase. The use of peroxidase as an enzyme has several advantages including its stability, wide availability, cost and the ability to easily quench endogenous tissue peroxidase activity (see section 3.2.5.). For the avidin-biotin method, three reagents are used; a primary antibody specific for the antigen to be localised, a secondary antibody capable of binding to the first which is conjugated to biotin, and a complex of peroxidase conjugated avidin and biotin. The free sites of the avidin molecule allow binding to the biotin on the secondary antibody. The peroxidase enzyme and therefore the original antigen are visualised with an appropriate chromagen. The strong affinity of avidin for biotin gives this method great sensitivity.

Unlabelled antibody-enzyme bridge techniques utilise a bridging antibody (unconjugated) between the primary antibody and the final antibody. The final antibody is also unconjugated and raised to horseradish peroxidase in the same species as the primary antibody. This layer is followed by horseradish peroxidase which is then bound by an antigen-antibody reaction and can then be developed. The avoidance of chemical conjugation means that the immunological reactivity of all the antibodies is kept to a maximum.

Optimal specific staining depends not only on the characteristics of the primary antibody and the antigen to be localised but also appropriate tissue fixation and preparation, the optimal dilution of primary and secondary antibodies, incubation times and temperatures. These conditions were investigated in order to achieve reproducible specific staining with minimal background staining. Positive staining of a specimen not a result of antigen-antibody binding is termed non-specific staining. The main causes of this and their solutions are considered in detail in section 3.2.5.

3.2 Materials and methods

3.2.1 Tissue Collection

All gestations were calculated from the date of the last menstrual period and confirmed by ultrasound and/or clinical assessment. Decidual and trophoblastic tissues were obtained from patients undergoing therapeutic surgical termination of pregnancy in the first trimester for social reasons by suction curettage, with or without pretreatment with the synthetic prostaglandin analogue gemeprost (16,16 dimethyl transΔ2 PGE_1 methylester) between 2 and 4 hours prior to the procedure. Tissues were also obtained from patients undergoing medical therapeutic termination of pregnancy for social reasons in the first and second trimester using mifepristone 600mg orally 48 hours prior to receiving a 1mg gemeprost vaginal pessary. All patients aborted within 6 hours. Placenta and fetal membranes were also collected from spontaneous vaginal deliveries preterm (between 24-35 weeks gestation, at term (36-42 weeks gestation) by elective caesarean section, spontaneous vaginal delivery, and by induction of labour using prostaglandin E_2 (all patients studied recieved 2mg of PGE_2 and all delivered within 24 hours). There was no clinical evidence of infection such as pyrexia in any patient, nor any histological evidence of neutrophil infiltration in any tissue studied. For immunocytochemistry, all tissues were divided into blocks of approximately 1.5cm^3 and placed in 4%(w/v) buffered paraformaldehyde (pH 7.4) within 30 mins of delivery.

3.2.2 Tissue Fixation Time

The optimal fixation time of the cross-linking fixative, 4% paraformaldehyde was evaluated over the range 8-48 hours.
After fixation tissue was processed through graded ethanol in a standard tissue processor and embedded in paraffin wax. The paraffin wax blocks were stored in a cool place.

3.2.3 Tissue Pretreatment

Tissue sections of 5μm were cut on a microtome and floated onto clean glass slides and baked overnight at 56°C. To facilitate tissue penetration by the antibody, the slides were pretreated with 0.1% (w/v) trypsin (Type III from bovine pancreas, Sigma Chemical Company, Dorset) and the optimal incubation time and temperature was evaluated. The slides were also washed in 0.5% (v/v) Triton-X 100 before application of the primary antibody.

3.2.4 Antibodies

3.2.4.1. CRH immunostaining

The primary antibody used for CRH immunostaining was a polyclonal CRH antisera raised in rabbits against an ovine CRH-thyroglobulin conjugate (CRH2G kindly supplied by Dr A.N. Brooks) The secondary antibody used for CRH immunostaining was biotinylated swine anti-rabbit immunoglobulin G (DAKO E353 Lot no. 053)

A checkerboard titration was used to determine the optimal dilution of both primary and secondary antibody.

	Primary antibody				
	1:100	1:500	1:1000	1:1500	1:3000
Secondary antibody					
1:200					
1:500					

The Avidin-Biotin Complex (DAKO K33 Lot no. 033) was used as per manufacturers instuctions.

3.2.4.2. ACTH immunostaining

The primary antibody used for ACTH immunostaining was a polyclonal anti CLIP antiserum raised in a rabbit which shows 100% cross-reactivity with ACTH (kindly supplied by Professor L. Rees St Bartholomew's Hospital London).

Two different staining methods were used to stain for ACTH:
1. An avidin-biotin method similar to that used for CRH immunostaining using biotinylated swine anti-rabbit immunologlobulin G as the secondary antibody and avidin biotin complex.
2.An alkaline phoshatase anti alkaline phosphatase bridge technique with nitroblue tetrazolium as the chromagen. The linking antibody used was a swine anti rabbit immunoglobulin G (DAKO Z196 Lot no.022)The secondary antibody used was alkaline phosphatase anti alkaline phoshatase (DAKO D651Lot no.091)

The optimal dilution of primary and secondary antibody for the avidin-biotin technique was determined using a checkerboard.

		Primary antibody		
		1:100	1:500	1:1000
Secondary antibody	1:200			
	1:500			

The dilutions of linking antibody and secondary antibody were determined using a checkerboard.

3.2.5 Non-specific background staining

Non-specific background staining is staining of a specimen not due to antigen-antibody binding. The most common cause is attachment of antibody to highly charged collagen and connective tissue elements of the tissue. In addition, the substrate-chromagen reaction used to visualise peroxidase cannot distinguish between the enzyme immunologically localising the cellular antigen and similar enzymatic activity present in the specimen before staining. This is especially problematic in tissues such as placenta that contain a lot of red blood cells. If this endogenous peroxidase activity is not removed before adding the marking enzyme non-specific background staining will occur.

The following steps were taken to eliminate background staining:
1. Inhibition of endogenous peroxidase activity by incubating the sections for 30 mins at room temperature in a solution of 3% (v/v) hydrogen peroxide in 100% methanol.
2.Reduction of ionic interaction of tissue with antibody by addition of 0.9% NaCl in 0.05% M Tris /HCl buffer (Tris Buffered Saline, TBS. See Appendix 5)
3.The use of a blocking solution of 25%(v/v) normal swine serum and 5%(w/v) bovine serum albumin in TBS for 30 minutes at room temperature.

3.2.6. Controls

3.2.6.1. CRH immunostaining

The following negative controls were used; CRH primary antibody was substituted by antibody dilution buffer, non-immunised rabbit serum (1:100) dilution or CRH antibody (1:1000) dilution that had been preabsorbed for 18 hours at 4°C with 5μM human CRH (Bachem (UK)Ltd Saffron Walden Essex). The positive control used in each

case was term placenta (previously shown to stain positvely for CRH using this antibody by Riley et al 1991).

3.2.6.2. ACTH immunostaining

The following negative controls were used; anti CLIP antibody was substituted by antibody dilution buffer, non-immunised rabbit serum or anti-CLIP antibody (1:1000) that had been preabsorbed overnight with 5μM human ACTH for 18 hours at 4°C. The positive control used in each case was adult sheep pituitary.

3.3 Results and Discussion

3.3.1 Fixation

The results of immuncytochemistry can only be as good as the tissue used. It is therefore of great importance that the tissue is correctly fixed and processed and that the antigen being localised is not only fixed, but also available and accessible to the primary antibody. One of the most commonly used fixatives for immunocytochemistry of paraffin-embedded sections is paraformaldehyde. Due to its cross-linking properties it is especially good for fixing small molecules such as hormones. However fixation time is critical to prevent antigen masking . Small blocks of tissue (maximum $2cm^2$) should be placed in a minimum of 200 mls of formaldehyde. The formaldehyde used was buffered to pH 7.4 as acidic solutions can cause structural disturbances and poor morphology. A phosphate buffer was used to make up the formaldehyde to maintain tonicity. Early and term placentas were fixed for 8 hours, 22 hours and 48 hours in 4% buffered paraformaldehyde, providing a wide range of fixation times including that already used for tissues of this type in this laboratory. In 8 hours insufficient fixative had permeated the tissue, it was too soft to be sectioned and its morphological integrity was destroyed. In contrast, tissue fixed for

48 hours showed no positive immunostaining even after a trypsin digestion stage. The optimum fixation time was found to be 22 hours and subsequently all tissues were left at room temperature for this time before processing. (see plate no. 3.1)

3.3.2. Tissue pretreatment

As described above, fixation time is critical to prevent antigen masking. Formaldehyde forms cross linkages with basic amino acids, mainly lysine and arginine hindering penetration by antibody. Treatment with trypsin can digest these excess linkages and expose the antigen. To improve staining a trypsin digestion stage was added to the protocol. The times and temperatures to which the sections were exposed to trypsin were determined. Slides were incubated for 15 minutes, 30 minutes and 1 hour at 37°C and room temperature. In all cases, a 0.1% (w/v) Trypsin (Type III from bovine pancreas Sigma Chemicals Poole Dorset) solution in 0.1% $CaCl_2$ was used. It was found that a 30 minute incubation at 37°C gave the strongest specific staining, with minimal background. The 15 minute digestion did not improve the staining intensity, and the 1 hour digestion caused an unacceptable level of background staining. (See plate 3.1)
In addition to trypsin digestion, washing the section with a solution of Triton-X 100 is known to improve tissue permeability to antigen by solubilising the lipid component of the cell membrane. Therefore, a 15 minute wash of a 0.5% solution of Triton-x was added to the protocol.

3.3.3. Antibodies

The optimal dilution to be used depends on a number of different factors:

In polyclonal serum such as that used for both the CRH and ACTH immunostaining high levels of substances other than the specific

antibody will be present. This necessitates high dilutions to prevent non-specific background staining.
Generally, the longer the incubation time, the more dilute the antibody can be used.
As mentioned above, the fixation time, tissue processing and pretreatment protocol, including trypsin digestion, all affect optimal antibody dilution.
In both the CRH and ACTH staining methods, the antigen is localised via a system involving more than one antibody. The concentrations of both antigens are critical to the success of the system. The outcome should be the greatest possible intensity of staining with the lowest possible background.

3.3.3.1. CRH immunostaining

The optimal primary antibody dilution was found to be 1:1000. The biotinylated swine anti rabbit immunoglobulin G was used at a dilution of 1:500. (See Plate 3.2. for primary and secondary antibody dilutions.)

3.3.3.2. ACTH immunostaining

Two different methods were tried to achieve the best specific staining of ACTH within the tissues. The first was an avidin-biotin method such as that used for the CRH immunolocalisation. Unfortunately this did not produce the intensity of staining seen for CRH. Presumably this was due to the much smaller quantities of ACTH present. The avidin-biotin method with DAB as a chromagen gives a very specific result but tends to produce a fainter stain. It was therefore decided to try an alkaline phosphatase anti alkaline phosphatase bridge technique using nitroblue tetrazolium as the chromagen. (See Plate 3.3 for the results of staining using both techniques). This bridge technique uses alkaline phosphatase as a central part. The placenta contains a large quantity of endogenous alkaline phosphatase and it might be expected that this would result

in problems with non-specific background staining. However, the staining was seen to be consistently localised to the same cells and no staining was seen in the preabsorbed controls suggesting specific staining.

3.3.4. Controls

In both the CRH immunostaining and the ACTH imunostaining, there was no specific staining where primary antibody was substituted for by antibody dilution buffer, non-immunised rabbit serum or primary antibody that had been preabsorbed ar 4°C for 18 hours with 5μM human CRH (in the case of CRH immunostaining), or 5μM ACTH (in the case of CLIP immunostaining) (See plates 3.2 and 3.3). In contrast specific immunostaining was seen in the positive controls. These results confirm that both the CRH immunostaining method, and the CLIP immunostaining method were specific.

3.4. Conclusion

The optimal immunostaining methods were therefore as follows

3.4.1. CRH Immunostaining

5μm sections were cut, floated onto microscope slides and then baked overnight at 56°C. The next day they were deparaffinised in xylene substitute (Histoclear, National Diagnostics New Jersey) and rehydrated in graded ethanol. The sections were incubated for 30 minutes in 3% (v/v) hydrogen peroxide (BDH chemicals Ltd, Poole Dorset, U.K.) in methanol (BDH) to block endogenous peroxidase activity, then washed in 0.05M Tris-HCl buffer (TBS; 0.05M Tris(hydroxymethyl)methylamine/l; 0.9% (w/v) NaCl, pH 9.6) twice for 5 minutes, The sections were then incubated with 0.1% Trypsin (Type III from bovine pancreas, Sigma Chemical Company Poole Dorset) in 0.1%(w/v) $CaCl_2$ (BDH) pH7.4 at 37°C for 30 mins to facilitate penetration of the tissue by the antibody and then washed twice for

5 minutes in TBS before a wash in 0.5% (v/v)Triton-X 100 in distilled water for 15 minutes. After two 5 minute washes in TBS, sections were incubated with 25% (v/v) normal swine serum and 5% (w/v) bovine serum albumin in TBS for 30 minutes at room temperature to reduce non-specific staining. Sections were then incubated overnight at 4°C with primary antibody in a humidified chamber. The sections were then washed twice in TBS for 5 minutes and then incubated for 30 minutes with secondary antibody, biotinylated swine anti-rabbit immunoglobulin G (DAKOPATTS, DAKO Ltd) diluted (1:500) in TBS/NSS/BSA. Following two further washes in TBS for 5 minutes, peroxidase-conjugated avidin-biotin complex (DAKOPATTS, DAKO Ltd) was applied according to the manufacturers instructions for 30 minutes. The sections were then twice washed in TBS for 5 minutes. Hormone was localised by the detection of peroxidase activity, effected by a 5 minute incubation with a freshly prepared solution containing 3,3-diaminobenzidinetetrachloride (DAB 0.4mg/ml 0.05M Tris-HCl) to which was added 0.01% hydrogen peroxide. Sections were counterstained with haematoxylin, dehydrated through graded ethanol, cleared in xylene and mounted in Eukitt (Kindler. Gmbh+Co Freiburg Germany) for microscopic examination.

3.4.2. ACTH immunostaining

5µm sections were cut, deparaffinised in xylene substitute (Histoclear, National Diagnostics, New Jersey) and rehydrated in graded ethanol. The sections were washed in 0.5 M Tris-HCl buffer (TBS; 0.5 mol Tris(hydroxymethyl)methylamine; 0.9% NaCl (w/v) (pH 7.6) for 10 mins and then incubated with 0.1% Trypsin (Type III from bovine pancreas, Sigma chemical company Dorset) in 0.1% (w/v) $CaCl_2$ (BDH) pH 7.4 at 37°C for 30 mins to facilitate penetration of the tissue by the antibody and then washed for a further 10 mins in TBS before a wash in 0.5% Triton-X-100 (vol/vol) in distilled water for 15 mins. After two 5 min washes in TBS, sections were incubated with 25%(vol/vol) normal swine serum and 5% bovine serum albumin in TBS for 30 mins at room temperature to reduce non-specific staining. Sections were then incubated overnight with primary antibody at a

dilution of 1:1000 at 4°C in a humidified chamber. The sections were then washed twice in TBS for 5 mins and then incubated for 30 mins with secondary antibody swine antirabbit immunoglobulin G (DAKOPATTS, DAKO Ltd) diluted (1:60) in TBS/NSS/BSA. Following two further washes in TBS for 5 mins the sections were incubated for a further 30 mins with alkaline phosphatase anti-alkaline phosphatase antibody diluted (1:100) in NSS/TBS/BSA. The sections were then washed twice in TBS and then once in TBS/$MgCl_2$ (100mM tris pH 9.5, 50mM $MgCl_2$, 100mM NaCl). Substrate solution was then added (10 mls Tris/$MgCl_2$ pH 9.5, 45µl NBT, 35µl X-phosphate, 10µl levimasole) The slides were then incubated in the dark overnight. Sections were dehydrated and mounted in Eukitt for microscopic examination.

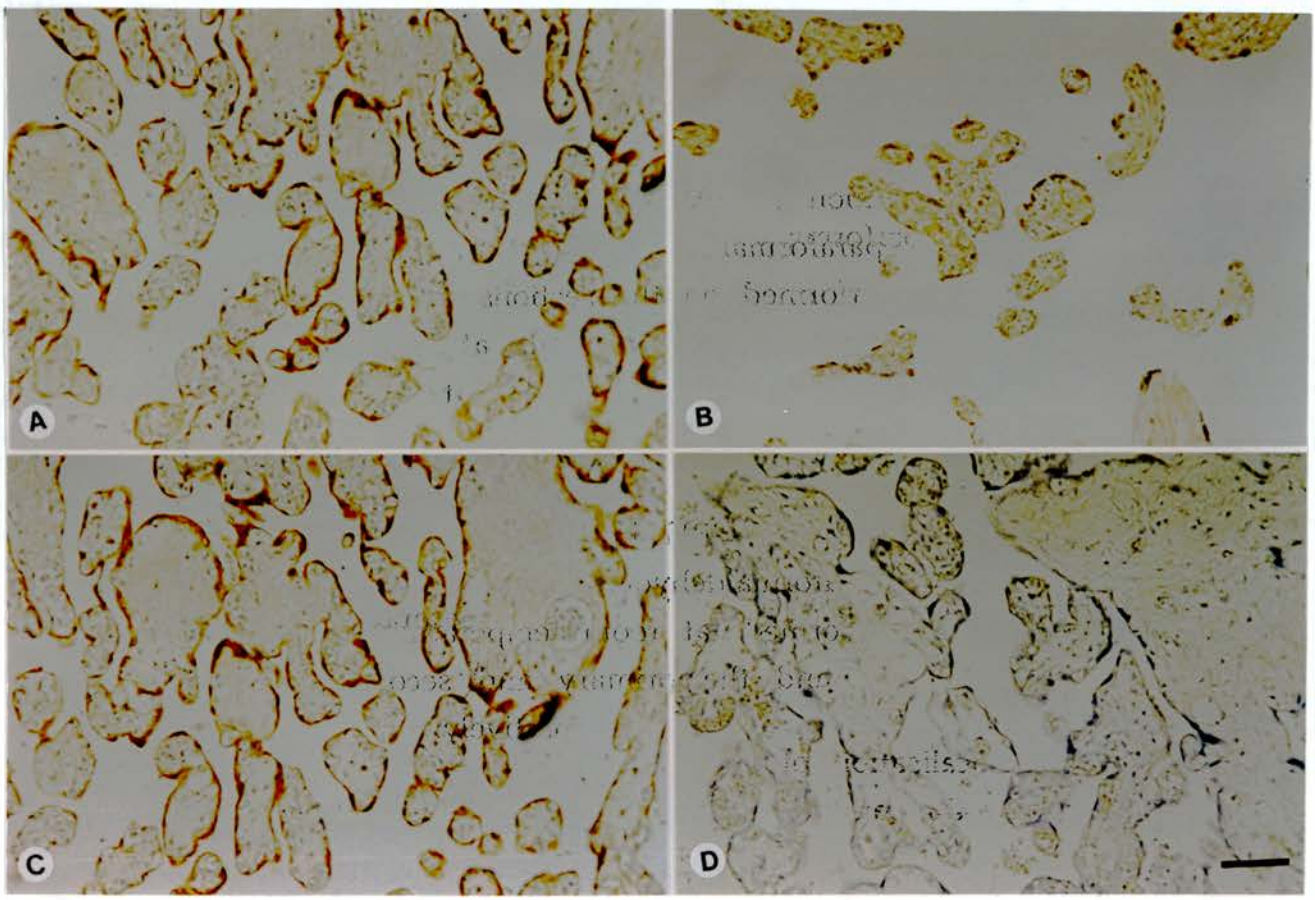

A
B
C
D

Plate 3.1. Development of an immunocytochemistry method to localise human corticotrophin releasing factor within the placenta and fetal membranes.

3.1.A. Immunolocalisation of CRH in human placenta fixed for 48 hours in 4% paraformaldehyde. Predigestion with 0.1% (w/v) trypsin was performed on the sections and the primary and secondary antibodies were used at 1:1000 and 1:500 respectively.

3.1.B. Immunolocalisation of CRH in human placenta fixed for 22 hours in 4% paraformaldehyde. Predigestion with 0.1% (w/v) trypsin was performed on the sections and the primary and secondary antibodies were used at 1:1000 and 1:500 respectively. Note the increased intensity of staining compared with 3.1.A.

3.1.C. Immunolocalisation of CRH in human placenta fixed for 22 hours in 4% paraformaldehyde. Predigestion with 0.1% (w/v) trypsin was performed at room temperature for 15 minutes on the sections and the primary and secondary antibodies were used at 1:1000 and 1:500 respectively.

3.1.D. Immunolocalisation of CRH in human placenta fixed for 22 hours in 4% paraformaldehyde. Predigestion with 0.1% (w/v) trypsin was performed at 37° C for 30 minutes on the sections and the primary and secondary antibodies were used at 1:1000 and 1:500 respectively. Note the increased immunostaining compared to 3.1.C.

The bar indicates 50μm

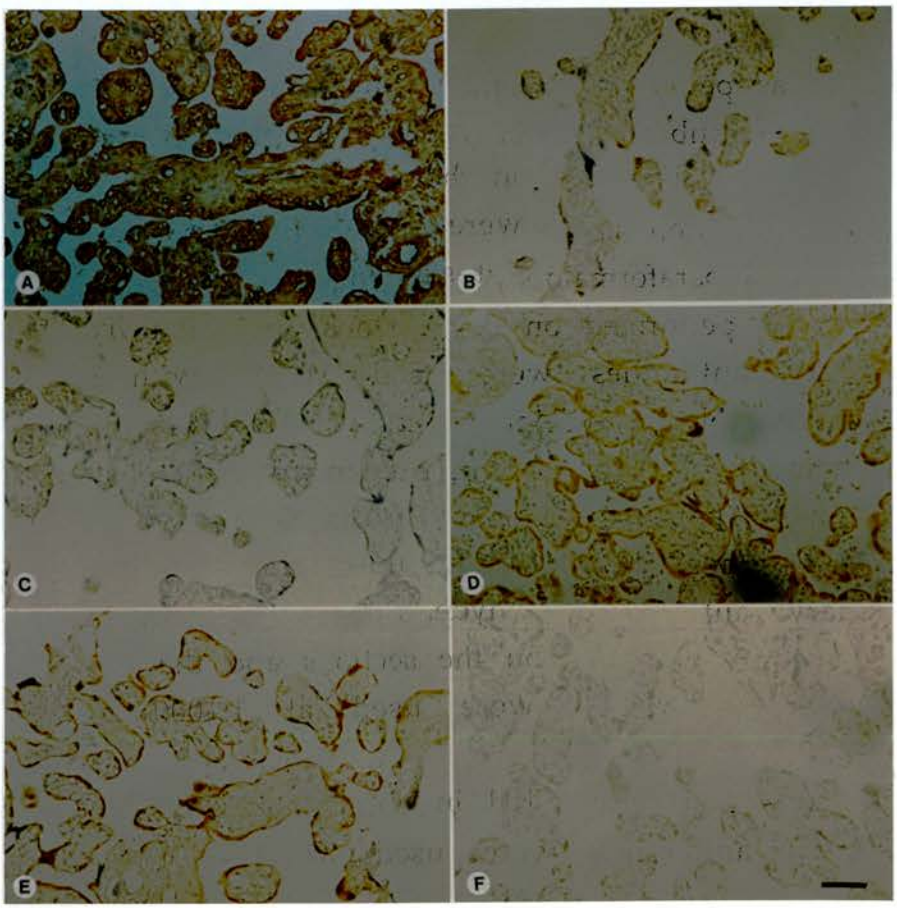

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Plate 3.2. Determination of optimal primary and secondary antibody concentration for CRH immunostaining

3.2.A. Immunolocalisation of CRH in human placenta fixed for 22 hours in 4% paraformaldehyde. Predigestion with 0.1% (w/v) trypsin was performed on the sections and the primary and secondary antibodies were used at 1:500 and 1:500 respectively. Note the very high level of non-specific staining.

3.2.B. Immunolocalisation of CRH in human placenta fixed for 22 hours in 4% paraformaldehyde. Predigestion with 0.1% (w/v) trypsin was performed on the sections and the primary and secondary antibodies were used at 1:1500 and 1:200 respectively. Note the faint staining.

3.2.C. Immunolocalisation of CRH in human placenta fixed for 22 hours in 4% paraformaldehyde. Predigestion with 0.1% (w/v) trypsin was performed on the sections and the primary and secondary antibodies were used at 1:1500 and 1:500 respectively. Note the faint staining.

3.2.D. Immunolocalisation of CRH in human placenta fixed for 22 hours in 4% paraformaldehyde. Predigestion with 0.1% (w/v) trypsin was performed on the sections and the primary and secondary antibodies were used at 1:1000 and 1:200 respectively.

3.2.E. Immunolocalisation of CRH in human placenta fixed for 22 hours in 4% paraformaldehyde. Predigestion with 0.1% (w/v) trypsin was performed on the sections and the primary and secondary antibodies were used at 1:1000 and 1:500 respectively. Note the specific staining of the syncytiotrophoblast with minimal background staining.

3.2.F. Control tissue. Human placenta fixed for 22 hours in 4% paraformaldehyde. Predigestion with 0.1% (w/v) trypsin was performed on the sections. The secondary antibody was used at 1:500. The primary antibody at a dilution of 1:1000 had been preabsorbed overnight at 4°C with 5μM human CRH.

The bar indicates 50μm

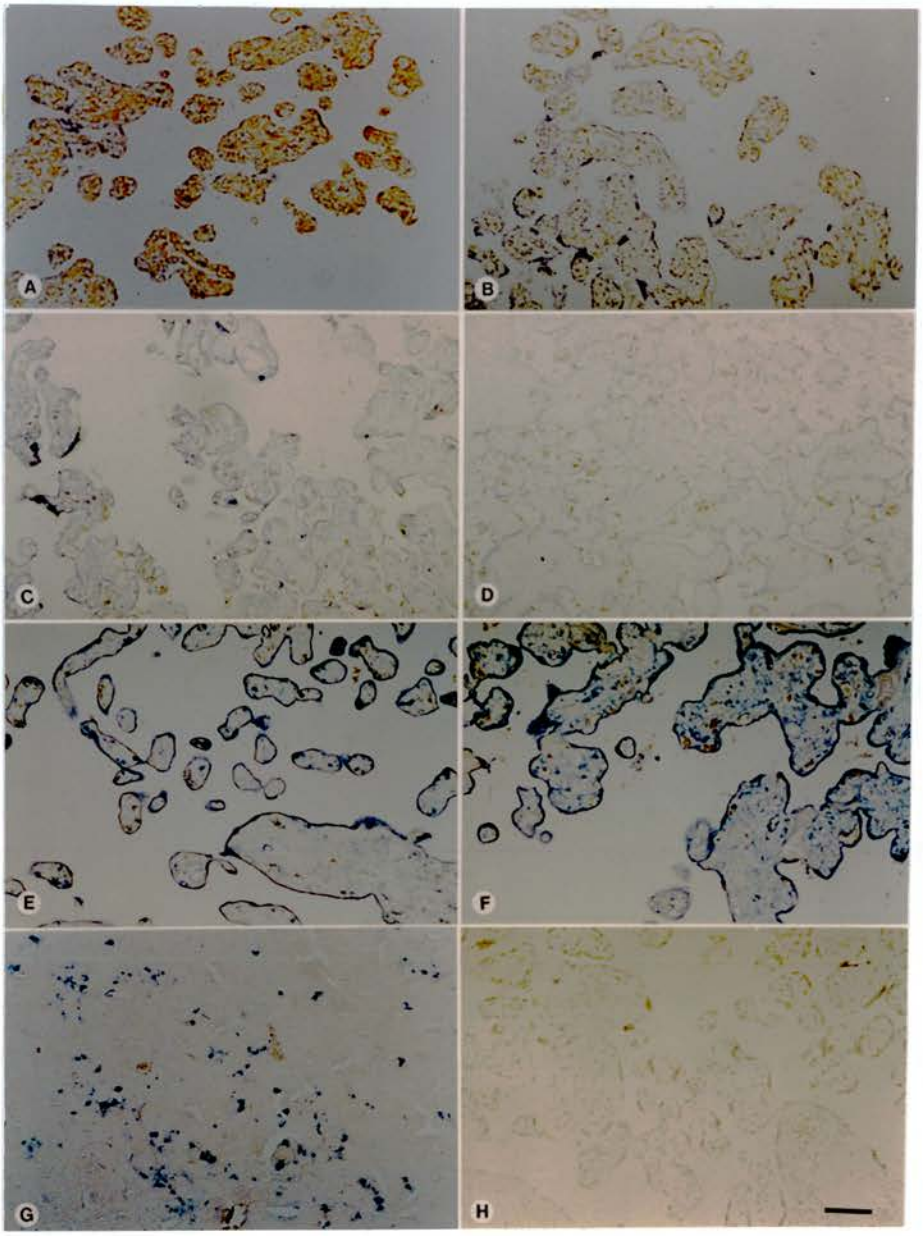
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Plate 3.3. Determination of optimal conditions for the immunolocalisation of ACTH in human placenta and fetal membranes. (The bar indicates 50μm)

3.3.A Avidin-biotin immunolocalisation of ACTH in human placenta. Predigestion with 0.1% (w/v) trypsin was performed on the sections and the primary and secondary antibodies were used at 1:500 and 1:500 respectively.

3.3.B. Avidin-biotin immunolocalisation of ACTH in human placenta. The primary and secondary antibodies were used at 1:200 and 1:500 respectively. Note the low level of generalised non-specific staining. It was therefore decided to use an alkaline phosphatase anti alkaline phosphatase bridging technique.

3.3.C. Immunolocalisation of ACTH in human placenta using the APAAP method. Predigestion with 0.1% (w/v) trypsin was performed on the sections and the primary and secondary and APAAP antibodies were used at 1:500 and 1:60 and 1:100 respectively.

3.3.D. Immunolocalisation of ACTH in human placenta as in 3.3.C The primary and secondary and APAAP antibodies were used at 1:1500 and 1:60 and 1:100 respectively.

3.3.E. Immunolocalisation of ACTH in human placenta as in 3.3.C. The primary and secondary and APAAP antibodies were used at 1:1000 and 1:60 and 1:100 respectively.

3.3.F. Immunolocalisation of ACTH in human placenta as in 3.3.C. The primary and secondary and APAAP antibodies were used at 1:2000 and 1:60 and 1:100 respectively.

3.3.G. Positive control. Immunolocalisation of ACTH in adult sheep pituitary as in 3.3.C. The sections and the primary and secondary and APAAP antibodies were used at 1:1000 and 1:60 and 1:100 respectively.

3.3.H. Negative control. Immunolocalisation of ACTH in human placenta as in 3.3.C. The sections and the primary and secondary and APAAP antibodies were used at 1:1000 and 1:60 and 1:100 respectively. The primary antibody was preabsorbed overnight with 5μM human ACTH.

CHAPTER 4

DEVELOPMENT OF IN SITU HYBRIDISATION METHODS TO LOCALISE CRH mRNA AND POMC mRNA WITHIN THE PLACENTA AND FETAL MEMBRANES.

IN SITU HYBRIDISATION

4.1. Introduction

In situ hybridisation techniques allow specific nucleic acid sequences to be detected in individual cells. The development of in situ hybridisation methods, in combination with the immunocytochemical techniques developed in chapter 3 for CRH and ACTH would allow both the mRNA transcribed from the gene itself and the gene product translated from the mRNA to be identified.

The technique was originally developed in the 1960's (Pardue and Gall 1969; John et al 1969; Buongiorno-Nardelli and Amaldi 1969) and was used primarily for the localisation of DNA sequences. More recently it has been used in the detection of specific mRNA sequences as well as for chromosomal gene mapping.

A probe is a nucleic acid sequence of either DNA or RNA, labelled by incorporation with either a radioactive or non-radioactive marker. The single stranded probe hybridises (anneals) to its complementary single-stranded nucleic acid sequence in the tissue section to form a new double stranded molecule that incorporates the label. The sites of hybridisation are then detected and visualised. The detection method depends on the nature of the reporter molecule, which may be radioactive or non-radioactive. Both DNA and RNA probes can be used to detect mRNA and hence gene expression in tissue sections, but DNA probes are more commonly used as they are less susceptible to degradation. The best probe length for in situ hybridisation is about

100-300 bases, for optimal nucleic acid hybrid stability and tissue penetration. DNA probes may be produced by cloning and purification of any DNA sequence using recombinant technology, or oligonucleotide sequences can be synthesised de novo. Probes produced by both these methods were used in the development of the in situ hybridisation techniques described in this chapter. To clone nucleic acids, the DNA is inserted into a vector, commonly a plasmid, and amplified in appropriate host cells. For RNA probes (riboprobes) the sequence of interest is inserted into a vector containing transcription initiation sites for bacteriophage RNA polymerases. These sites enable transcription to be initiated in vitro in the presence of labelled and unlabelled nucleotides as substrates. This method was used in the in situ hybridisation method for the detection of POMC mRNA (see section 3.2.3.3.). Riboprobes tend to form more stable hybrids than do oligonucleotides because of their much longer length, however this may impede tissue penetration. Synthetic oligonucleotides are sequences of nucleotides usually 10-50 base pairs long which are synthesised on a DNA synthesiser. They are usually labelled by an end labelling reaction such as that described in section 4.2.3.1. and appendix 3. The short length of oligonucleotide probes has the advantage of good tissue penetration, but even a few mismatched base pairs will significantly reduce the stability of the nucleic acid hybrids.

The sensitivity of in situ hybridisation depends on the effect of tissue preparation on retention and accessibility of cellular (target) DNA or RNA; the type of probe construct, efficiency of probe labelling and sensitivity of the method used for signal detection; the effect of hybridisation conditions on the efficiency of hybridisation. In developing an in situ hybridisation method, all these factors must be taken into consideration.

The aim of this study was to develop an in situ hybridisation method, using an oligonucleotide probe to CRH mRNA and a riboprobe to POMC mRNA, to investigate the cellular localisation of the respective mRNA within the placenta and fetal membranes. The technique would then be used to see if gene expression and/or histological distribution

was affected by gestational age, administration of exogenous prostaglandins or the antiprogestin mifepristone, or by labour.

4.2 Materials and Methods

4.2.1. Collection and Fixation of Tissue

The tissues were collected and fixed as for the immunocytochemistry studies. See Section 3.2.1- 3.2.2. Development of an immunocytochemical method to localise CRH and ACTH within the placenta and fetal membranes.

The fixed tissue was processed on a standard tissue processor and embedded in parrafin wax. 5μm sections were cut on a microtome using a clean blade that had been dipped in xylene to avoid RNAse contamination and then floated onto TESPA-coated (to improve adherance of the sections) slides (see Appendix 1. for details of preparation of TESPA coated slides) and then baked overnight at 56°C.

4.2.2. Tissue pretreatment

(All solutions used for in situ hybridisation are detailed in Appendix 2.)

Sections were dewaxed in xylene for 10 minutes and then rehydrated in clean, graded ethanol. They were then pretreated with 0.2M HCl for 20 minutes at room temperature before two 5 minute washes in Stillplus water. Thereafter they were incubated at 37°C for 30 minutes in 2μg/ml proteinase K. After a wash in 0.2% glycine for 20 minutes they were washed in TEA before acetylation with acetic anhydride.

4.2.3. Probes

4.2.3.1. Oligonucleotide probe specific for human CRH mRNA labelled with Digoxigenin.

Three synthetic oligodeoxyribonucleotides (two antisense and a sense probe, for sequences see figure 4.1.) were made on a 391 DNA Synthesizer (Applied Biosystems PCR-MATE). The sequences were chosen from the full sequence of the human corticotrophin releasing factor precursor gene as published (Shibahara et al 1983). A primary requirement of an oligonucleotide probe is that it must hybridise specifically to the target gene sequence and not to unrelated sequences. To this end, three sequences were chosen by Dr P Saunders (MRC Reproductive Biology Unit) using the Genejockey programme written by Dr Phil Taylor, (MRC Reproductive Biology Unit), featuring well conserved regions of the molecule. After synthesis overnight, the oligonucleotide probes were deprotected with ammonia (see Appendix 6). Oligonucleotide concentration was calculated by measuring the O.D 260 of 10µl of oligonucleotide solution in 1ml of water. The probes were then 3' end-labelled with Digoxigenin-dUTP using terminal transferase, the aim being to add 3-4 bases of digoxigenin-dUTP. The efficiency of the labelling reaction was checked using 16% polyacrylamide gel electrophoresis (PAGE). (For details of the labelling reaction and the PAGE see Appendix 3).

The efficacy of these probes for in situ hybridisation was compared with oligonucleotides of the same sequence commercially labelled with digoxigenin dUTP (British Biotechnology products Ltd Abingdon Oxon OX14 3YS UK).

4.2.3.2. Oligonucleotide probes specific for human CRH mRNA labelled with ^{35}S.

The oligonucleotides used were those used for digoxigenin labelling. The protocol used was a 3' end-labelling reaction with ^{35}Sd ATP

(W. Scott Young III In situ hybridisation with oligodeoxyribonucleotide probes in In Situ Hybridisation, A Practical Guide Bios Scientific publications) using terminal transferase. Terminal transferase from two different sources were compared (Boeringer Mannheim and Promega). For details of the method see Appendix 3.

4.2.3.3. POMC Riboprobe labelled with ^{35}S

The POMC riboprobe was kindly donated by Dr Pamella Kolb, department of Obstetrics and Gynaecology University of Washington Seattle. The POMC insert was contained within a plasmid (pSP64 and pSP65) with transcription initiation sites for the RNA polymerases SP6 and T7. These sites enabled transcription to be initiated in vitro in the presence of labelled and unlabelled nucleotides resulting in the formation of sense and antisense single stranded RNA riboprobes. The labelled nucleotide used was ^{35}S rCTP (Lot no. 89413 Amersham) and the SP6 and T7 polymerase were obtained from Boehringer Mannheim (lot no. 126/8820-03). The detailed reaction method is in Appendix 3.

4.2.4. Composition of Hybridisation buffer

The degree of specificity of hybridisation reactions can be controlled accurately by varying the reaction conditions. The temperature at which hybridisation occurs depends on not only the probe construction, but the concentration of salt and formamide in the hybridisation buffer.

4.2.4.1. Oligonucleotide probe hybridisation buffer.

The same hybridisation buffer was used for both digoxigenin-labelled and ^{35}S-labelled probes. Because the two antisense probes had different GC nucleotide contents they required different buffer compositions to enable their hybridisations to be performed at the same temperature.

4.2.4.1.1. Antisense 1 hybridisation buffer

3xSSC
10% Dextran sulphate
0.1% Denhardt's
125µg/ml salmon sperm DNA
125µg/ml Yeast tRNA
43% formamide

4.2.4.1.2. Antisense 2 / Sense hybridisation buffer

3xSSC
10% Dextran sulphate
0.1% Denhardt's
125µg/ml salmon sperm DNA
125µg/ml Yeast tRNA
37% formamide

4.2.4.2. Riboprobe hybridisation buffer

See appendix 3

Prehybridisation buffer was prepared as for hybridisation buffer except the dextran sulphate was replaced by Stillplus water to reduce the viscosity of the solution and facilitate its removal from the slides after the prehybridisation stage. The sections were incubated in 100µl of prehybridisation buffer for 2.5 hours at a range of temperatures.

The hybridisation was performed in hybridisation buffer at a range of temperatures. Each section was covered by 50µl of hybridisation buffer containing probe at a concentration of $1x\ 10^6$ counts for radioactively labelled probes, or 1600, 800 or 400 pg/ml for digoxigenin-labelled probe. The hybridisation time used in all cases was 18 hours.

The treatment of the slides after hybridisation depended on probe type and labelling method.

4.2.5. Washes

4.2.5.1. Digoxigenin -labelled oligonucleotides

Different washing steps were compared to assess the effect on background levels. The slides were first placed in 2xSSC to remove the cover slips, and then washed in 0.1x SSC for 15 minutes, four times at a range of temperatures between 30 and 40°C.

4.2.5.2. ^{35}S-labelled oligonucleotides

Again, different washing steps were compared to assess the effect on background noise. The cover slips were removed in 2x SSC and the slides washed four times in 0.1xSSC for 15 minutes each, again at a range of temperatures. The slides were then rinsed in a range of washes of differing stringencies in order to improve signal/noise ratio.

4.2.5.3. ^{35}S-labelled POMC Riboprobes.

Three protocols were tried;

4.2.5.3.1
- 12x15 minute washes in 4XSSC
- 30 minute wash in RNAse 20µg/l at 37°C
- 30 minute wash in RNAse buffer at 37°C
- 30 minute wash in 2xSSC at room temperature
- 30 minute wash in 2xSSC at 45°C
- 30 minute wash in 0.5xSSC at room temperature

4.2.5.3.2
- 2x 15 minute washes in 4xSSC
- 30 minute wash in RNAse 20µg/l at 37°C
- 30 minute wash in RNAse buffer at 37°C
- 30 minute wash in 2xSSC at room temperature
- 30 minute wash in 0.1xSSC at room temperature

4.2.5.3.3 2x15 minute washes in 4xSSC
30 minute wash in RNAse 20µg/l at 37°C
30 minute wash in RNAse buffer at 37°C
30 minute wash in 1xSSC at room temperature
30 minute wash in 0.1xSSC at room temperature

4.2.6. Detection of the in situ hybrids

Following the washes the slides were treated differently according to the type of reporter used;

4.2.6.1 Digoxigenin- labelled probes.

1. The slides were treated with a blocking solution of normal sheep serum (NSS) and tris-buffered saline (TBS) (1:5) for 30 minutes in a humid conainer.
2. Antidigoxigenin-alkaline phosphatase Fab fragments in NSS/TBS (1:5) at a dilution of 1:300 was applied and the slides left at room temperature for 2 hours.
3. The slides were washed in TBS twice for 15 minutes.
4. The slides were washed for 2 minutes in digoxigenin detection buffer
5. Chromagen (NBT; for details see appendix 2) was added and the slides were left to incubate overnight in the dark in a humid box at room temperature for the colour to form.
6. After a further wash in digoxigenin detection buffer the slides were dehydrated in absolute alcohol, cleared in xylene and mounted in Eukitt.

4.2.6.2. ^{35}S-labelled probes.

With both oligonucleotide and riboprobes, the procedure was the same. The slides were dehydrated by passing through an alcohol series of 50% and 85% (for 20 seconds each) containing 300mM of ammonium acetate each to stabilise the hybrids, and then a 20 second wash in absolute ethanol. The slides were left to air dry before dipping in photographic emulsion (for method, see appendix 4a). The slides were left in dark

boxes to expose for 3-6 weeks. After being developed (for details see appendix 4b) the slides were counterstained with haematoxylin.

4.3. Results and Discussion

4.3.1 Fixation of Tissue

Optimal fixation conditions should preserve the maximum amount of cellular RNA without disrupting normal morphology and at the same time allow access for the probe. In vivo, messenger RNA is steadily synthesised and degraded enzymatically. Out of the body, mRNA is rapidly degraded, and consequently tissue should be fixed as soon as possible. To this end all tissues used were placed in paraformaldehyde within 30 minutes of delivery. Paraformaldehyde has previously been successfully used to fix tissue for in situ hybridization in several studies (Brigati et al 1983; Hafen et al 1983; MacAllister and Rock 1985; Hofler et al 1986). Although it is a cross-linking fixative, it does not cross-link proteins so extensively as to prevent tissue penetration by probes. Other fixatives may preserve more RNA than paraformaldehyde but may impair the accessibility of probes. Glutaraldehyde, has been reported to preserve 20% more RNA but reduces accessibility to 40% (Singer et al 1987). It was decided to use 4% paraformaldehyde as a fixative because it preserved morphology and antigenicity for immunocytochemistry particularly when the tissue was fixed for the optimum twenty-two hours, and yet would still allow probe penetration for in situ hybridisation.

Tissue sections must adhere well to the glass slides during the rigorous hybridisation procedure. To this end, the slides were first coated with TESPA (See Appendix 1) (Burns et al 1987) which facilitates tissue adherence.

Pretreatment of sections with proteinase digestion is a standard procedure in almost all in situ hybridisation protocols, particularly where cross-linking fixatives have been used. The combination of proteinase K digestion with 0.2M HCl wash increases the permeability of tissue to probe, the protease by digesting the protein surrounding the

nucleic acid sequence, and the HCl by partial hydrolysis of the target sequences. The acetylation step is used to reduce non- specific electrostatic binding of the probe by neutralising positively charged molecules and prevents non-specific binding of the probe to the slide. Prehybridisation blocks non-specific protein and nucleic acid interaction with the probe.

4.3.2. Probes

4.3.2.1. Oligonucleotide probes for CRH mRNA.

Synthetic oligonucleotides are conveniently prepared by DNA synthesisers. The length of synthetic oligonucleotides (in this case 24-mer) is far shorter than riboprobes which may be 0.5-5 kb. This has important consequences for hybrid stability and probe specificity. Their main disadvantage is low specific activity when compared to riboprobes. Shorter probes have an increased probability of finding a perfect match, by chance alone. However, in situ hybridisation rarely discrimminates between perfect and near perfect matches resulting in a lack of specificity. This fact coupled with relatively low expression of CRH mRNA, particularly in early placental tissue may explain the difficulties encountered with the in situ hybridisation for CRH. The problems encountered were twofold. Firstly, labelling of the oligonucleotide with both radioactive and digoxigenin reporters was inconsistent and therefore irreproducible (see section 4.3.3.). Secondly, when hybridisation was attempted with labelled probe, there was considerable non-specific binding of probe to all areas of the section, and there was no significant difference between sense and antisense probes. It was difficult to explain the inconsistency of the labelling since the conditions were the same each time. The non-specificity of the probes may have been secondary to the labelling difficulties, but may also be a function of the low specificity of oligonucleotide probes. A specific riboprobe for hCRHmRNA, such as that used for POMC may give better results. Alternatively, a mixture of oligonucleotide probes can be used to improve hybridisation specificity.

4.3.2.2. POMC riboprobe

The POMC riboprobe is a single stranded RNA probe and was synthesised and labelled simultaneously by in vitro transcription. This reaction involves synthesis of the RNA strand by RNA polymerase from radioactive and non-radioactive nucleotides. The polymerase used depends on the RNA polymerase promoter found on either side of the cloning site. The high specificity of each polymerase for its promoter enables ssRNA probes to be generated complementary to the coding (sense) or non-coding (antisense) strands. These probes have a higher specific activity than DNA probes and also greater hybrid stability. These factors favour increased sensitivity and consistency of reactions. Furthermore, the use of RNAse in the post hybridisation wash will result in digestion of unhybridised probe and so a reduction in non-specific background noise. So, despite its increased length (sense was 927 basepairs, antisense was 930 basepairs) when compared with the oligonucleotide probe (24 basepairs), these factors probably explain its increased specificity.

4.3.3. Probe labelling

Probe labelling may be either direct, with direct attachment of the 'reporter' molecule to DNA or RNA, or indirect, where a hapten e.g digoxigenin is attached to the probe and detected by a labelled binding protein or the probe-target hybrid is detected by a specific antibody.

Radioactively labelled probes are widely used, for several reasons; the efficiency of probe labelling can be easily monitored, radioisotopes are readily incorporated into the synthesised DNA and RNA, and autoradiography with liquid emulsions is a fairly sensitive detection system. ^{35}S was chosen to label both the CRH oligonucleotide probe and the POMC riboprobe because it produces reasonable resolution with fairly short exposure particularly with low levels of gene expression. The terminal transferase tailing method used to label the CRH

oligonucleotide probe with ^{35}S gave inconsistent and therefore unreliable degrees of incorporation of radioactivity even when the same batch of enzyme was used. Poorly labelled oligonucleotide resulted in low signal in the resulting in situ hybridisation even when an attempt was made to improve hybridisation and post-hybridisation conditions. It was therefore decided to label the CRH oligonucleotides with the non-radioactive label, digoxigenin. The digoxigenin system was developed in the 1990's (Kessler 1990) and has been modified since. The method is based on a steroid naturally found only in the foxglove, digitalis purpura, and therefore there is no binding of the anti-DIG antibody in other biological materials. Digoxigenin is linked to uridine nucleotides and incorporated by terminal transferase to the 3' end of the oligonucleotide. Detection is mediated by high affinity anti-digoxigenin antibodies conjugated to alkaline phosphatase. The benefits of this method over radioactive labelling are of speed of development as well as eliminating the problems of safety and disposal of radioactive material. However, the low level of expression and the problems of using a single oligonucleotide probe may account for the complete lack of staining achieved with this method of probe labelling for in situ hybridisation. Using different concentrations of probe made no difference to the result. A commercially obtained oligonucleotide probe, labelled with digoxigenin (British Biotechnologies Ltd) was also unsuccessful.

4.3.3.1. POMC riboprobe labelling with ^{35}S

The POMC riboprobe was labelled with [^{35}S] dCTP using SP6 polymerase using in vitro transcription. The detailed labelling reaction is discussed in section 4.3.2.2. and the method given in Appendix 3. The specific activity of the probe was assessed directly by counting 1µl of probe on a scintillation counter. Labelling of the riboprobe was more successful than that of the CRH oligonucleotide probe. The appropriate probe concentration for in situ hybridisation is one which produces the largest signal to noise ratio. A concentration of $1x10^6$cpm per slide was used for the in situ hybridisation as this gave the optimal signal without increasing the background. See Plate 4.1.

4.3.4. Hybridisation

The optimum conditions for hybridisation were sought. This depends on several factors including the composition of hybridisation buffer, the probe concentration, hybridisation temperature as well as the pretreatment of the tissue already discussed. Hybridisation depends on the ability of DNA to anneal with complementary strands in an environment just below their melting point (Tm). The maximum hybridisation rate is, however, about 15-20°C below the Tm. The Tm depends on the length of the probe, its content of the bases guanine and cytosine as well as the concentration of formamide and salt in the hybridisation buffer. Higher salt concentrations increase the stability of the hybrids.
The folowing equation has been given for the dependence of Tm on the length (l) and G/C content of the probe, and the molar salt concentration (M)

$$Tm = 16.6 \log M + 0.41 (G/C) + 81.5 - (820/l)$$

However, increasing the salt concentration above 0.4M NaCl has little effect on hybridisation or stability and the above formula does not hold (Meinkoth and Wahl 1984).
The addition of formamide to the hybridisation solution, reduces the melting temperature by 0.7°C for every 1% formamide added. It reduces the thermal stability of double stranded polynucleotides, so that hybridisation can be performed at lower temperatures. By varying the amount of formamide used, the optimum temperature can be achieved for hybridisation, whilst retaining good tissue morphology.
In aqueous solutions dextran sulphate is strongly hydrated producing a volume exclusion effect leading to an apparent increase in probe concentration and consequently higher hybridisation rates.
For the POMC riboprobe, the hybridisation was carried out at 50°C and 50% formamide as these conditions favoured the stability of well-matched hybrids.

The CRH oligonucleotide probes were hybridised at room temperature with 43% formamide for antisense 1 and 37% formamide for antisense 2 and sense as theoretically these should be the most favourable conditions. However, because of problems with labelling, results for these probes were poor.

4.3.5. Post- hybridisation washes

The aim of this is to help to reduce background noise by removing poorly matched hybrids. Labelled probe can hybridise non-specifically to sequences which are similar but not entirely homologous to the probe sequence. Such hybrids are less stable than perfectly matched hybrids. They may be removed by washing with more stringent washes. As high salt concentrations increase the stability of the hybrid, lowering the concentration (ie a more stringent wash) will favour better matched and therefore more stable hybrids. Equally, increasing the temperature of the wash increases the stringency.
For the POMC riboprobe, increasing the stringency of the washes and the addition of an RNAse wash to remove unhybridised probe decreased the background, and the optimum post-hybridisation conditions were found to be those described in section 4.2.5.3.3. See plates 4.1. and 4.2.

4.4. Conclusion

The final protocol used for all in situ hybridisation for POMC mRNA in this thesis is as follows:
(for details of all solutions see Appendix 2)

1. 5µm paraffin sections were cut and floated onto TESPA coated slides and baked overnight at 56°C.
2. The sections were dewaxed in xylene for 10 minutes and then rehydrated in graded ethanols (100%, 95%, 75%).
3. The sections were washed in 0.2M HCl for 20 minutes at room temperature.
4. The sections were washed twice in stillplus water at room temperature for 5 minutes each.

5. The sections were digested with 2µg/ml proteinase K in proteinase K buffer for 30 minutes at 37°C.
6. The sections were placed in 0.2% glycine for 10 minutes at 4°C to stop proteinase K action.
7. The sections were rinsed in TEA for 5 minutes and then acetylated for 10 minutes with acetic anhydride in TEA.
8. Prehybridisation. The sections were incubated at 50°C for 2.5 hours with prehybridisation buffer.
9. Hybridisation. The hybridisation was performed at 50°C for 18 hours in hybridisation buffer containing 50% formamide and labelled ^{35}S-POMC cDNA probe at a concentration of $1x10^6$ cpm per slide.
10. Washes. After hybridisation the slides were immersed in 4x SSC to remove the coverslips and then washed in the following solutions:
    2X 15 min washes in 4XSSC
    1X 30 min wash in RNAse 20µg/L at 37°C
    1X 30 min wash in RNAse buffer at 37°C
    1X 30 min wash in 1XSSC at room temperature
    1X 30 min wash in 0.5XSSC at 45°C
    1X 30 min wash in 0.1X SSC at room temperature.
11. Following the washes, the slides were dehydrated by passing through an alcohol series (50%, 85%) containing 300mM ammonium acetate to stabilise the hybrids, and one change of 100% alcohol. The slides were coated with emulsion and left for 3 weeks to expose.
12. After exposure, the slides were developed. (see appendix 4b)

Figure 4.1 Nucleotide sequence of the human CRH oligonucleotide probes

1. Antisense 1.

5' GAGGTGGAAGGTGAGATCCAGGGA 3'

2. Antisense 2.

5' TTCCTGTTGCTGTGAGCTTGCTGT 3'

3. Sense equivalent to antisense 2.

5' ACAGCAAGCTCACAGCAACAGGAA 3'

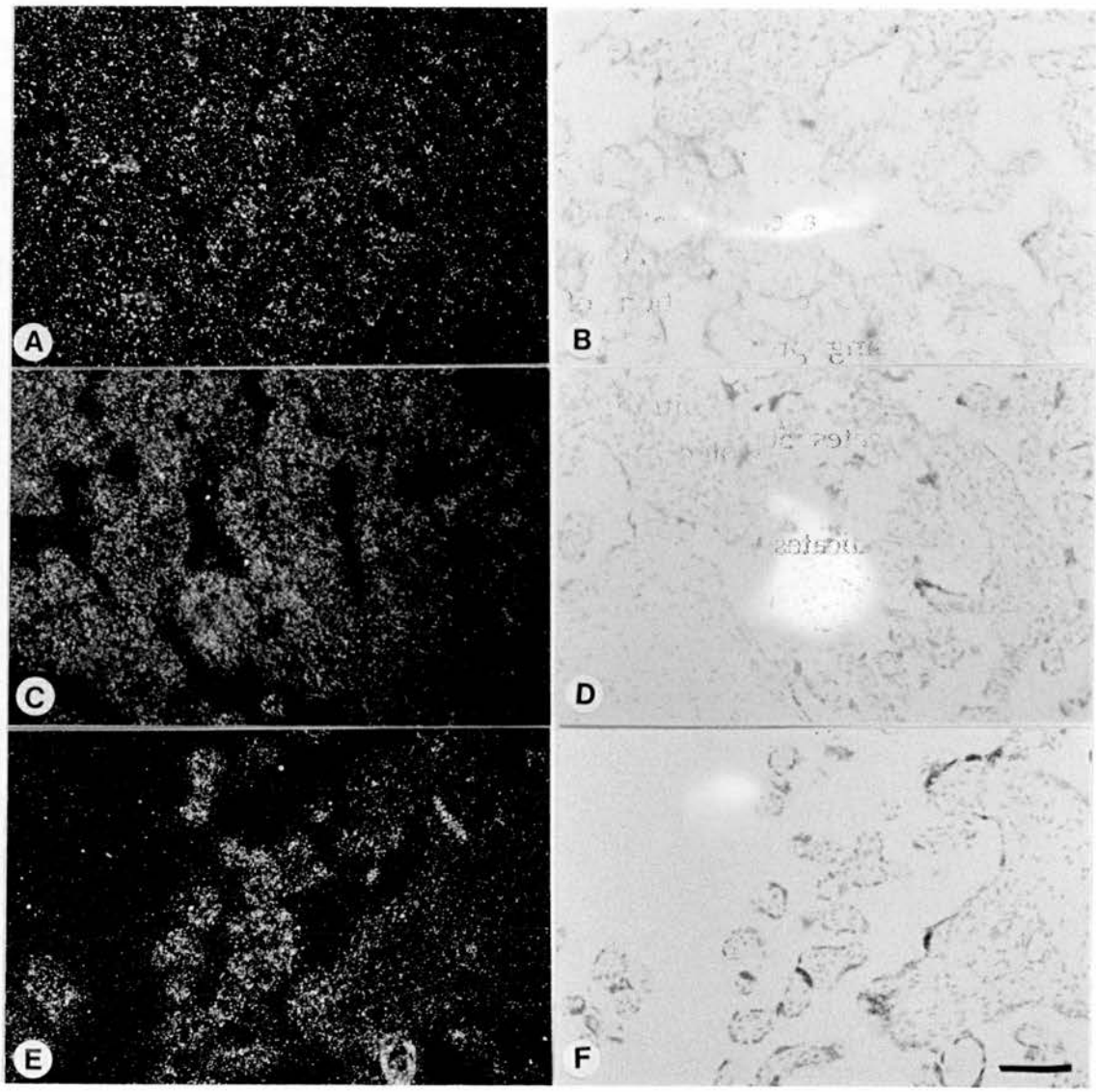

A
B
C
D
E
F

Plate 4.1. Determination of optimal conditions for the identification of POMC mRNA in human placenta (1)

4.1.A & B Dark (A) and light (B) field micrographs of in situ hybridisation of POMC mRNA in term placenta. Poorly labelled probe. Note general non-specific effect.

4.1.C & D Dark (C) and light (D) field micrographs of in situ hybridisation of POMC mRNA in term placenta. Probe used at a concentration of 0.5×10^6 counts per slide.

4.1.E & F Dark (E) and light (F) field micrographs of in situ hybridisation of POMC mRNA in term placenta. Probe used at a concentration of 1×10^6 counts per slide and washed using protocol 4.2.5.3.1.

The bar indicates 50μm

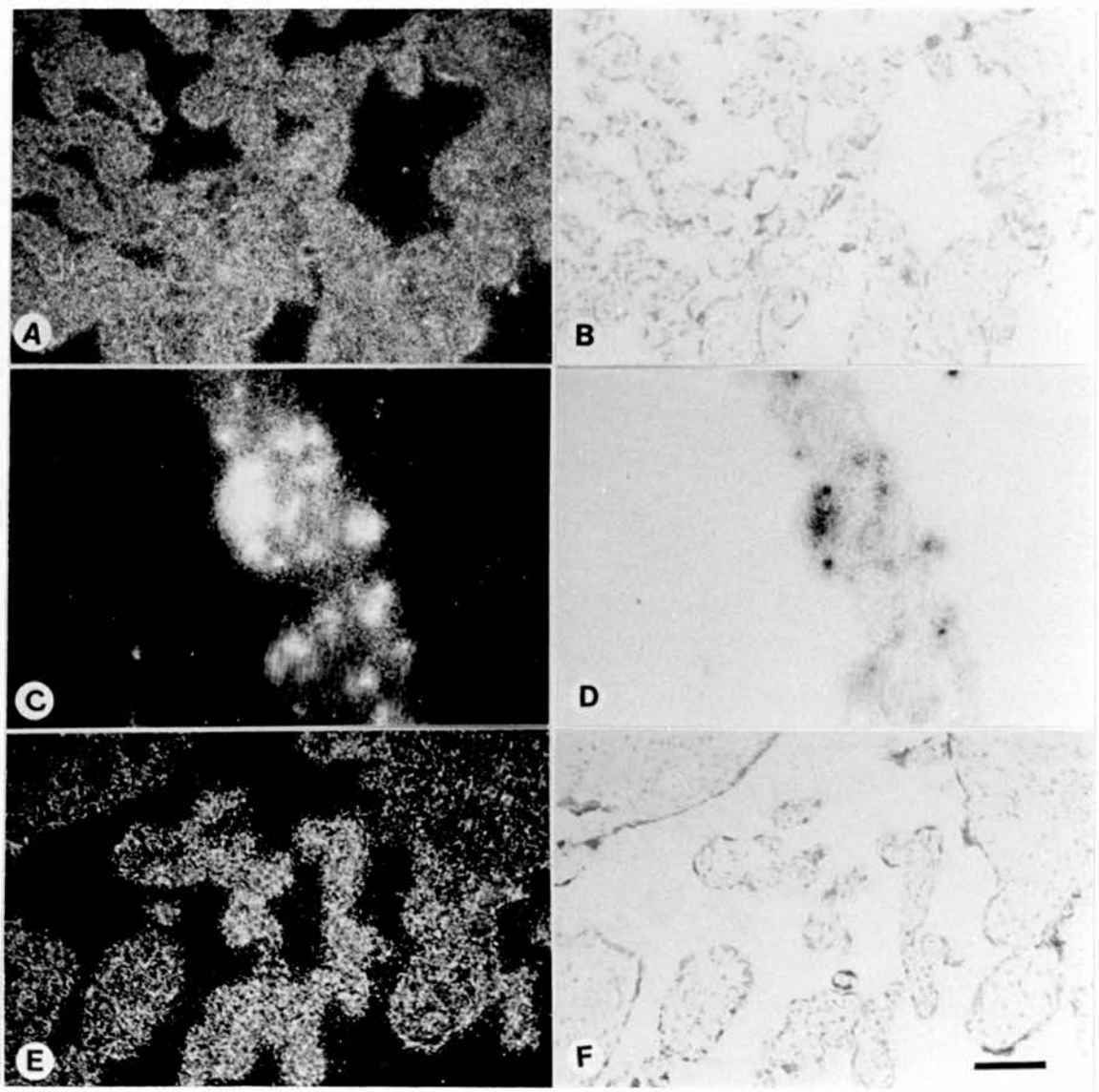

A
B
C
D
E
F

Plate 4.2. Determination of optimal conditions for the identification of POMC mRNA in human placenta (2)

4.2.A & B. Dark (A) and light (B) field micrographs of in situ hybridisation of POMC mRNA in term placenta. The washing conditions used were those detailed in section 4.2.5.3.2. Probe used at $1x10^6$ per slide.

4.2.C & D. Positive control. Dark (C) and light (D) field micrographs of in situ hybridisation of POMC mRNA in piuitary. Probe used at $1x10^6$ per slide.

4.2.E & F Dark (E) and light (F) field micrographs of in situ hybridisation of POMC mRNA in term placenta. The washing conditions used were those detailed in section 4.2.5.3.3. Probe used at $1x10^6$ per slide.

The bar indicates 50μm

CHAPTER 5

THE ONTOGENY OF CRH IN PLACENTA AND FETAL MEMBRANES

5.1. Introduction

As described in chapter 1, Corticotrophin-releasing hormone (CRH) is a 41 aminoacid hypothalamic peptide (Vale et al 1981), present in the placenta from the first trimester onwards (Shibasaki et al 1982). Placental peptide content (Sasaki et al 1987), CRHmRNA levels (Frim et al 1988) and maternal plasma concentrations (Sasaki et al 1984; Goland et al 1986; Campbell et al 1987; Laatikainen et al 1987b; Okamoto et al 1989) all rise throughout gestation and fall rapidly after delivery. These observations suggest that the placenta both synthesises and releases increasing amounts of CRH throughout pregnancy.

Localisation of CRH in the placenta has been examined using immunohistochemical techniques, but earlier reports are in conflict with more recent observations. Two studies suggested that CRH immunoreactivity was located in placental cytotrophoblast cells (Petraglia et al 1987b; Saijonmaa et al 1988). However this cell type decreases in number reaching relatively small numbers at term (Gosseye and Fox 1984) when the concentration of CRH is at its highest in the placenta, maternal plasma, and amniotic fluid (Maser-Gluth et al 1987; Laatikainen et al 1988). More recently, Riley et al (1991) have demonstrated CRH immunoactivity in the syncytiotrophoblast cells of the placenta and also in the amnion, chorion and decidua. These results are consistent with the large numbers of syncytiotrophoblasts present at term. In addition, CRH is released when cells from these tissues are cultured in vitro (Jones et al 1989; Jones and Challis 1989 Jones and Challis 1990a).

The object of this part of the study was to examine the ontogeny of CRH in the placenta and fetal membranes during pregnancy. This was achieved by measuring the CRH content of placental and fetal membrane extracts prepared from tissues collected at all gestational ages. Specific cellular localisation within the same tissues was accomplished by immunocytochemistry. An attempt was made to determine the exact location of the expressed CRH gene using in situ hybridisation.

5.2. Materials and methods

5.2.1. Tissue collection

Tissues were collected as described in Chapter 2. Briefly, all gestations were calculated from the date of the last menstrual period and confirmed by ultrasound and/or clinical assessment. Decidual and trophoblastic tissues were obtained from patients undergoing therapeutic termination of pregnancy in the first trimester for social reasons by curettage, with (n=5) and without (n=5) pretreatment with the synthetic prostaglandin analogue gemeprost (16,16, dimethyl transΔ^2 PGE_1 methylester) between 2 and 4 hours prior to the procedure and also from patients undergoing therapeutic termination in the first trimester(n=5) and second trimester induced by mifepristone and prostaglandin (600mg of mifepristone orally 48hours prior to receiving a 1mg gemeprost vaginal pessary) (n=5). Placenta and fetal membranes were also collected from spontaneous vaginal deliveries at term (n=7). There was no clinical evidence of infection in any patient nor was there any histological evidence (no inflammatory infiltrate) in any of the tissues studied.

5.2.2. Tissue fixation

All tissues were divided into blocks of approximately 1.5 cm^3 and placed in 4% (w/v) buffered paraformaldehyde (pH 7.4) within 30 minutes of delivery. Tissues were fixed at room temperature for 22

hours, as this had been found to be the time required for good tissue preservation and optimal staining (see chapter 2), processed in a standard tissue processor and embedded in paraffin wax. Additional tissue blocks were frozen in liquid nitrogen for later extraction and measurement of CRH content.

5.2.3. Immunocytochemistry

Immunocytochemistry was carried out on 5μm sections of all tissues as described in detail in chapter 3. A polyclonal CRH antisera raised in rabbits against an ovine CRH-thyroglobulin-conjugate was used at an optimum dilution of 1:1000. The following negative controls were used; CRH primary antibody was substituted by antibody dilution buffer, non-immunised rabbit serum (1:1000 dilution) or CRH antibody (1:1000) dilution that had been preabsorbed with 5μM human CRH (Bachem UK Ltd Saffron Walden Essex WG 750) for 18 hours at 4°C. The positive control used in each case was human term placenta.

5.2.4. Peptide Extraction

This was performed on all frozen tissue specimens as described in chapter 2. Samples of placental extract were saved for protein estimation (prior to centrifugation). 3ml aliquots were lyophilized, and stored for radioimmunoassay and chromatography.

5.2.5. Radioimmunoassay

Each of the 3 ml lyophilized samples was reconstituted in 500μl of CRH assay buffer. The concentration of CRH in each sample was measured using a radioimmunoassay kit for human CRH (Penninsula Laboratories, Westside Industrial Estate St Helens Merseyside England). For details see chapter 2.

5.2.6. Protein Estimation

Total protein content of the placental extracts was estimated using a Biorad Protein assay (see chapter 2). CRH content of the extracts was expressed in terms of total protein.

5.2.7. Sephadex Chromatography

To provide further evidence that the peptide in the placental extract detected by radioimmunoassay was in fact CRH, placental extracts and standard CRH, both reconstituted in CRH radioimmunoassay buffer, were applied to a 50x1cm Sephadex G-50 Fine column (Pharmacia Fine Chemicals Uppsala Sweden) equilibrated with CRH assay buffer. Sixty 1ml fractions were collected at a constant flow rate of approximately 4ml/hour. Blue dextran was used to determine the void volume of the column. All the fractions from the column were collected and stored at -20°C for radioimmunoassay.

5.2.8. Data analysis

Tissue concentrations of CRH were compared by one-way analysis of variance and Duncan's multiple range test with significance set at $p<0.05$ (CLR ANOVA programme)

5.3. Results

5.3.1. Immunocytochemistry

Positive immunostaining for CRH was found in all placentas between five and 42 weeks gestation regardless of method of termination of pregnancy or delivery (Plate 5.1). There was a wide tissue-tissue variation in the intensity of the staining which was not related to the stage of gestation or method of termination or delivery, although the distribution of the staining within the tissues was always the same. In the control slides, no positive CRH immunoreactivity was found where

primary antibody was substituted for antibody dilution buffer, non-immunised rabbit serum or antibody that had been preabsorbed with human CRH (see Plate 2.2F).

Between five and 12 weeks gestation positive CRH staining was found in syncytiotrophoblast cells with faint staining in cytotrophoblast cells. Positive staining was also seen in the cells of the decidual stroma. There was no difference in staining in tissue obtained after suction curettage, with or without prostaglandin pretreatment compared with tissue obtained after termination with mifepristone and prostaglandins.

From the second trimester onwards (13-42 weeks gestation) positive CRH staining was seen in syncytiotrophoblast but not cytotrophoblast cells. In the amnion the staining was particularly pronounced in the epithelial and subepithelial layers. The chorion stained in the trophoblast and reticular layers. Cells of the decidual stroma also stained positively throughout gestation. (see plate 5.1)

5.3.2. Radioimmunoassay

Radioimmunoassay of the placental extracts revealed a significant ($p<0.01$) increase in CRH content with advancing gestation (Fig. 5.1). Average CRH concentrations were 62.69 ± 6.62 pg/mg protein in first trimester placenta (n=15); 237.5 ± 38.31pg/mg protein in second trimester placenta (n=5) and 741.47 ± 70.47pg/mg protein in term placenta (n=17). There was no significant difference in placental CRH concentration with different modes of delivery (Fig. 6.2), or the administration of prostaglandin E_2 or the antiprogestin mifepristone (Fig. 6.1), and therefore all the results from each trimester were grouped together regardless of mode of delivery (Figure 5.1). There was a significantly ($p<0.01$) greater concentration of CRH in placenta than in amnion 75.2 ± 12.5 pg/mg protein (n=17), chorion 131.9 ± 24 pg/mg protein (n=17) or decidua 114.97 ± 25 pg/mg protein (n=17). (Fig. 5.2)

5.3.3. Chromatography

Standard CRH and CRH in placental extracts eluted with an identical retention time suggesting that extracted placental CRH had the same molecular weight as standard CRH (See Figure 5.3)

5.4. Discussion

This study clearly demonstrates that CRH immunoreactivity is present in the placenta and its localisation is constant throughout pregnancy. Positive CRH immunostaining was observed in the syncytiotrophoblast and decidua as early as five weeks gestation and in the amnion and chorion from 13 weeks gestation (the earliest available time these membranes could reliably be collected), and these results are consistent with the detection of CRH by radioimmunoassay in placental extracts at this stage of gestation. The localization of CRH in placenta so early in gestation is in contrast to a recent report in which CRH could not be detected until the second trimester (Riley et al, 1991). However, CRH is detectable in maternal plasma during the first trimester, albeit at low levels, and CRH mRNA has been demonstrated in the placenta by Northern analysis as early as seven weeks gestation (Frim et al, 1988). In addition, *in vitro* studies of cultured placental and amnion cells at 12 weeks gestation have shown a significant output of CRH, data which supports the finding of positive CRH immunostaining in the first trimester (Jones & Challis, 1990a). In previous studies positive immunostaining has been reported in cytotrophoblasts (Petraglia et al., 1987a; Saijonmaa et al., 1988). Saijonmaa et al (1988) used a high concentration of antibody with no preabsorbed control and found immunostaining in the first trimester but not at term. These workers attributed this to the relatively small numbers of cytotrophoblast cells at term but did not account for the very high levels of CRH found in the placenta at this time. Petraglia et al (1987a) found CRH immunopositive staining in cytotrophoblasts at term but again this finding does not

account for high levels of circulating CRH. These data demonstrating CRH immunostaining in the large numbers of syncytiotrophoblasts at term is consistent with the high maternal plasma and amniotic fluid levels and agrees with the immunohistochemical findings of Riley et al (1991). Furthermore, *in vitro* studies of cultured placental, amnion, chorion and decidual cells at term have demonstated significant CRH output at this time. The difference between these studies are difficult to explain but may relate to different methods of tissue collection and fixation, or to the antibodies used.

Immunostaining is not a reliable method for quantification as intensity of staining does not necessarily reflect peptide content. In addition there was much variability both within and between tissues of intensity of staining, although the distribution of the staining was always the same. In view of this, the CRH peptide content of the same groups of tissues was measured. The results of this was entirely in agreement with previous findings of peptide content (Sasaki et al 1987). It was hoped that in situ hybridisation, in conjunction with immunocytochemistry, would provide further evidence for the site of synthesis of CRH. Unfortunately, problems with the specificity of the oligonucleotide probes made for this purpose affected the reproducibility of the results (see Chapter 4). More reliable results may be achievable by using a riboprobe and would provide valuable information of both the site of synthesis, and quantification of placental CRH.

Figure 5.1

Change in the concentration of CRH measured by radioimmunoassay in extracts of placenta collected at various stages of pregnancy. Each histogram represents the mean ± SEM of 15, 5 and 17 measurements in first, second and third trimester tissues, respectively. Values with different superscripts are significantly different ($p<0.01$).

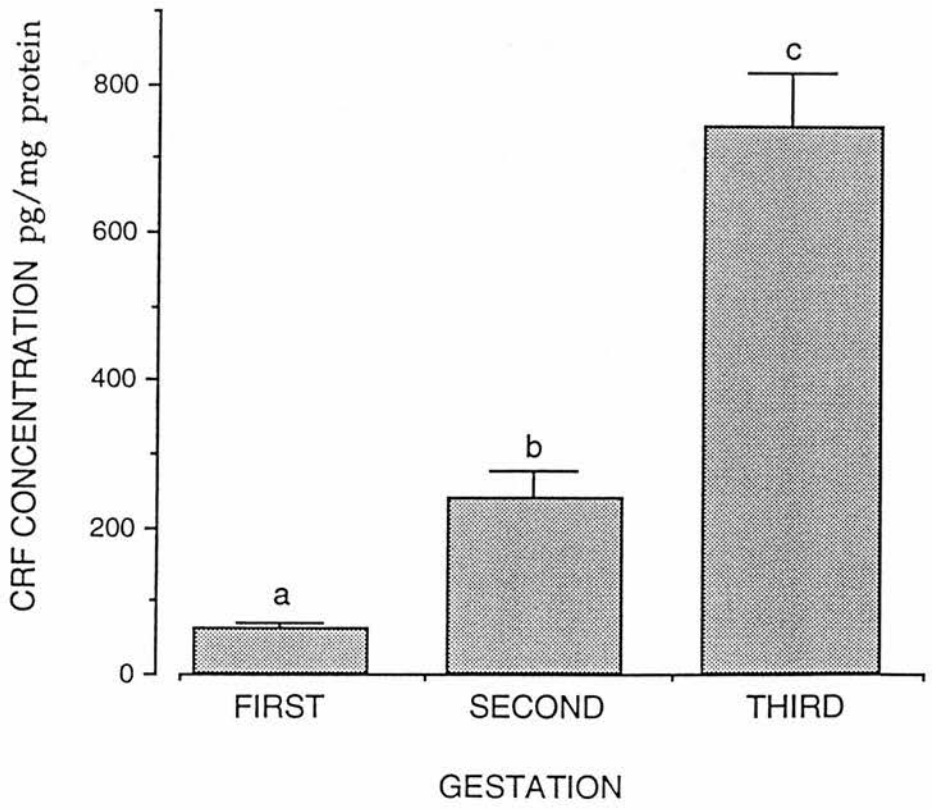


Figure 5.2.

Immunoreactive CRH concentration measured by radioimmunoassay in extracts of placenta, amnion, chorion and decidua collected after spontaneous delivery at term (n=7) Values are the mean ± SEM. Values with different superscripts are significantly different ($p<0.01$).

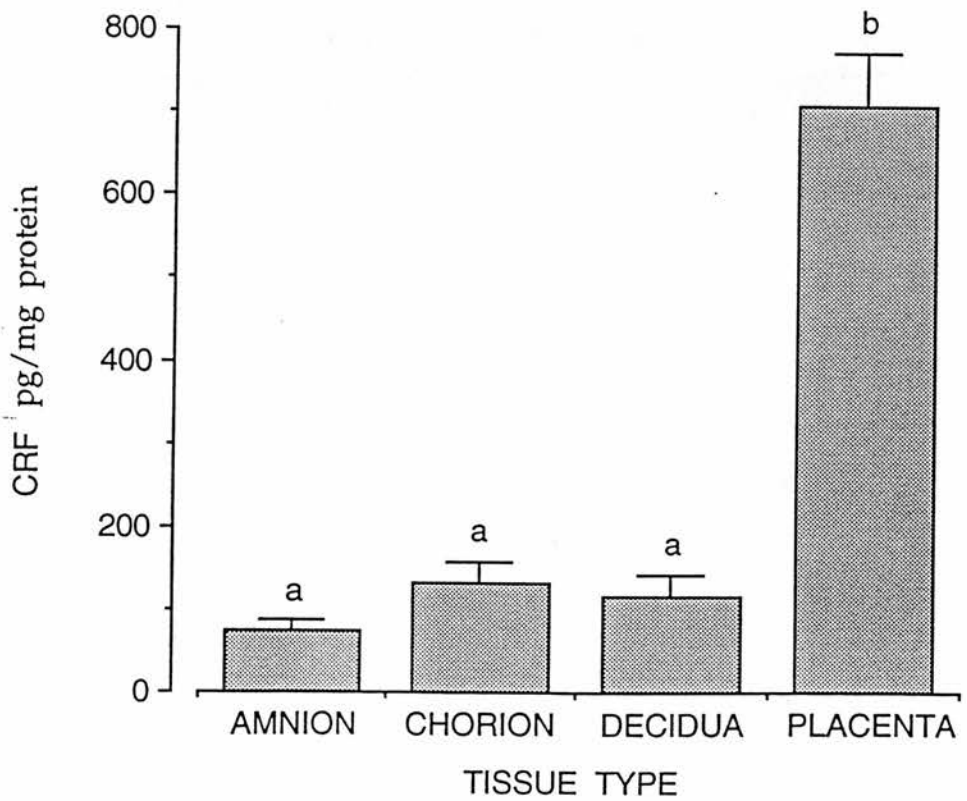
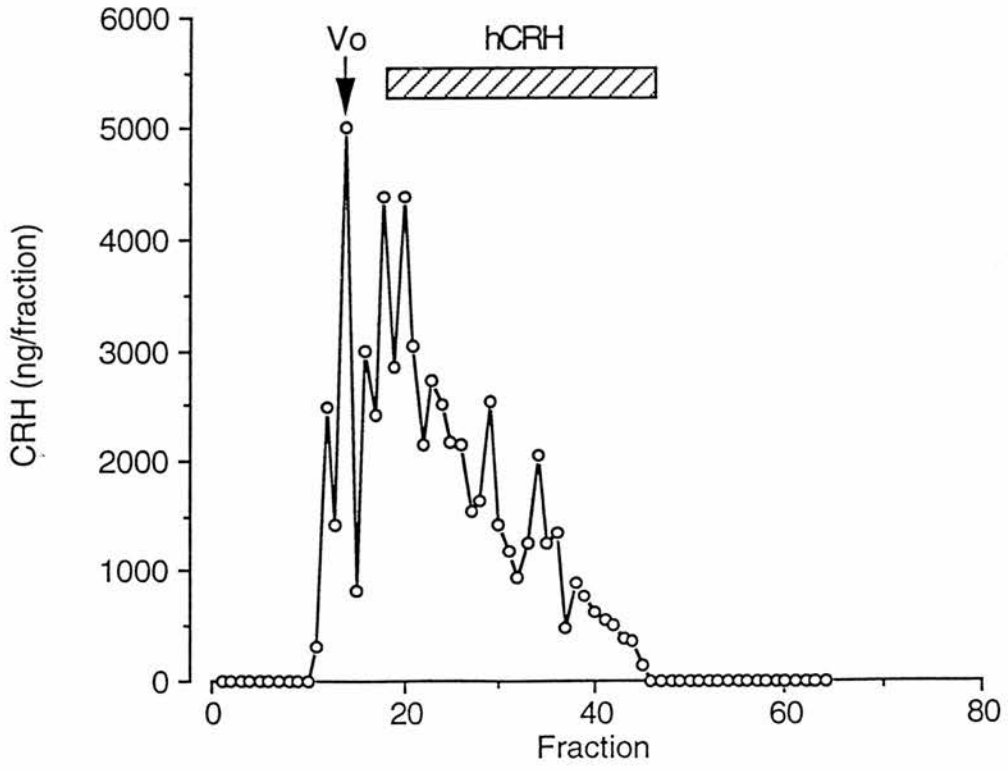


Figure 5.3. Immunoreactive CRH measured by radioimmunoassay in fractions of placental extract applied to a sephadex G-50 fine column. The bar indicates the fractions in which standard human CRH was eluted from the same column; placental CRH extract and standard human CRH eluted with an identical retention time. The void volume of the column (Vo) is indicated.



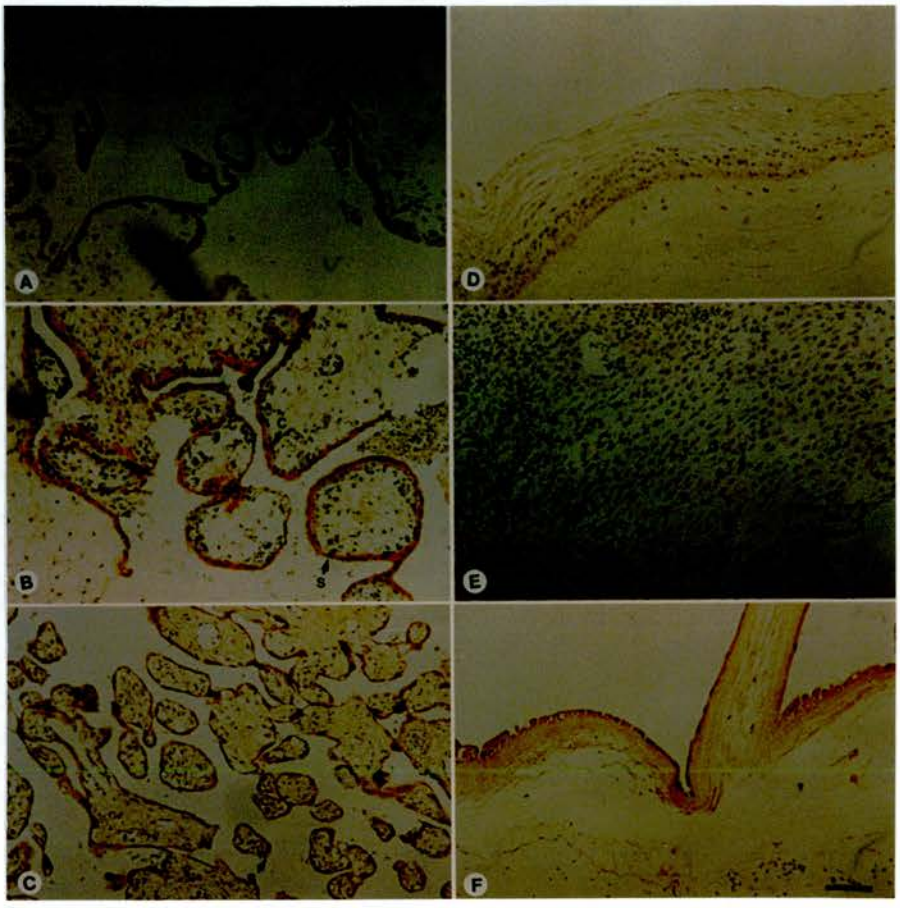

A
B
S
C
D
E
F

Plate 5.1. CRH immunolocalisation in placenta and fetal membranes throughout gestation

5.1.A. Placental villi obtained after suction termination of pregnancy at 8 weeks gestation. CRH immunostaining is seen in the syncytiotrophoblast.

5.1.B. Placental villi obtained after termination of pregnancy in the second trimester. CRH immunostaining is seen in the syncytiotrophoblast

5.1.C. Placental villi demonstrating positive CRH immunostaining in syncytiotrophoblast from a normal vaginal delivery at term.

5.1.D. Amnion from a normal term vaginal delivery demonstrating CRH immunostaining in the epithelial and subepithelial layers.

5.1.E. Chorion from a normal term vaginal delivery demonstrating CRH immunostaining in the reticular and cellular layers.

5.1.F. Decidua from a normal term vaginal delivery demonstrating CRH immunostaining in the decidual stromal cells.

The bar indicates 50μm
s=syncytiotrophoblast; c=cytotrophoblast

CHAPTER 6

ONTOGENY OF ACTH IN PLACENTA AND FETAL MEMBRANES

6.1. Introduction

Adrenocorticotrophic Hormone (ACTH) is a 39 amino acid peptide, which is synthesised as part of the proopiomelanocortin precursor molecule. Since the early 1950' s it has been suggested that the human placenta may influence the hypothalamopituitary adrenal axis during pregnancy by synthesising and secreting a wide range of peptides normally of hypothalamic or pituitary origin including ACTH (Jailer and Knowlton 1950; Silman and Bergman 1953; Assali and Hammermetz 1954). This placental ACTH could potentially stimulate the HPA axis in the mother or the fetus by stimulating adrenal steroidogenesis. This may be of particular significance in the fetus, since the fetal adrenal cortex supplies the placenta with androgens for oestrogen synthesis throughout pregnancy, and secretes cortisol which is essential for late fetal maturation.

Some studies have found the concentration of ACTH in maternal plasma to rise during pregnancy (Rees et al 1975; Carr et al 1981) and synthesis in vitro of both bioactive and immunoreactive ACTH by human placenta at term and in the first trimester has been reported. There is some disagreement as to whether placental ACTH levels rise or stay constant during pregnancy. Immunoreactive POMC- derived peptides have been detected in the human placenta and de novo synthesis of POMC and its derived peptides has been demonstated in cultures of human trophoblasts (Liotta et al 1977, Waddell et al 1993) Furthermore, POMC mRNA has been detected in human placenta at term (Cheng et al 1986). Immunoreactive ACTH has been localised immunocytochemically to the syncytiotrophoblast (Al-Timini & Fox 1986) and showed a similar intensity of staining in first and third trimester placentae, agreeing with those studies that indicated little or no variation in the levels of placental ACTH secretion throughout

pregnancy (Liotta et al, 1977, Odagiri et al 1979) In view of the potential role of ACTH in controlling the HPA axis throughout pregnancy and its possible role in the onset of parturition, this part of the study examined the localisation and content of IR-ACTH in placenta and fetal membranes throughout gestation and the expression of the POMC gene in the same tissues. Thus both the mRNA transcribed from the gene and the gene product translated from the mRNA could be identified. Since there is some disagreement over the quantification of ACTH during pregnancy, it was hoped that this information would provide strong evidence for both the site of synthesis and the control of expression of placental POMC peptides.

6.2. Materials and methods

For a detailed description of the development of these methods see chapters two, three and four.

6.2.1. Tissue collection and fixation

Tissues were collected and fixed as described in chapter 5.

6.2.2. Immunocytochemistry

Immunocytochemistry was carried out on 5μm sections of all tissues as described in detail in chapter 3. A polyclonal CLIP antisera raised in rabbits against a human CLIP-thyroglobulin-conjugate was used at an optimum dilution of 1:1000. This antibody shows 100% cross-reactivity with ACTH (1-39). The following negative controls were used; CLIP primary antibody was substituted by antibody dilution buffer, non-immunised rabbit serum (1:1000 dilution) or CLIP antibody (1:1000) dilution that had been preabsorbed with 5μM human ACTH (Cambridge Research Biochemicals lot Z1975) for 18 hours at 4°C. The positive control used in each case was adult sheep pituitary.

6.2.3. Peptide extraction and radioimmunoassay

Peptide was extracted from frozen blocks of tissue as described in chapter 4. An aliquot of each sample (prior to centrifugation) was stored at 4°C for later protein estimation. A further two 3ml aliquots were lyophilised and stored for radioimmunoassay and chromatography. Each of the 3 ml lyophilised samples were brought up to 500µl in ACTH assay buffer. The concentration of ACTH in each sample was measured using a direct radioimmuoassay as described in chapter 2. Total protein content of the placental extracts was estimated using a Biorad protein assay (see chapter 2). ACTH content of the extracts was expressed in terms of total protein.

6.2.4. Sephadex chromatography

Placental extracts and standard human ACTH were applied to a 50x1 cm glass chromatographic column containing sephadex G-50 Fine (Pharmacia Fine Chemicals Uppsala Sweden) equilibrated with ACTH assay buffer. 2mls of 10% bovine serum albumin solution were added to reduce non-specific binding. Sixty 1ml fractions were collected at a flow rate of approximately 4 ml/hr. Blue dextran was used to determine the void volume of the column. All the fractions from the column were collected and stored at -20°C for radioimmunoassay

6.2.5. Data analysis

Tissue concentrations of ACTH were compared by one-way analysis of variance and Duncan's multiple range test with significance set at $p<0.05$ (CLR ANOVA programme)

6.2.6. In situ hybridisation

In situ hybridisation was performed on 5µm paraffin embedded sections using the protocol developed as described in chapter 3. Sections of adult sheep pituitary were used as a positive control and a sense probe

complementary to antisense used as a negative control. All slides were counterstained with haematoxylin and eosin, dehydrated and mounted in Eukitt. In situ hybridisation signals from each study group were quantified using a computer-assisted image analysis system. Six areas of trophoblast, and six areas of tissue background from each slide were randomly chosen and the number of grains/cm^2 counted. The system consisted of a Charge-Coupled Device camera, microscope, and camera monitor linked to a IBM PC/AT microcomputer. The software program (cue-2) for densitometry was provided by Olympus Optical Company. An optimum threshold and filter combination were set to select only the silver grains. The grain count from the six areas was calculated as a mean and the mean background subtracted. The mean grain count between sense and antisense were compared, and the sense counts always approximated to antisense background values.

6.3. Results

6.3.1. Immunostaining

Positive immunostaining for ACTH was found in all placentas between 5 and 42 weeks gestation regardless of the method of termination of pregnancy or delivery (see plate 6.1). In control slides, no positive ACTH immunostaining was found where the primary antibody had been substituted by antibody dilution bufer or non-immunised rabbit serum or CLIP antibody at 1:1000 dilution which had been preabsorbed overnight with 5μM ACTH (see plate 2.3.).

In the first trimester (from 5-12 weeks gestation) positive staining was seen in the cytotrophoblast layer of cells, with none in the syncytiotrophoblast. Positive staining was also seen in the cells of the decidual stroma. The staining was faint when compared with tissue of more advanced gestation. From the second trimester onwards, positive ACTH immunostaining was seen in the syncytiotrophoblast. The staining increased in intensity with advancing gestation. In the amnion the staining was particularly pronounced in the epithelial layers with also some staining in the subepithelial layer. In the chorion

staining was seen mainly in the reticular layer. Cells of the decidual stroma stained positively throughout gestation. (see plate 6.1)

6.3.2. Radioimmunoassay

Radioimmunoassay of the placental extracts revealed a significant ($p<0.01$) increase in ACTH content in the third trimester, agreeing with the observation that the intensity of the staining appeared to increase (see figure 6.1). Mean ACTH in term placenta (n=17) was 29.39±4.59pg/mg protein, in second trimester placenta (n=5) was 10.22±1.81pg/mg protein and in first trimester placenta (n=15) was 7.35±0.83pg/mg protein. There was no significant difference in placental ACTH content with different modes of delivery (Fig 8.2), or the administration of prostaglandin E_2 or the antiprogestin mifepristone (Fig 8.1), so all term and all first trimester data were considered together. There was a significantly ($p<0.01$) greater concentration of ACTH in placenta than in amnion 13.5±1.9 pg/mg protein (n=17), chorion 16.6±2.3 pg/mg protein (n=17) or decidua 11.39±1.34 pg/mg protein (n=17) (Fig. 6.2).

6.3.3. Chromatography

Sephadex G50 chromatography revealed that ACTH from placental extracts and human ACTH (1-39) standard eluted with an identical retention time. indicating that they have the same molecular weight (see Fig 6.4).

6.3.4. In situ hybridisation

In common with the immunocytochemical results, in situ hybridisation localised POMC mRNA to the trophoblast cells at all stages of pregnancy. It was less easy to distinguish between cytotrophoblast and syncytiotrophoblast cells in the first trimester and it appeared that grains were present in both types of cell. In the second and third trimester, mRNA was present in the syncytiotrophoblasts (see plate 6.2). Image

analysis of grain density revealed that the intensity increased significantly between the first trimester(0.76±0.07grains/cm^2x10^5) and the third(2.97±0.2grains/cm^2x10^5) (Fig 6.3) suggesting an increase in POMC gene expression over this time. In situ hybridisation also localised POMC mRNA particularly to the epithelial layers of the amnion with also some expression in the subepithelial layer. In the chorion POMC gene expression was seen mainly in the reticular layer (see plate 6.3)

6.4. Discussion

These results demonstrate positive ACTH immunostaining and POMC gene expression in the placenta throughout pregnancy. ACTH immunostaining is seen in the cytotrophoblasts and decidual stroma as early as five weeks gestation and in the fetal membranes from the second trimester onwards (the earliest time these tissues could be reliably collected). Extraction of peptide from the placenta and radioimmunassay for ACTH revealed detectable levels at all stages of pregnancy. The concentrations of ACTH measured in placental extracts were similar to those reported previously by Liotta et al (1977), Rees et al (1975) and Odagiri et al (1989).

The intensity of the immunostaining increased throughout gestation but its localisation changed from the cytotrophoblast in the first trimester to the syncytiotrophoblast thereafter. The intensity of the in situ hybridisation signal increased in parallel but appeared to be present in both cytotrophoblast and syncytiotrophoblast in the first trimester. The placental ACTH content was also significantly greater at term than in the first trimester. Previous ACTH immunostaining (Al-Timini and Fox 1986) localised ACTH to the syncytiotrophoblast throughout gestation and found no change in the intensity or the localisation of the immunostaining, although they noted that it always occured at a low level. They used an anti-ACTH antiserum at very low dilution of 1:100 compared with the 1:1000 dilution of anti-CLIP antiserum used in this study. The use of antiserum to a smaller antigen (CLIP versus ACTH) may have affected tissue penetration of the antigen or altered the antigen-antibody binding, although the specificity of this binding was

unaltered as demonstrated by the loss of positive staining when antiserum preabsorbed with human ACTH was used. Use of a higher dilution of antibody reduces background staining, an advantage of using 1:1000 dilution of anti-CLIP antibody. Studies of placental ACTH content which do not show any variation in placental ACTH content throughout pregnancy (Liotta et al 1977; Rees et al 1975; Odagiri et al 1979) support the findings of Al-Timini and Fox. However, the findings of this study, demonstrating an increase in placental ACTH content with gestation, and the increase in POMC gene expression, strongly support the staining results seen using anti-CLIP antiserum. The demonstration of positive immunostaining of cytotrophoblast cells in the first trimester, and syncytiotrophoblasts thereafter is consistent with the changing numbers of these cells during pregnancy. Cytotrophoblasts are more numerous in the first trimester but in later pregnancy syncytiotrophoblasts (the mitotic end stage of cytotrophoblasts) predominate. This is consistent with the rising concentration of ACTH detected in the placenta in later pregnancy in this study. In addition, a progressive rise in plasma ACTH has been demonstrated during pregnancy (Rees et al 1975; Carr et al 1981) although most values lay within the normal range. If the placenta is the source of at least a proportion of this maternal plasma ACTH, then the change in plasma concentration may reflect increasing placental synthesis of ACTH.

The POMC gene appears to be expressed in both cytotrophoblasts and syncytiotrophoblasts in the first trimester, although the peptide is only detectable by immunocytochemistry in the cytotrophoblasts at this time. Perhaps the gene is transcribed from both types of cell but translation only occurs in the cytotrophoblasts. The syncytiotrophoblasts appear to be derived from cytotrophoblasts. At this early stage, these recently transformed cells may not have the full synthetic capability for the production of POMC derived peptides. This may be linked to their function. If their role is in the initiation of parturition, then high output would be unnecessary early in pregnancy.

However, the biological significance of this placental ACTH remains uncertain. Potentially placental ACTH could stimulate adrenal

steroidogenesis in the mother or the fetus and thus influence their respective hypothalamo-pituitary-adrenal axes. In the rat and rhesus monkey (Knobil and Briggs 1955; Hodgen et al 1975) pregnancy reduces the extent of adrenal atrophy that ocurs in response to maternal hypophysectomy. After removal of the fetus (but not the placenta) cortisol levels remained relatively high , but then fell dramatically after placental delivery (Hodgen et al 1975) suggesting direct placental support for the maternal adrenal cortex. Circumstantial evidence supports a role for the placenta in maintaining fetal or maternal adrenal function during human pregnancy. Growth of the fetal adrenal cortex appears to be normal in anencephalic fetuses up to mid-gestation (Benirschke 1956) and since ACTH does not cross the placenta (Dupouy et al 1980) this suggests that some factor other than fetal pituitary ACTH is trophic to the fetal adrenal. It is likely that at least some of this support could come from the placenta. The demonstration of ACTH immunoreactivity from early to late gestation suggests that the placenta may exert this effect throughout the whole of pregnancy. Its role in relation to labour is more speculative. It has been hypothesised that activation of the fetal hypothalamo-pituitary-adrenal axis may be linked with the onset of parturition, with placental CRH passing into the fetal circulation causing the release of ACTH from the fetal pituitary and an increase in plasma cortisol. This ACTH also increases DHEAS resulting in increased oestrogen output by the placenta. The cortisol may also stimulate placental CRH production in a positive feedback loop. The effects of placental CRH on prostaglandin production and myometrial contractility, as discussed in Chapter 1, could lead to ripening of the cervix and myometrial contractility (Challis and Hooper 1989) and ultimately birth. Placental ACTH may too pass into the fetal circulation and affect cortisol production by the fetal adrenal. The finding of a rise in placental ACTH content in the third trimester prior to labour supports the hypothesis that placental ACTH may also be involved in the activation of the fetal hypothalamo-pituitary-adrenal axis. In view of this, further studies were undertaken to investigate the modulation of placental ACTH by labour, the administration of prostaglandins and the administration of the antiprogestin mifepristone (see Chapter 8)

Figure 6.1.

Change in the concentration of ACTH measured by radioimmunoassay in extracts of placenta collected at various stages of pregnancy. Each histogram represents the mean ± SEM of 15, 5 and 17 measurements in first, second and third trimester tissues, respectively. Values with different superscripts are significantly different ($p<0.01$).

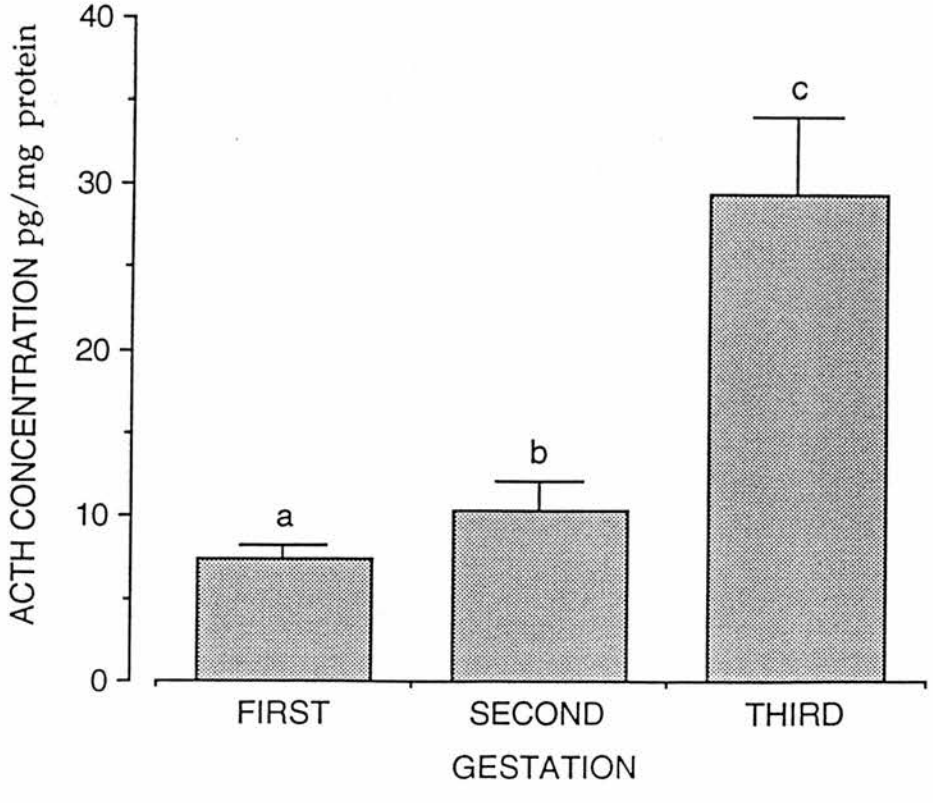


Figure 6.2.
Immunoreactive ACTH concentration measured by radioimmunoassay in extracts of placenta, amnion, chorion and decidua collected after spontaneous delivery at term (n=7) Values are the mean ± SEM. Values with different superscripts are significantly different ($p<0.01$).

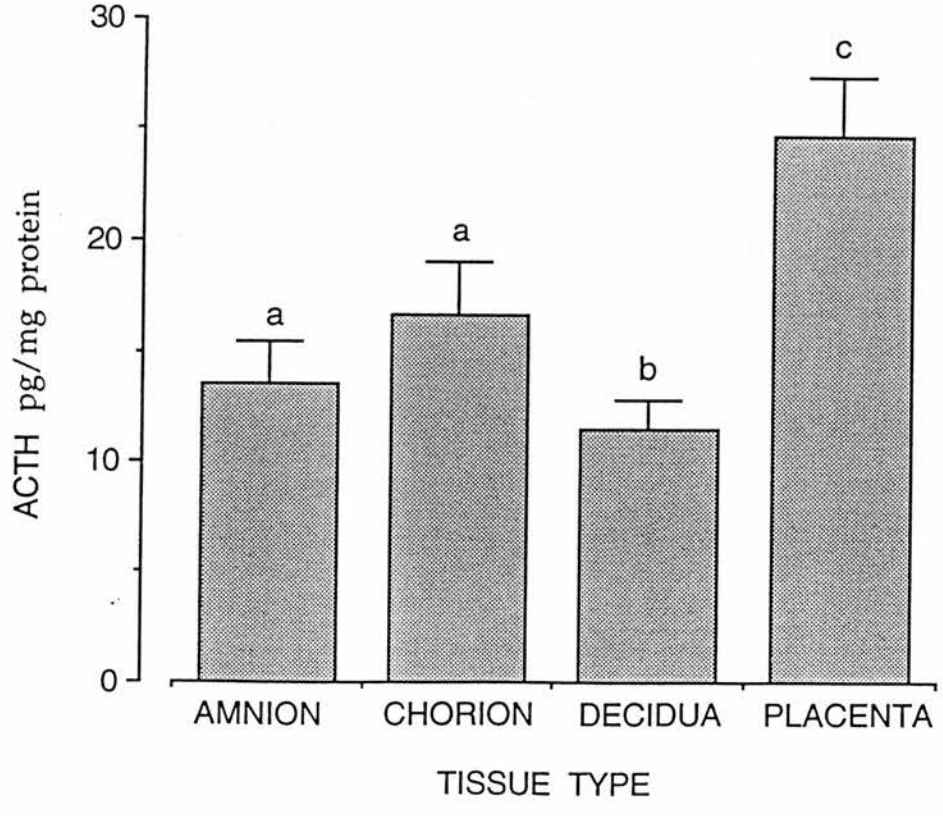


Figure 6.3.

Change in POMC gene expression measured by quantification of in situ hybridisation performed on sections of placentas collected at various stages of pregnancy. Each histogram represents the means of six random counts of the tissue grain density (background non-specific counts have been subtracted) of each of three tissues from the first and third trimesters respectively. Values with different superscripts are significantly different ($p<0.01$).

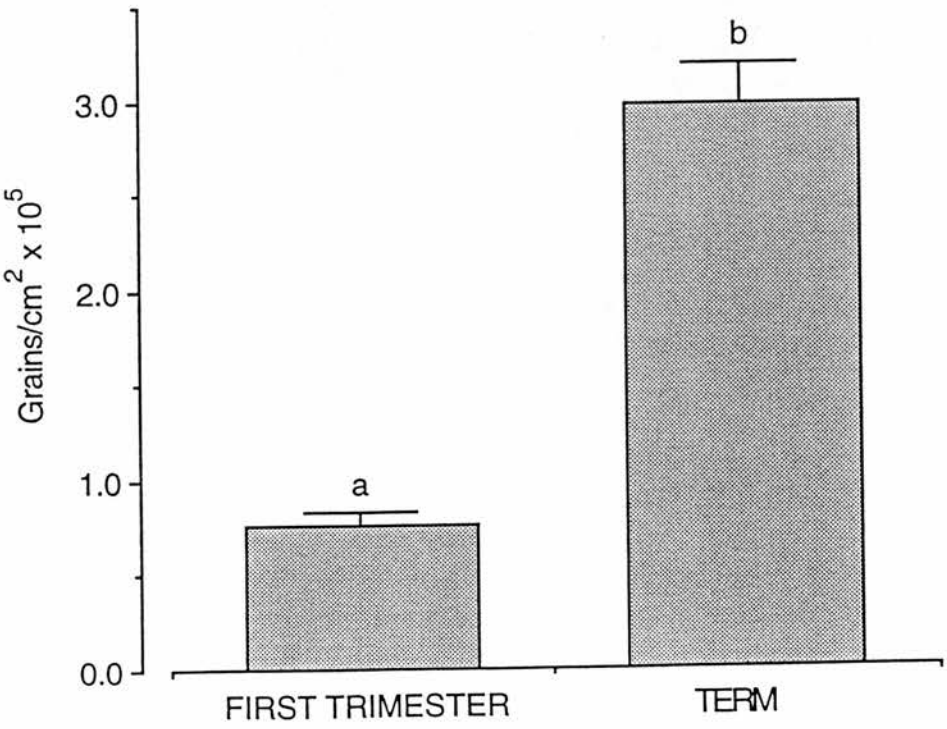
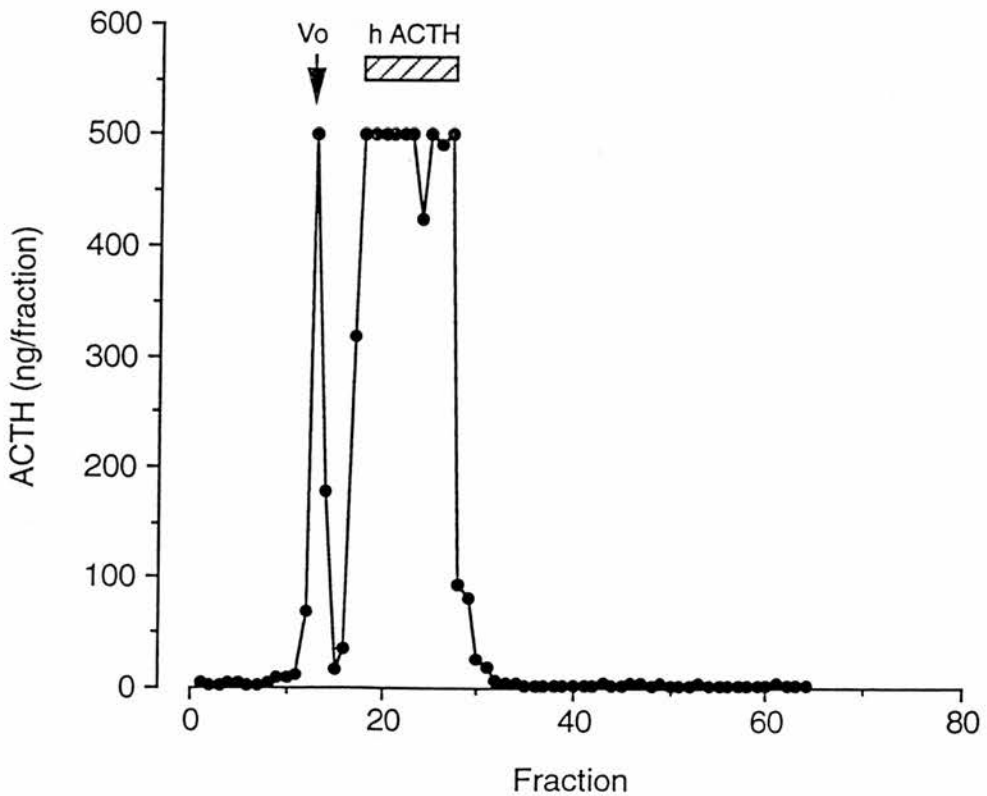


Figure 6.4. Immunoreactive ACTH measured by radioimmunoassay in fractions of placental extract applied to a sephadex G-50 fine column. The bar indicates the fractions in which standard human ACTH was eluted from the same column; placental CRH extract and standard human ACTH eluted with an identical retention time. The void volume of the column (Vo) is indicated.



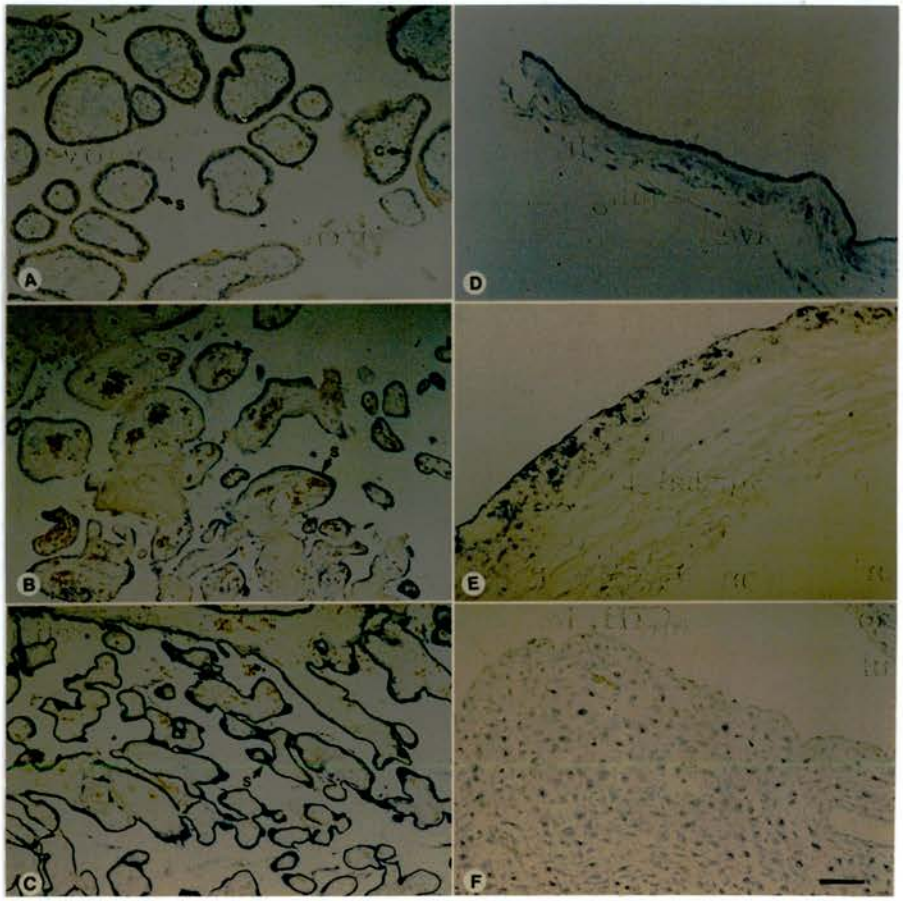

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Plate 6.1. ACTH immunolocalisation in placenta and fetal membranes throughout gestation

6.1.A. Placental villi obtained after suction termination of pregnancy at 8 weeks gestation. ACTH immunostaining is seen in the cytotrophoblast.

6.1.B. Placental villi obtained after termination of pregnancy in the second trimester. ACTH immunostaining is seen in the syncytiotrophoblast

6.1.C. Placental villi demonstrating positive ACTH immunostaining in syncytiotrophoblast from a normal vaginal delivery at term. Note the increased intensity of the staining.

6.1.D. Amnion from a normal term vaginal delivery demonstrating ACTH immunostaining in the epithelial and subepithelial layers.

6.1.E. Chorion from a normal term vaginal delivery demonstrating ACTH immunostaining in the reticular and cellular layers.

6.1.F. Decidua from a normal term vaginal delivery demonstrating ACTH immunostaining in the decidual stromal cells.

The bar indicates 50μm

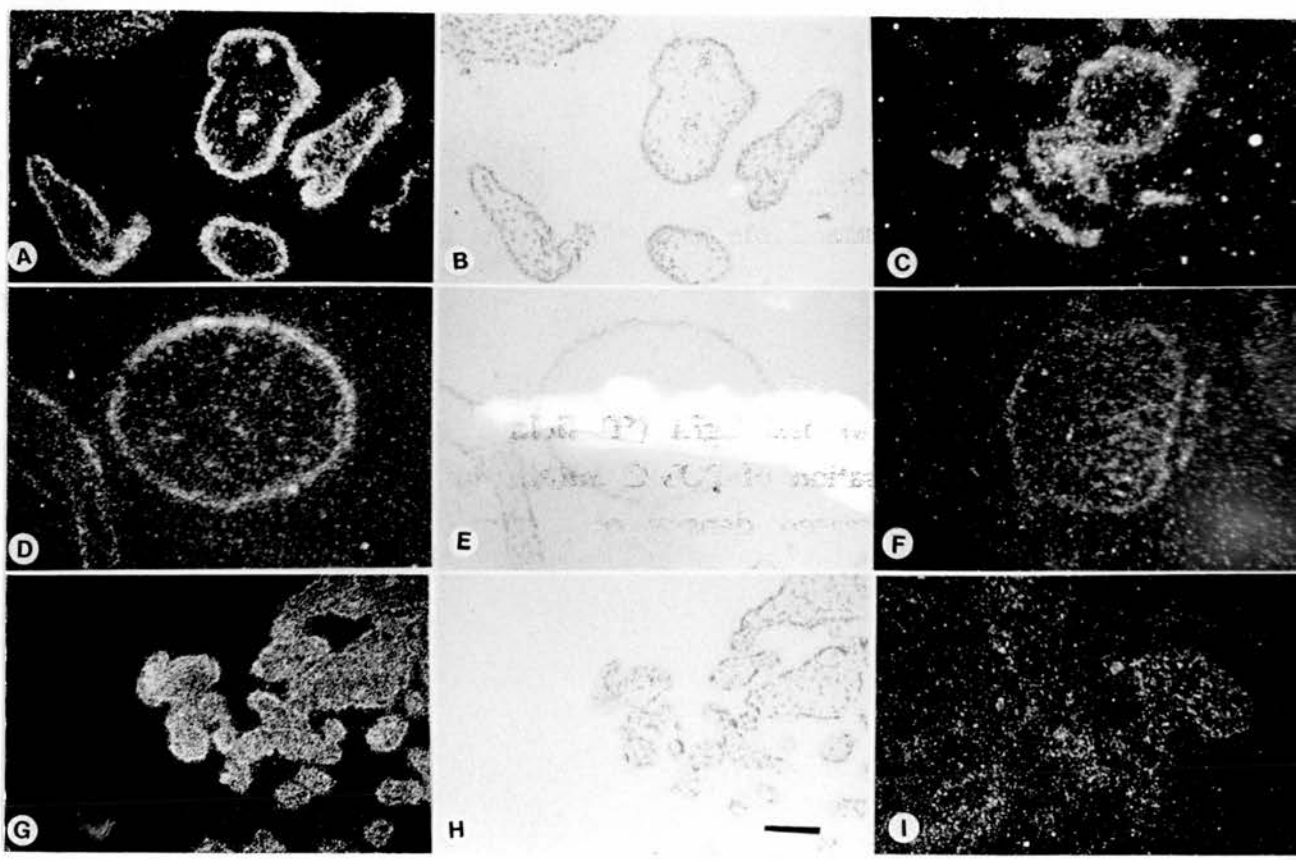
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Plate 6.2. In situ hybridisation of POMCmRNA within placenta throughout gestation

6.2.A & B Dark (A) and light (B) field micrographs of in situ hybridisation of POMC mRNA in first trimester placenta. Note the grains over the trophoblast cells.

6.2.C Negative control. Tissue shown in 6.2.A but using sense probe. Note low level of non specific hybridisation.

6.2.D & E Dark (D) and light (E) field micrographs of in situ hybridisation of POMC mRNA in second trimester placenta. Note the grains over the trophoblast cells.

6.2.F Negative control. Tissue shown in 6.2.D but using sense probe. Note low level of non specific hybridisation.

6.2.G & H Dark (G) and light (H) field micrographs of in situ hybridisation of POMC mRNA in term placenta. Note the increased density of grains over the trophoblast cells.

6.2.I Negative control. Tissue shown in 6.2.G but using sense probe. Note low level of non specific hybridisation.

The bar indicates 50μm

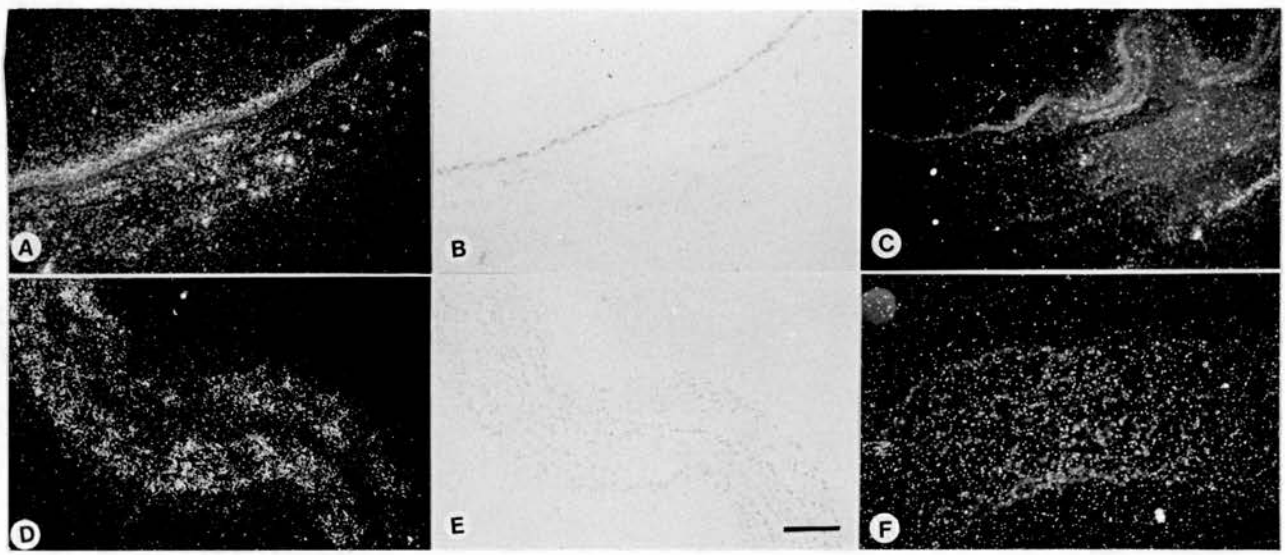
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Plate 6.3. In situ hybridisation of POMC in the fetal membranes

6.3.A &B Dark (A) and light (B) field micrographs of in situ hybridisation of POMC mRNA in amnion collected at term.

6.3.C Negative control. Tissue shown in 6.3.A but using sense probe. Note low level of non specific hybridisation.

6.3.D & E Dark (D) and light (E) field micrographs of in situ hybridisation of POMC mRNA in chorion collected at term.

6.3.F Negative control. Tissue shown in 6.3.D but using sense probe. Note low level of non specific hybridisation.

The bar indicates 50μm

CHAPTER 7

MODULATION OF PLACENTAL CRH PRODUCTION

7.1. Introduction

As discussed in Chapter 5, CRH is present in the placenta and fetal membranes from the first trimester onwards. The results of placental and membrane CRH content seen in chapter 5 are consistent with earlier findings that placental peptide content (Sasaki et al 1987), CRHmRNA levels (Frim et al 1988) and maternal plasma concentrations (Sasaki et al 1984; Goland et al 1986; Campbell et al 1987; Laatikainen et al 1987b; Okamoto et al 1989) all rise through gestation and fall rapidly after delivery. These observations suggest that the placenta both synthesises and releases increasing amounts of CRH throughout gestation. In addition maternal plasma CRH levels rise prior to delivery, and in women who subsequently go into preterm labour, abnormally high levels of CRH are seen several weeks before delivery (Campbell et al 1987), suggesting its possible invovement in the initiation of parturition. Furthermore, plasma levels of CRH are elevated during labour peaking at delivery, suggesting a possible role in the progress of labour (Schulte et al 1987).

In sheep, parturition is preceeded by a rise in the levels of myometrial stimulants oestrogens and prostaglandins combined with a fall in the level of the myometrial relaxant progesterone (Csapo et al 1961). There is no such change in plasma progesterone concentations in women at the time of labour. Paradoxically, antiprogestins are effective abortifacients in both animals and humans and have been shown to increase myometrial sensitivity to prostaglandins (Rodger & Baird, 1987; Rodger et al., 1988; Garfield & Baulieu, 1987). *In vitro* cell culture experiments using human amnion and placental tissue, have shown that progesterone decreases CRH output from amnion cells in early pregnancy (Jones & Challis, 1990a) and from both tissue types at term

(Jones et al., 1989). It has been suggested that progesterone has an inhibitory effect on placental CRH production during pregnancy until term when a local change, possibly at the receptor level, may remove this inhibition (Challis & Olson, 1988). Furthermore, CRH stimulates prostaglandin production (Jones & Challis, 1990b). Thus, rising CRH concentrations as a result of local progesterone withdrawal would cause an increase in prostaglandin production and lead to cervical ripening and myometrial contractility. In addition, CRH may act directly on the myometrium which contains specific binding sites for CRH. These increase in affinity for CRH during pregnancy which may influence parturition by sensitising the myometrium to uterine agonists (Hillhouse et al., 1993).

In view of the potential role of CRH in the control of parturition, the localisation of CRH in the placenta and fetal membranes from the first trimester onwards, and the CRH content of the same tissues, was examined. By comparing tissues collected after elective caesarean section, spontaneous and induced labours, the effect of labour and of the administration of exogenous prostaglandins, on both CRH immunolocalisation and on tissue content could be compared. In the first trimester, the antiprogestin mifepristone was used to determine whether progesterone has a regulatory effect on CRH production and the effect of exogenous prostaglandins was also studied.

7.2. Materials and Methods.

7.2.1. Tissue collection

All gestations were calculated from the date of the last menstrual period and were confirmed by ultrasound assessment and/or clinical assessment. Decidual and trophoblast tissues were obtained from patients undergoing therapeutic termination of pregnancy in the first trimester by curettage with (n=5) and without (n=5) pretreatment with the synthetic prostaglandin analogue gemeprost (16,16, dimethyltrans Δ^2 PGE_1 methylester) between 2 and 4 hours prior to the procedure and also

from pateints undergoing therapeutic termination in the first (n=5) and second (n=5) trimesters using mifepristone 600mg orally 48 hours prior to receiving a 1mg gemeprost vaginal pessary. All patients aborted within 6 hours. Placenta and fetal membranes were also collected from spontaneous vaginal deliveries at term (36-42 weeks gestation) (n=7), by elective caesarean section (n=5), and by induction of labour using prostaglandin E_2 (n=5) (all patients studied recieved 2mg of PGE_2 and all delivered within 24 hours). There was no clinical or histological evidence of infection in any patient.

The materials and methods used were identical to those used in chapter 5. Briefly, tissues were collected, fixed in 4% paraformaldehyde and examined immunocytochemically. Peptide content in placental extracts was measured by radioimmunoassay and sephadex chromatography was used to examine the molecular size forms of placental CRH.

7.3. Results

Positive immunostaining for CRH was found in all placentas between five and 42 weeks gestation regardless of method of termination of pregnancy or delivery (see plate 5.1). There was some tissue-tissue variation in the intensity of the staining which was not related to the stage of gestation or method of termination or of delivery, although the distribution of the staining within the tissues was always the same. In the control slides, no positive CRH immunoreactivity was found where primary antibody was substituted for antibody dilution buffer, non-immunised rabbit serum or antibody that had been preabsorbed with human CRH. (see plate 3.2)

Between five and 12 weeks gestation positive CRH staining was found in syncytiotrophoblast cells with faint staining in cytotrophoblast cells. Positive staining was also seen in the cells of the decidual stroma. There was no difference in staining in tissue obtained after suction curettage, with or without prostaglandin pretreatment compared with

tissue obtained after termination with mifepristone and prostaglandins (see plate 7.1).

From the second trimester onwards (13-42 weeks gestation) positive CRH staining was seen in syncytiotrophoblast but not cytotrophoblast cells. In the amnion the staining was particularly pronounced in the epithelial and subepithelial layers. The chorion stained in the trophoblast and reticular layers. Cells of the decidual stroma also stained positively throughout gestation. There was no difference in staining in tissue obtained from elective caesarean section (not in labour) at term compared with that from spontaneous vaginal delivery at term. There was also no difference in staining in tissue obtained after labour induced with prostaglandins compared with that from spontaneous labour (see plates 7.2 and 7.3).

Radioimmunoassay of the placental extracts revealed a significant ($p<0.01$) increase in CRH content with advancing gestation (see chapter 5). There was no significant difference in placental CRH concentration with the administration of prostaglandin E_2 or the antiprogestin mifepristone (Fig 7.1) in the first trimester, or with different modes of delivery at term (Fig 7.2). Average CRH concentrations in first trimester placental extract collected after curettage with (61.52±11.99pg/mg protein) and without (61.36±8.51pg/mg protein) pretreatment with prostaglandin, and (65.20±15.53pg/mg protein) in first trimester placental extracts collected after termination with mifepristone. Placental CRH concentrations in extracts collected after spontaneous, induced and caesarean deliveries were 794.14±131.61pg/mg protein, 713.4±109.18pg/mg protein, and 694.4±130.32pg/mg protein respectively. There was also no significant difference in the content of CRH of the fetal membranes and decidua with different modes of delivery at term (Fig 7.3). Thus, the content of CRH in the fetal membranes was also not affected by labour, prostaglandins or mifepristone.

Standard CRH and CRH in placental extracts eluted with an identical retention time suggesting that extracted placental CRH had the same molecular weight as standard CRH (See Figure 5.3)

7.4. Discussion

This study clearly demonstrates that CRH immunoreactivity is present in the placenta and its localisation is constant throughout pregnancy. There was no difference in the intensity of the staining in tissues obtained after elective caesarean section compared to those collected after spontaneous or induced labour at term. This was consistent with the finding that the CRH content of these tissues was not affected by labour. This is in contrast to previous investigations of the effects of labour on placental CRH content (Saijonmaa et al 1988). This study found CRH immunostaining in first trimester placenta, but were unable to immunolocalise CRH in term placentae. However, using the same antibody in a radioimmunoassay, they found the mean concentration of CRH in placental tissue to be significantly larger after spontaneous vaginal delivery than after elective caesarean section.

The hypothesis that progesterone may have a regulatory effect on placental CRH production was tested using the antiprogestogen, mifepristone. There was no difference in intensity of staining in placentas from patients pretreated with mifepristone compared to those who were not, and in addition to this, there was no significant difference in the CRH content of these placentas compared with those obtained after suction termination. Forty-eight hours of exposure to mifepristone is likely to be a sufficient duration to produce an increase in peptide synthesis, however the anti-glucocorticoid side effects of mifepristone may counteract its anti-progestogenic effects since glucocorticoids have been shown to stimulate CRH release by the placenta and fetal membranes *in vitro* (Jones et al., 1989; Jones & Challis, 1990a). Another explanation for the lack of effect of mifepristone on CRH concentration may relate to the early stage of gestation when these studies were conducted. *In vitro* studies have demonstrated that progesterone inhibits CRH release by the placenta at term (Jones et al.,

1987) but has no effect in early pregnancy (Jones & Challis, 1990a). It may be that mifepristone given later in pregnancy would affect output of CRH whereas in the first trimester it does not. The logical next step would be to investigate the effect of mifepristone on placental CRH content at term when used as an agent for the induction of labour. Nonetheless, treatment with mifepristone results in a clinical effect producing abortion. It would appear from the data that this effect of mifepristone is not mediated via the induction of CRH synthesis as CRH appeared to be constitutively present in all tissues studied. This does not preclude more subtle changes in the synthesis or export of CRH which might be important in the physiological or pharmacological control of uterine contractility. In addition, the constitutive presence of CRH in syncytiotrophoblast suggests that it may play a "housekeeping" role in the placenta and membrane unit rather than simply being related to parturition.

Figure 7.1.

Immunoreactive CRH concentrations measured by radioimmunoassay in first trimester placental extracts collected after termination of pregnancy induced by mifepristone (RU486) (n=5), curettage with prostaglandin (STOP+C) (n=5), or curettage without prostaglandin (STOP-C) (n=5). Values are the mean ± SEM. There was no significant difference between the groups.

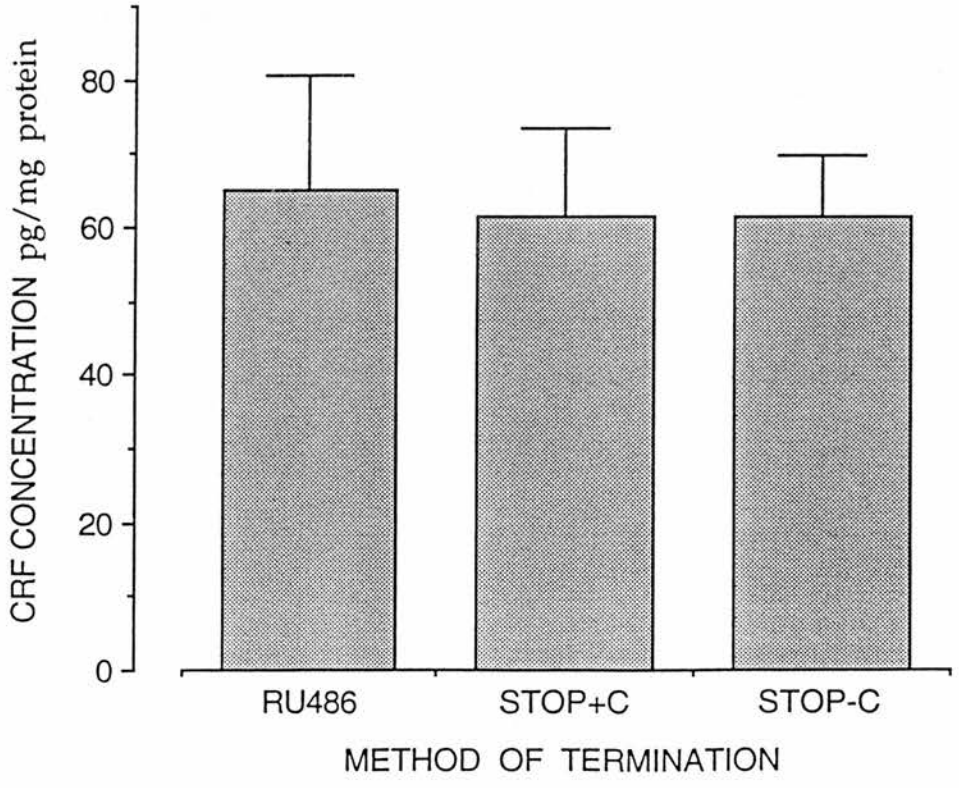


Figure 7.2.

Immunoreactive CRH concentration measured by radioimmunoassay in extracts of placenta collected after spontaneous (n=7), induced (n=5) or caesarean delivery (n=5) at term. Values are the mean ± SEM. There was no significant difference between the groups.

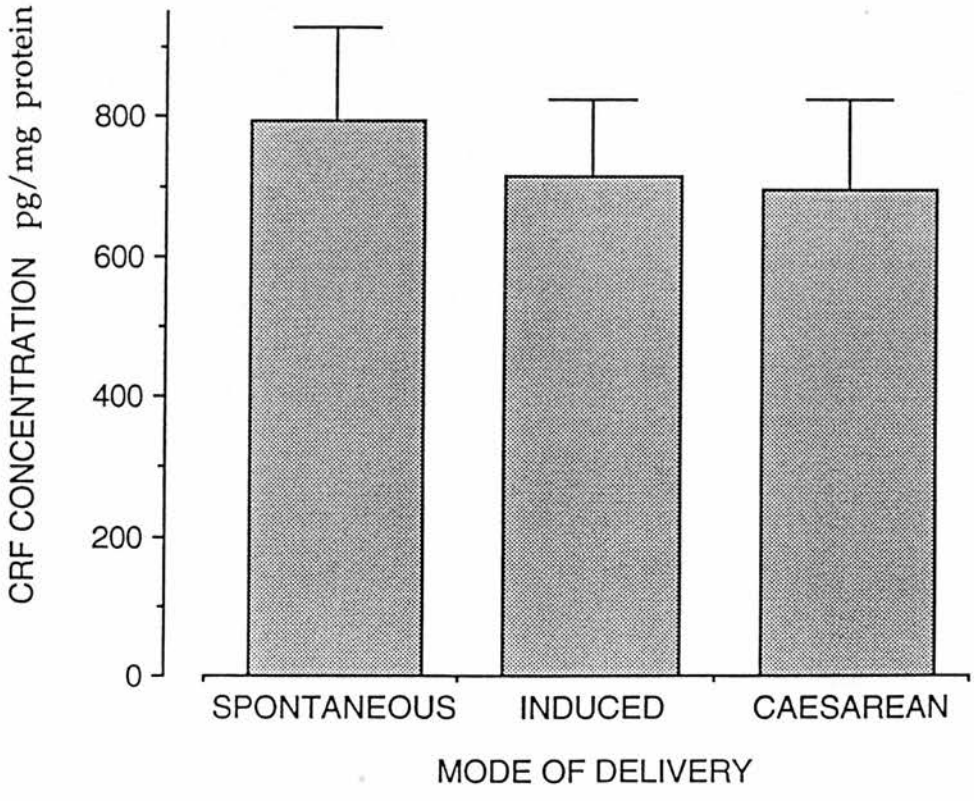
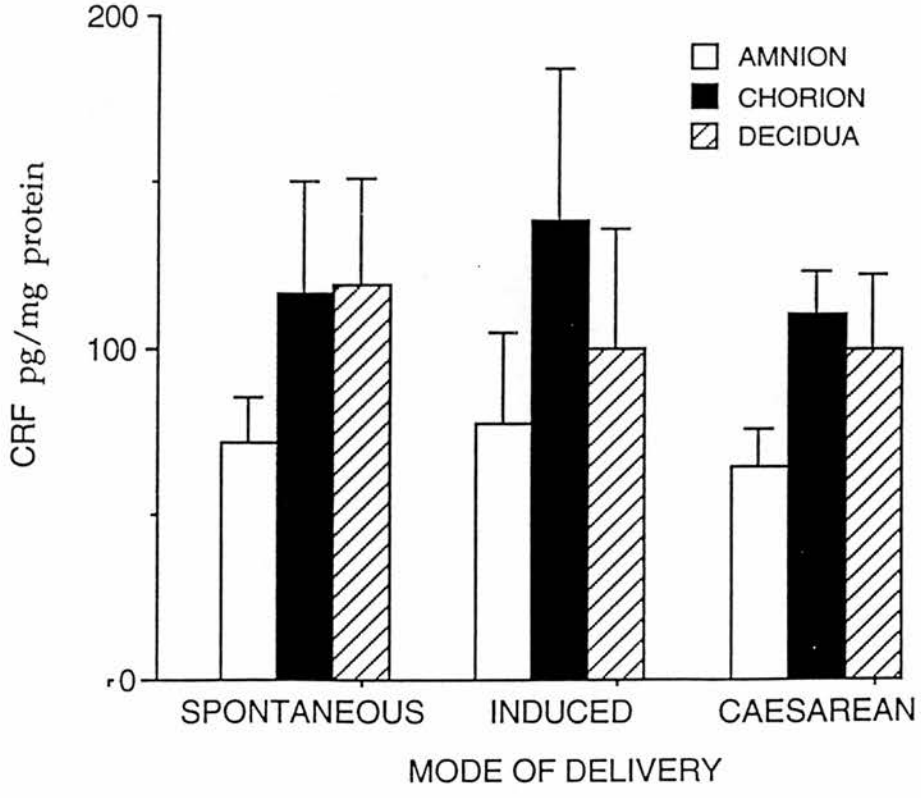


Figure 7.3.
Immunoreactive CRH concentration measured by radioimmunoassay in extracts of amnion, chorion and decidua collected after spontaneous (n=7), induced (n=5) or caesarean delivery (n=5) at term. Values are the mean ± SEM. There was no significant difference between the groups.



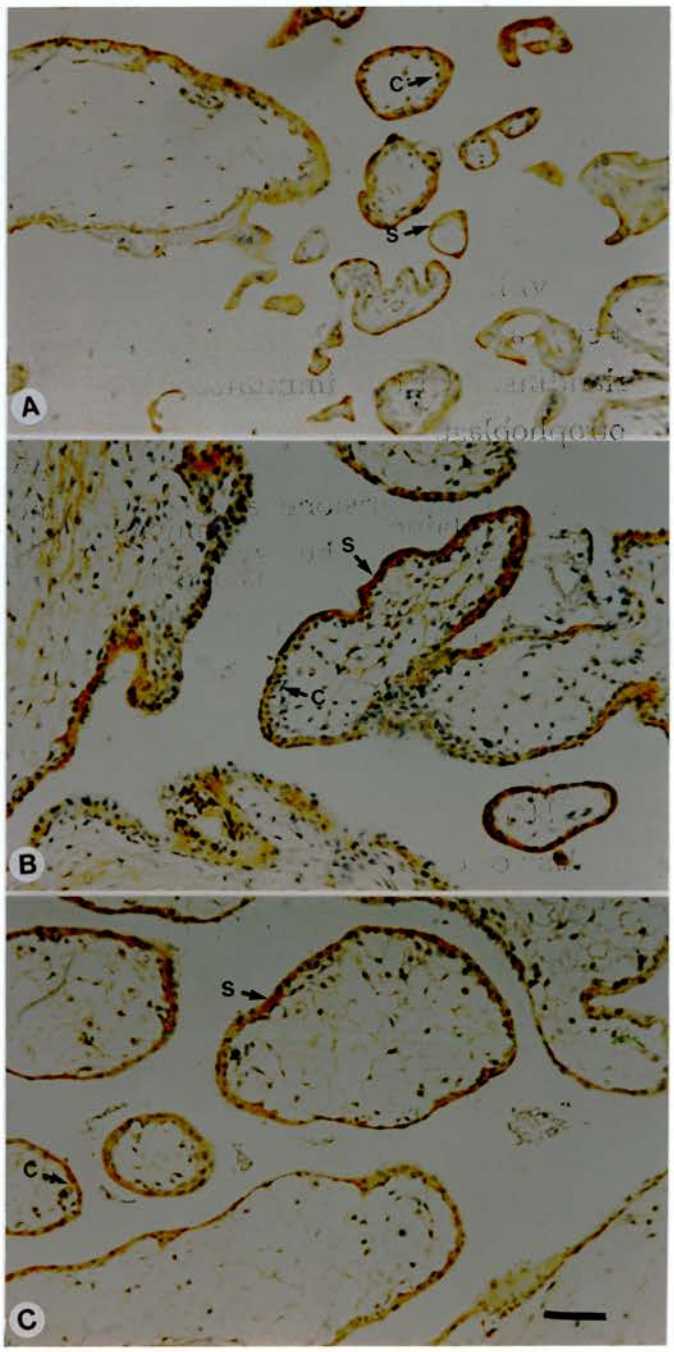

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Plate 7.1. Modulation of CRH immunostaining of first trimester placenta

7.1.A. Placental villi obtained after suction termination of pregnancy at 8 weeks gestation without pretreatment with prostaglandins. CRH immunostaining is seen in the syncytiotrophoblast.

7.1.B. Placental villi obtained after suction termination of pregnancy at 8 weeks gestation pretreated with prostaglandins. CRH immunostaining is seen in the syncytiotrophoblast.

7.1.C. Placental villi obtained after termination of pregnancy at 8 weeks gestation with mifepristone and prostaglandins. CRH immunostaining is seen in the syncytiotrophoblast. Note there is no difference in the distribution or intensity of the staining between the three modes of termination.

The bar indicates 50μm
s=syncytiotrophoblast; c= cytotrophoblast

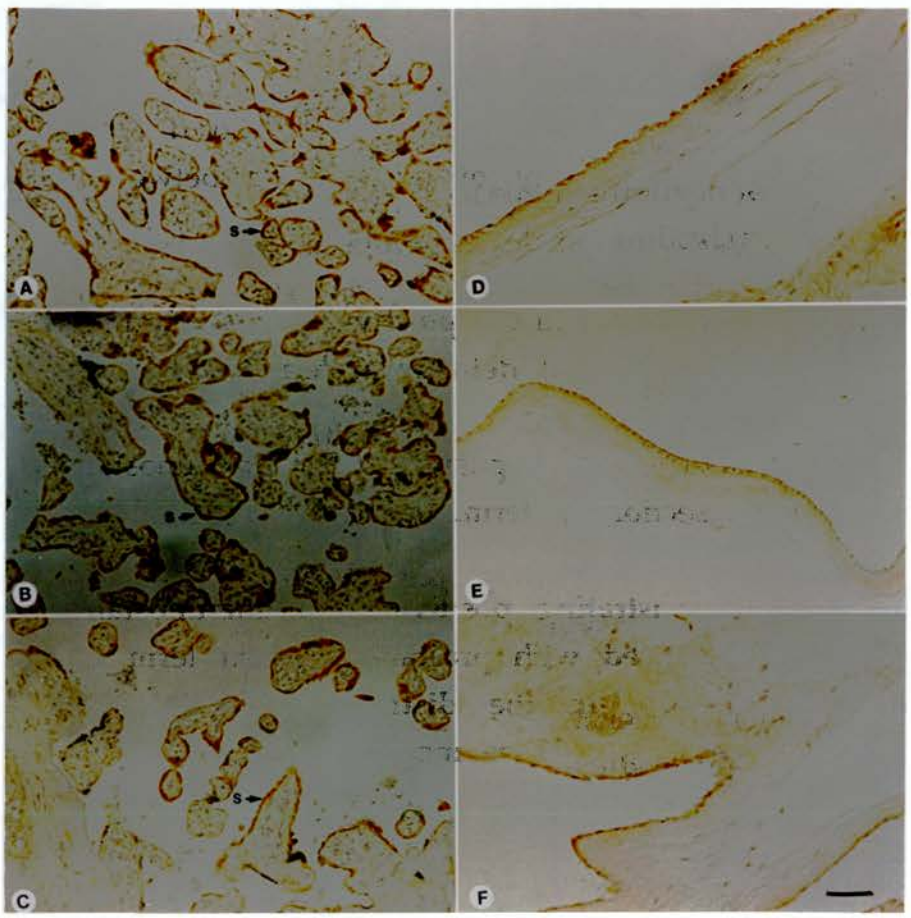
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Plate 7.2. Modulation of CRH immunostaining of placenta and fetal membranes at term (1)

7.2.A. Placental villi demonstrating positive CRH immunostaining in syncytiotrophoblast from a normal vaginal delivery at term.

7.2.B. Placental villi demonstrating positive CRH immunostaining in syncytiotrophoblast from a caesarean section at term.

7.2.C. Placental villi demonstrating positive CRH immunostaining in syncytiotrophoblast from a delivery induced with prostaglandins at term. Note there is no difference in the distribution or intensity of the staining between the three modes of delivery

7.2.D. Amnion demonstrating positive CRH immunostaining from a normal vaginal delivery at term.

7.2.E. Amnion demonstrating positive CRH immunostaining from a caesarean section at term.

7.2.F. Amnion demonstrating positive CRH immunostaining from a delivery induced with prostaglandins at term. Note there is no difference in the distribution or intensity of the staining between the three modes of delivery

The bar indicates 50μm
s=syncytiotrophoblast; c= cytotrophoblast

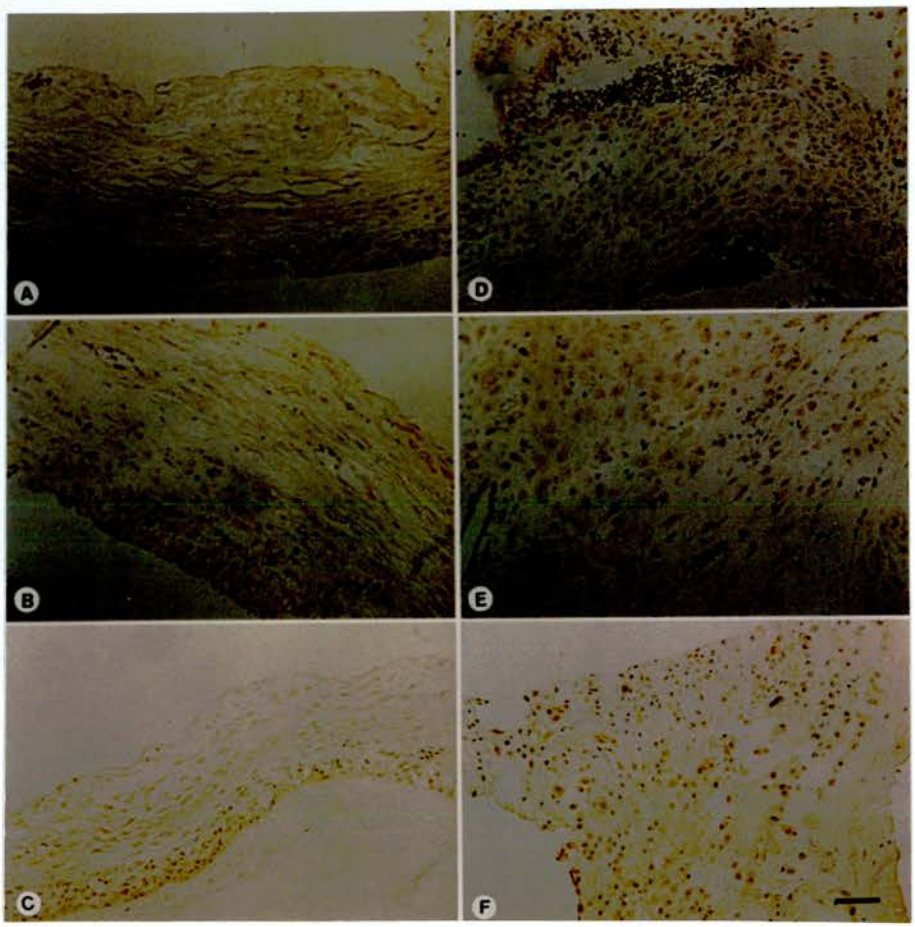
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Plate 7.3. Modulation of CRH immunostaining of placenta and fetal membranes at term (2)

7.3.A. Chorion demonstrating positive CRH immunostaining from a normal vaginal delivery at term.

7.3.B. Chorion demonstrating positive CRH immunostaining from a caesarean section at term.

7.3.C. Chorion demonstrating positive CRH immunostaining from a delivery induced with prostaglandins at term. Note there is no difference in the distribution or intensity of the staining between the three modes of delivery

7.3.D. Decidua demonstrating positive CRH immunostaining from a normal vaginal delivery at term.

7.3.E. Decidua demonstrating positive CRH immunostaining from a caesarean section at term.

7.3.F. Decidua demonstrating positive CRH immunostaining from a delivery induced with prostaglandins at term. Note there is no difference in the distribution or intensity of the staining between the three modes of delivery

The bar indicates 50μm

CHAPTER 8

MODULATION OF PLACENTAL ACTH PRODUCTION

8.1 Introduction

As discussed in Chapter 6, ACTH is present in the placenta throughout gestation. A role in influencing maternal and adrenal function during pregnancy has been suggested (Jailer and Knowlton 1950; Silman and Bergman 1953; Assali and Hammermetz 1954), potentially affecting the HPA axis in the mother or the fetus by stimulating adrenal steroidogenesis. This may be of particular significance in the fetus, since the fetal adrenal cortex supplies the placenta with androgens for oestrogen synthesis throughout pregnancy, and secretes cortisol which is essential for late fetal maturation. It has also been suggested that this fetal maturation, and in particular maturation of the hypothalamopitutaryadrenal axis, may be involved with the onset of parturition.

In view of the potential role of ACTH in controlling the HPA axis throughout pregnancy and its possible role in the onset of parturition, this part of the study examined the localisation and content of IR-ACTH in placenta and fetal membranes, and looked at the effects of labour, exogenous prostaglandins and the administration of the antiprogestin mifepristone on staining and peptide content. The POMC gene expression in the same tissues was also investigated in terms of localisation and quantification by in situ hybridisation.

8.2. Materials and methods

8.2.1. Tissue collection

All gestations were calculated from the date of the last menstrual period and were confirmed by ultrasound and/or clinical assessment. Decidual and trophoblastic tissues were obtained from patients undergoing therapeutic termination of pregnancy in the first trimester by curettage with or without pretreatment with the synthetic prostaglandin analogue gemeprost (16,16 dimethyl transΔ^2 PGE_1methylester) between 2 and 4 hours prior to the procedure and also from patients undergoing therapeutic termination in the first and second trimester using mifepristone 600mg orally 48 hours prior to receiving a 1mg gemeprost vaginal pessary. All patients aborted within 6 hours. Placenta and fetal membranes were also collected from spontaneous vaginal deliveries at term (36-42 weeks gestation) by elective casarean section (n=5), spontaneous vaginal delivery (n=7), and by induction of labour using prostaglandin E_2 (n=5) (all patients studied received 2mg of PGE_2 and all delivered within 24 hours). There was no clinical evidence of infection in any patient, and there was no histological evidence of inflammation in any of the tissues studied.

The materials and methods used were identical to those used in chapter 6. Briefly, tissues were collected, fixed in 4% paraformaldehyde and examined immunocytochemically and by in situ hybridisation analysis. Peptide content in placental extracts was measured by radioimmunoassay and sephadex chromatography was used to examine the molecular size forms of placental ACTH.

8.3. Results

Positive immunostaining for ACTH was found in all placentas between 5 and 42 weeks gestation regardless of the method of termination of pregnancy or delivery. In control slides, no positive ACTH immunostaining was found where the primary antibody had been

substituted by antibody dilution buffer, non-immunised rabbit serum or CLIP antibody which had been preabsorbed with 5μM human ACTH.

In the first trimester (from 5-12 weeks gestation) positive staining was seen in the cytotrophoblast layer of cells, with none in the syncytiotrophoblast. Positive staining was also seen in the cells of the decidual stroma. The staining was faint when compared with tissue of more advanced gestation. There was no difference in staining (see plate 8.1) or hybridisation signal (see plate 8.4), either in intensity or localisation, between tissue obtained after suction curettage with or without prostaglandin pretreatment compared with tissue obtained after termination with mifepristone and prostaglandins.

From the second trimester onwards, positive ACTH immunostaining was seen in the syncytiotrophoblast. The staining increased in intensity withn advancing gestation. In the amnion the staining was particularly pronounced in the epithelial layers with also some staining in the subepithelial layer. In the chorion staining was seen mainly in the reticular layer. Cells of the decidual stroma stained positively throughout gestation.

There was no difference in staining (see plates 8.2. and 8.3.) or hybridisation signal (see plate 8.5) in tissues obtained after elective caesarean section (not in labour) at term compared with spontaneous delivery at term. There was no difference in staining or signal intensity in tissue obtained after labour induced with prostaglandins compared with that from spontaneous delivery

Radioimmunoassay of the placental extracts revealed a significant ($p<0.01$) increase in ACTH content in the third trimester. There was no significant difference in placental ACTH content at term with different modes of delivery (Fig 8.2), or in the first trimester after the administration of prostaglandin E_2 or the antiprogestin mifepristone (Fig 8.1). Mean value with standard errors are as follows; first trimester termination using curettage with (6.91±1.54pg/mg protein) and without

(7.1±0.97 pg/mg protein) pretreatment with prostaglandins, termination using mifepristone and prostaglandins (8.03±1.92 pg/mg protein). (see figure 8.1) Mean values of placental ACTH content for spontaneous delivery were 32.11±5.78 pg/mg protein, for elective caesarean section were 28.37±6.02 pg/mg protein and for induced labour were 26.6±13.26 pg/mg protein. Mode of delivery also had no effect on the ACTH content of fetal membranes (see fig 8.3).

Sephadex G50 chromatography revealed that ACTH from placental extracts and human ACTH (1-39) standard eluted with an identical retention time indicating that both had an identical molecular weight (see fig 6.4).

Image analysis of the in situ hybridisation studies showed that there was no significant difference in grain density, and therefore POMC gene expression, between first trimester placentas collected after termination with curettage with($0.8\pm0.085 grains/cm^2 x 10^5$) and without ($0.83\pm0.18 grains/cm^2 x 10^5$) prostaglandins and with mifepristone and prostaglandins (0.65 ± 0.06 $grains/cm^2 x 10^5$) (see fig 8.4). At term there was no difference in placentas collected after spontaneous ($3.08\pm0.18 grains/cm^2 x 10^5$), induced ($2.81\pm0.075 grains/cm^2 x 10^5$) and caesarean delivery ($2.52\pm0.45 grains/cm^2 x 10^5$) (see fig 8.5).

8.4. Discussion

As discussed in chapter 6 positive ACTH immunostaining in the placenta throughout pregnancy. ACTH immunostaining is seen in the cytotrophoblasts and decidual stroma as early as five weeks gestation and in the fetal membranes from the second trimester onwards (the earliest time these tissues could be collected). Extraction of peptide from the placenta and radioimmunassay for ACTH revealed detectable levels at al stages of pregnancy investigated. The intensity of this staining and POMC mRNA levels were unaffected by method of termination of

pregnancy consistent with the finding that there was no significant difference in peptide content between the different groups of placenta. The concentrations of ACTH were similar to those reported previously by Liotta et al (1977), Rees et al (1975) and Odagiri et al (1989).

The intensity of the immunostaining increased throughout gestation but the localisation changed from the cytotrophoblast in the first trimester to the syncytiotrophoblast thereafter. The placental ACTH content and POMC gene expression were also significantly greater at term than in the first trimester, but, like staining intensity, they were unaffected by labour or the administration of exogenous prostaglandins. Previous studies of placental ACTH immunostaining (Al-Timini and Fox 1986) localised ACTH to the syncytiotrophoblast and found no change in the intensity or localisation of the immunostaining throughout gestation. These studies did not investigate the effect of labour, prostaglandins or mifepristone on ACTH immunostaining. Furthermore, some studies of placental ACTH content have not shown any variation in placental ACTH content throughout pregnancy (Liotta et al 1977; Rees et al 1975; Odagiri et al 1979) (as discussed in chapter 6) but again did not consider the effects of mode of delivery on placental ACTH content. The finding of increased POMC gene expression strongly supports the placental ACTH content results which were in conflict with these previous studies.

This is the first study to localise and quantify POMC gene expression in the placenta throughout gestation using in situ hybridisation. Previous studies using northern analysis (Cheng et al 1986) has identified POMC mRNA in human placenta but only at term. In rat placenta, the expression of POMC gene appears to be constitutive as the concentration of POMC mRNA does not change during pregnancy (Cheng et al 1986). This study clearly showed that the POMC gene is expressed in the first trimester and that this expression increases in the final trimester prior to labour. Labour itself did not appear to affect POMC gene expression, as there was no significant difference between hybridisation signal in placentas collected after labour and those collected at elective caesarean section. The POMC gene is also expressed in the fetal membranes,

consistent with the finding of positive immunostaining and the extraction of ACTH from these tissues. In the first trimester there was no difference in POMC gene expression with method of termination, suggesting that, at least in the short term, prostaglandins and mifepristone have no effect on placental ACTH output.

The increase in peptide content, increase in intensity of immunostaining and increase in POMC gene expression as seen with in situ hybridisation support the hypothesis that placental ACTH rises prior to labour. The biological significance of this is uncertain. The demomstration of ACTH immunoreactivity from early to late gestation suggests that the placenta may exert an effect on the maternal and/or fetal adrenal throughout the whole of pregnancy. In addition placental ACTH may be involved with placental CRH in the control of the onset of parturition. It has been suggested that activation of the fetal hypothalamo-pituitary-adrenal axis is linked to the onset of parturition. Placentally produced peptides may have a key role in this. Placental CRH could pass to the fetal circulation, stimulating the release of ACTH from the fetal pituitary. This in turn would cause the release of cortisol from the adrenal. This cortisol may both increase DHEAS resulting in increased output by the placenta, and also stimulate placental CRH production in a positive feedback loop. The effects of placental CRH to increase prostaglandin production (see chapter 1) could lead to ripening of the cervix and myometrial contractility (Challis and Hooper 1989) And ultimately birth. Placental ACTH also passes into the fetal circulation, and may affect fetal cortisol. The finding of a rise in placental ACTH in the third trimester supports the hypothesis that it may be involved in this process. However the finding that its production is unaffected by labour suggests that it may be involved in the maturation of the fetal HPA axis through pregnancy rather than being a trigger for birth.

Figure 8.1.

Immunoreactive ACTH concentrations measured by radioimmunoassay in first trimester placental extracts collected after termination of pregnancy induced by mifepristone (RU486) (n=5), curettage with prostaglandin (STOP+C) (n=5), or curettage without prostaglandin (STOP-C) (n=5). Values are the mean ± SEM. There was no significant difference between the groups.

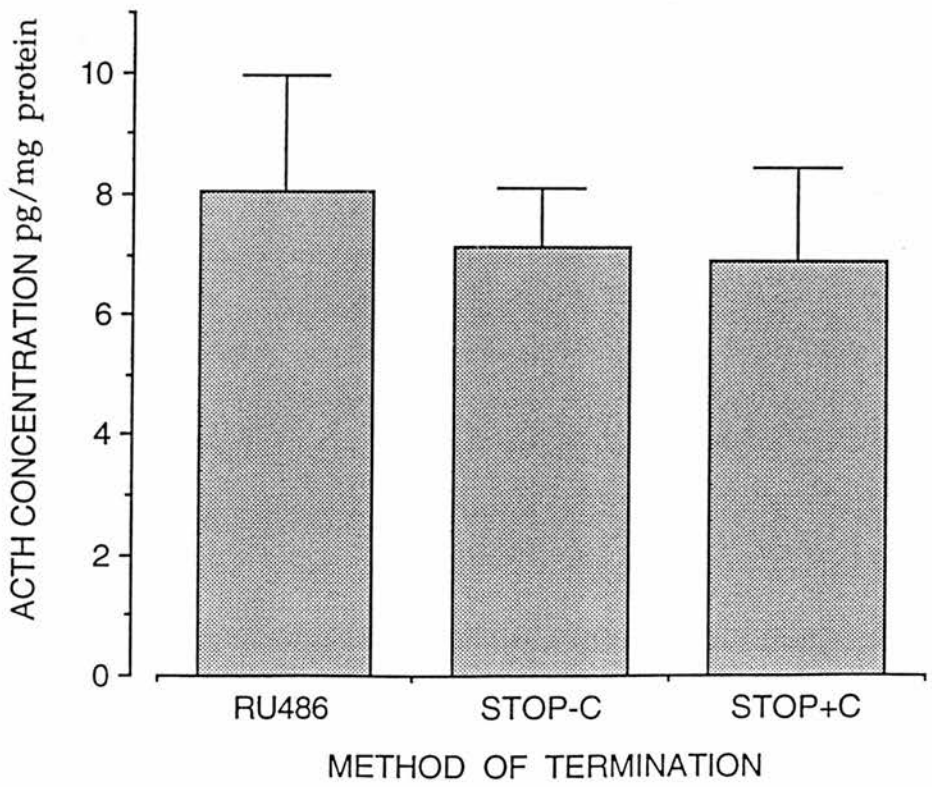


Figure 8.2.

Immunoreactive ACTH concentration measured by radioimmunoassay in extracts of placenta collected after spontaneous (n=7), induced (n=5) or caesarean delivery (n=5) at term. Values are the mean ± SEM. There was no significant difference between the groups.

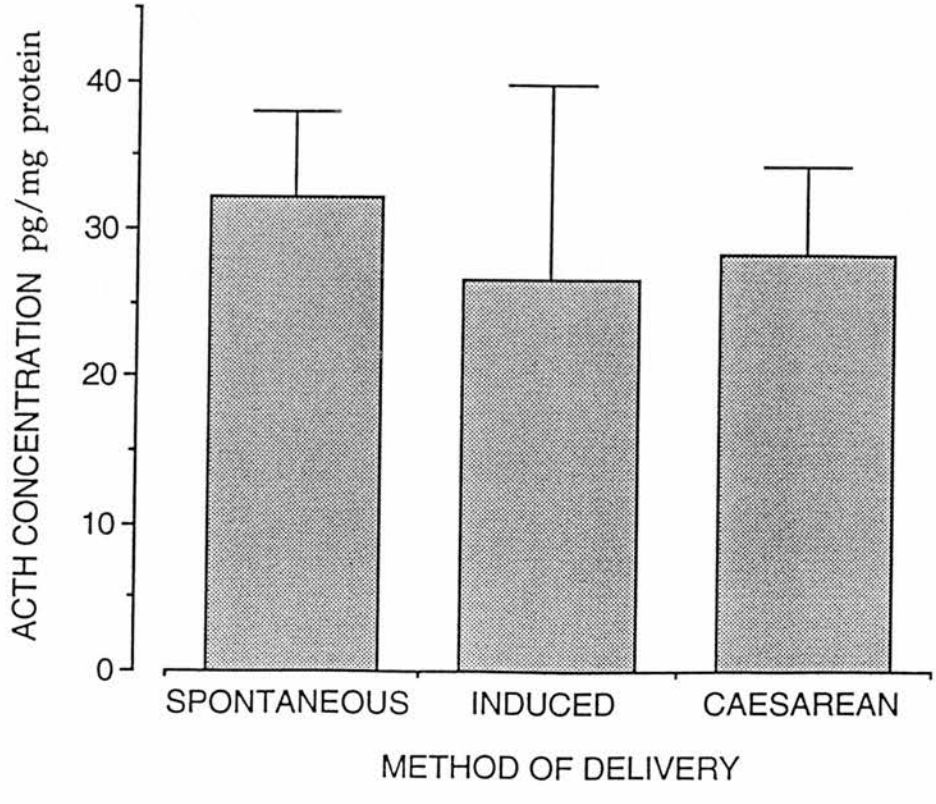


Figure 8.3.
Immunoreactive ACTH concentration measured by radioimmunoassay in extracts of amnion, chorion and decidua collected after spontaneous (n=7), induced (n=5) or caesarean delivery (n=5) at term. Values are the mean ± SEM. There was no significant difference between the groups.

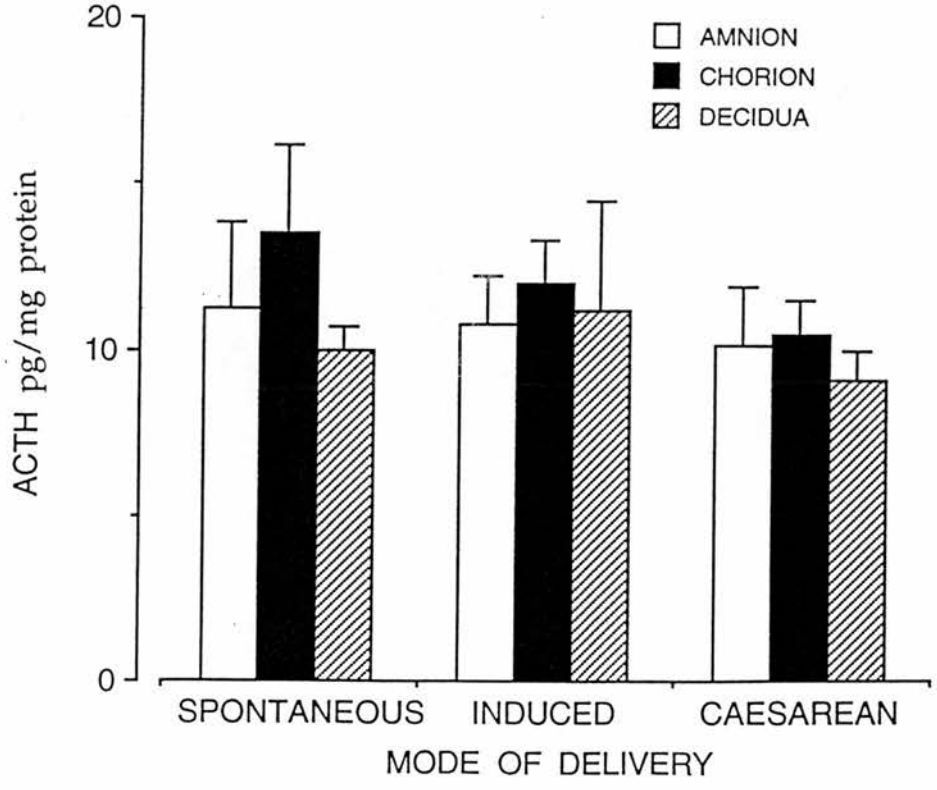


Figure 8.4.

POMC gene expression measured by quantification of in situ hybridisation performed on sections of placentas collected after termination of pregnancy in the first trimester induced by mifepristone (RU486) (n=3), curettage with prostaglandin (STOP+C) (n=3), or curettage without prostaglandin (STOP-C) (n=3). Each histogram represents the means of six random counts of the tissue grain density (background non-specific counts have been subtracted) of each of three groups of tissues.Values are the mean ± SEM. There was no significant difference between the groups.

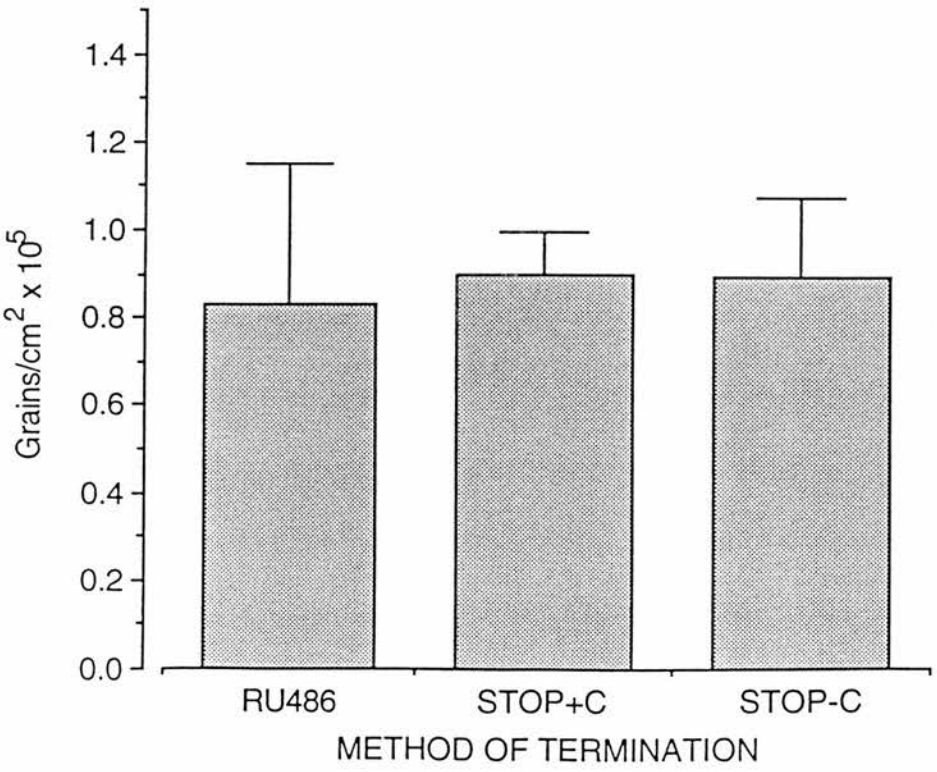
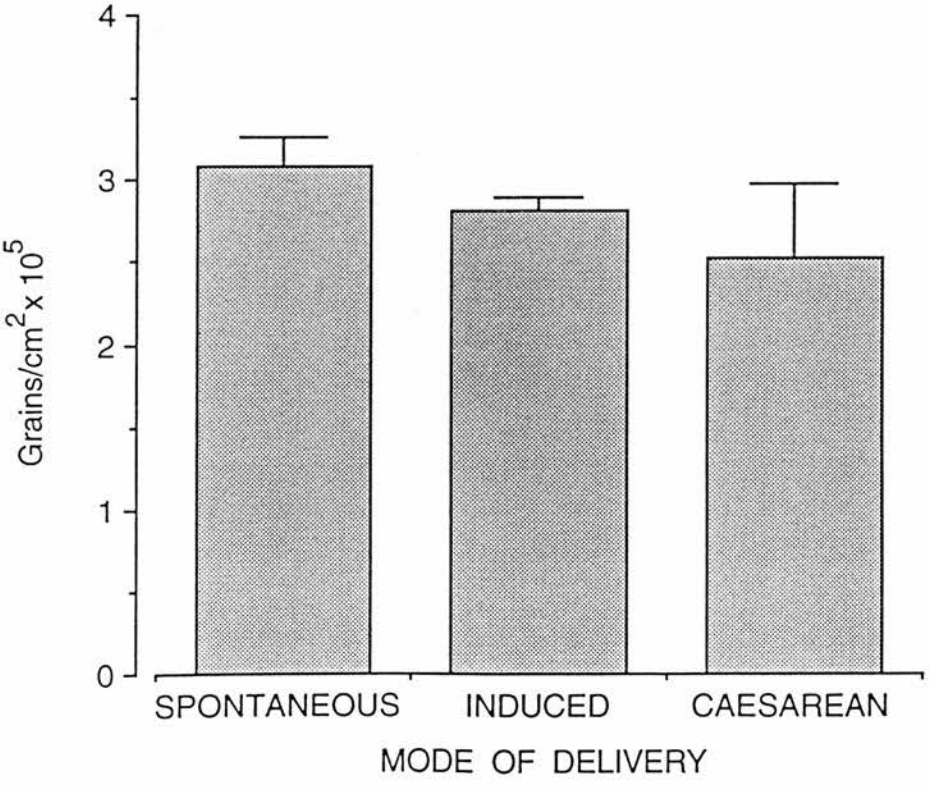


Figure 8.5.

POMC gene expression measured by quantification of in situ hybridisation performed on sections of placentas collected after spontaneous (n=3), induced (n=3) or caesarean (n=3) delivery at term. Each histogram represents the means of six random counts of the tissue grain density (background non-specific counts have been subtracted) of each of three groups of tissues. Values are the mean ± SEM. There was no significant difference between the groups.



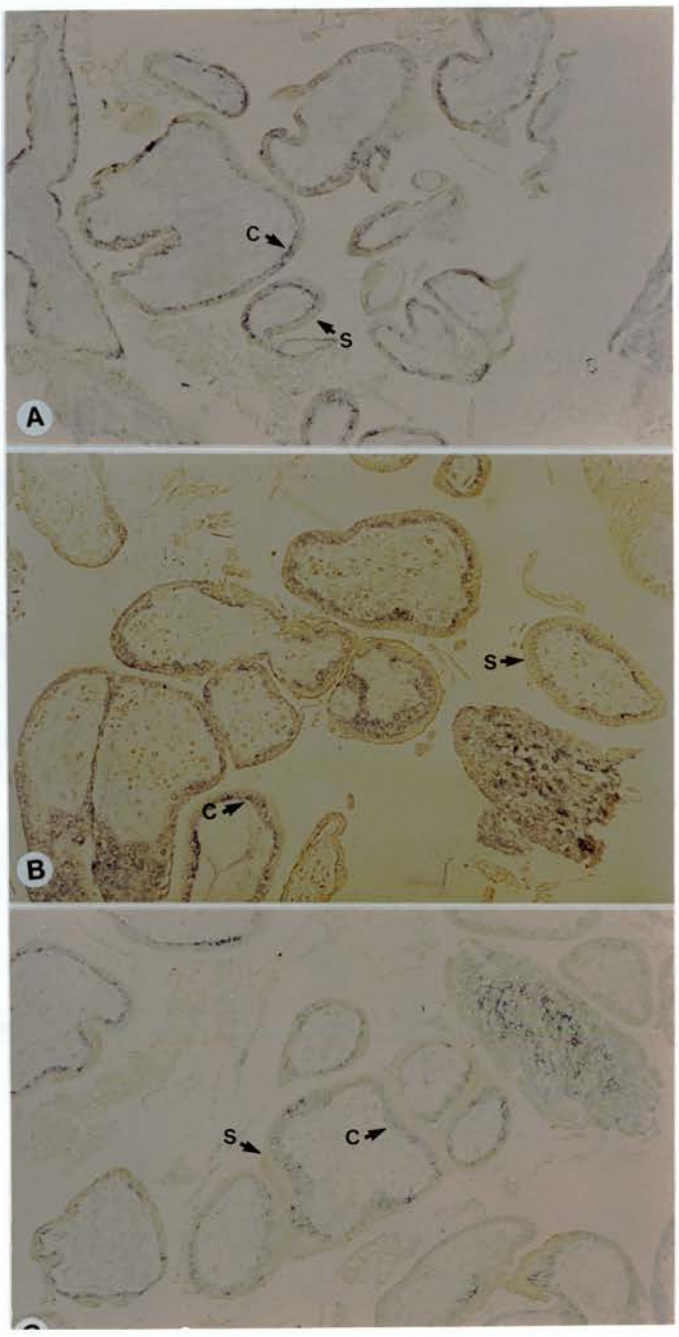

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Plate 8.1. Modulation of ACTH immunostaining of first trimester placenta

8.1.A. Placental villi obtained after suction termination of pregnancy at 8 weeks gestation without pretreatment with prostaglandins. ACTH immunostaining is seen in the cytotrophoblast.

8.1.B. Placental villi obtained after suction termination of pregnancy at 8 weeks gestation pretreated with prostaglandins. ACTH immunostaining is seen in the cytotrophoblast.

8.1.C. Placental villi obtained after termination of pregnancy at 8 weeks gestation with mifepristone and prostaglandins. ACTH immunostaining is seen in the cytotrophoblast. Note there is no difference in the distribution or intensity of the staining between the three modes of termination.

The bar indicates 50μm
s=syncytiotrophoblast; c= cytotrophoblast

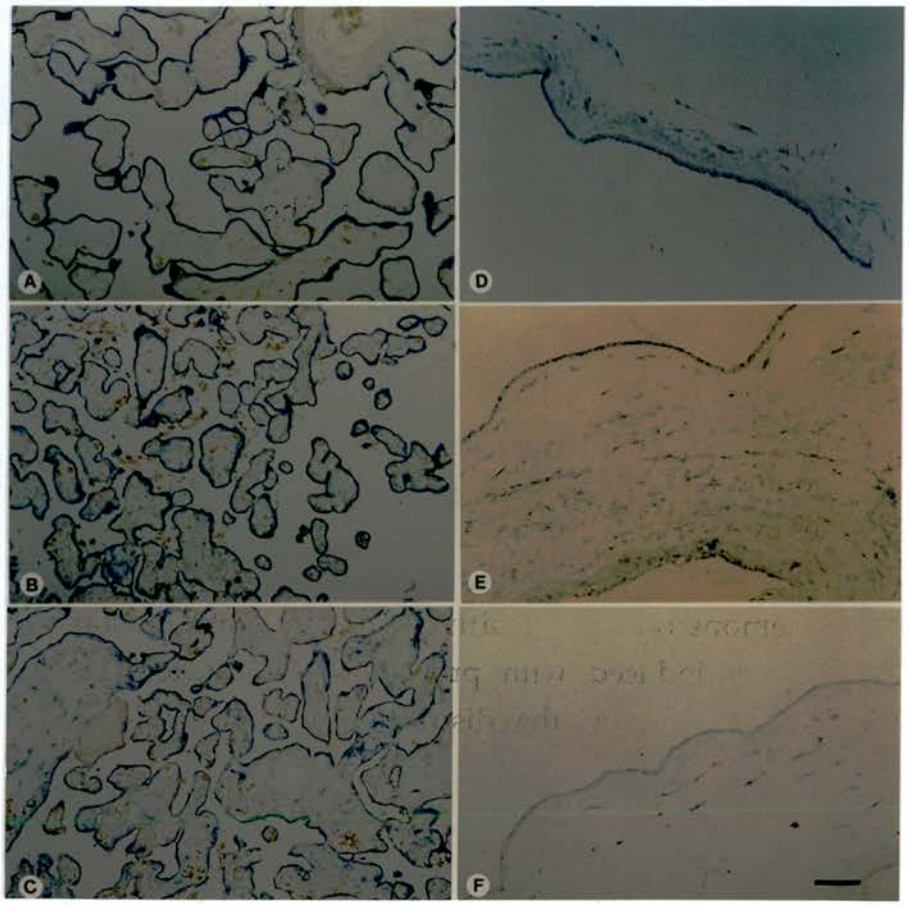
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Plate 8.2. Modulation of ACTH immunostaining of placenta and fetal membranes at term (1)

8.2.A. Placental villi demonstrating positive ACTH immunostaining in syncytiotrophoblast from a normal vaginal delivery at term.

8.2.B. Placental villi demonstrating positive ACTH immunostaining in syncytiotrophoblast from a caesarean section at term.

8.2.C. Placental villi demonstrating positive ACTH immunostaining in syncytiotrophoblast from a delivery induced with prostaglandins at term. Note there is no difference in the distribution or intensity of the staining between the three modes of delivery

8.2.D. Amnion demonstrating positive ACTH immunostaining from a normal vaginal delivery at term.

8.2.E. Amnion demonstrating positive ACTH immunostaining from a caesarean section at term.

8.2.F. Amnion demonstrating positive ACTH immunostaining from a delivery induced with prostaglandins at term. Note there is no difference in the distribution or intensity of the staining between the three modes of delivery

The bar indicates 50μm
s=syncytiotrophoblast; c= cytotrophoblast

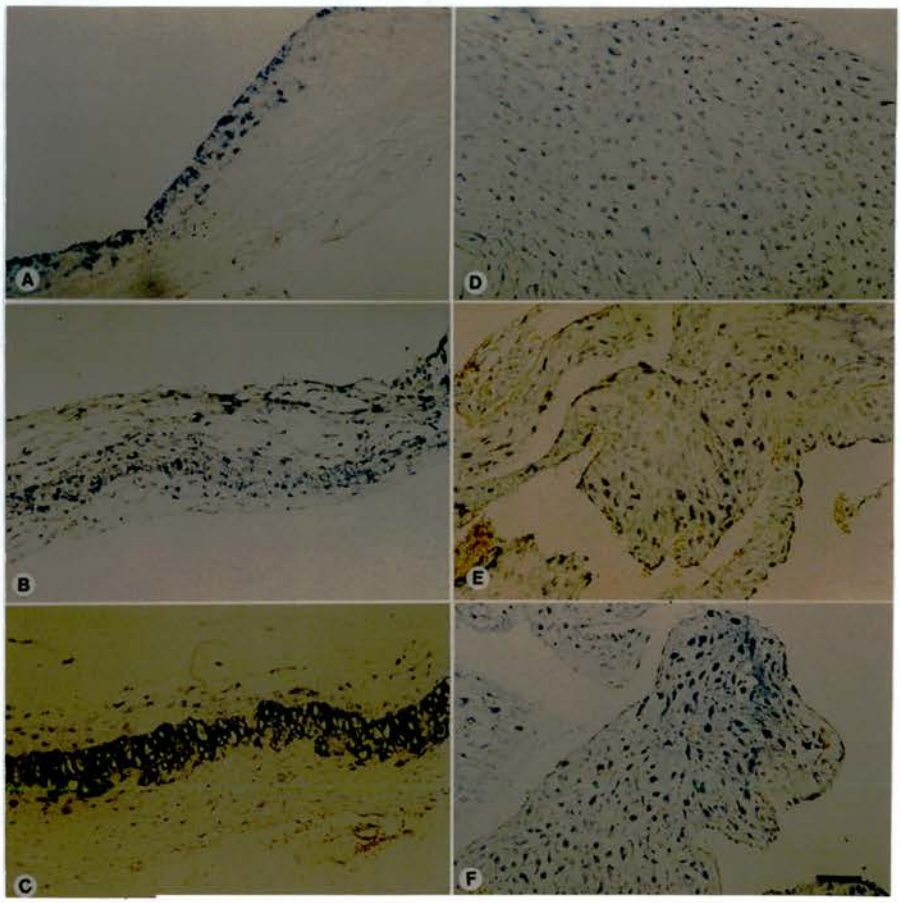
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Plate 8.3. Modulation of ACTH immunostaining of placenta and fetal membranes at term (2)

8.3.A. Chorion demonstrating positive ACTH immunostaining from a normal vaginal delivery at term.

8.3.B. Chorion demonstrating positive ACTH immunostaining from a caesarean section at term.

8.3.C. Chorion demonstrating positive ACTH immunostaining from a delivery induced with prostaglandins at term. Note there is no difference in the distribution or intensity of the staining between the three modes of delivery

8.3.D. Decidua demonstrating positive ACTH immunostaining from a normal vaginal delivery at term.

8.3.E. Decidua demonstrating positive ACTH immunostaining from a caesarean section at term.

8.3.F. Decidua demonstrating positive ACTH immunostaining from a delivery induced with prostaglandins at term. Note there is no difference in the distribution or intensity of the staining between the three modes of delivery

The bar indicates 50μm

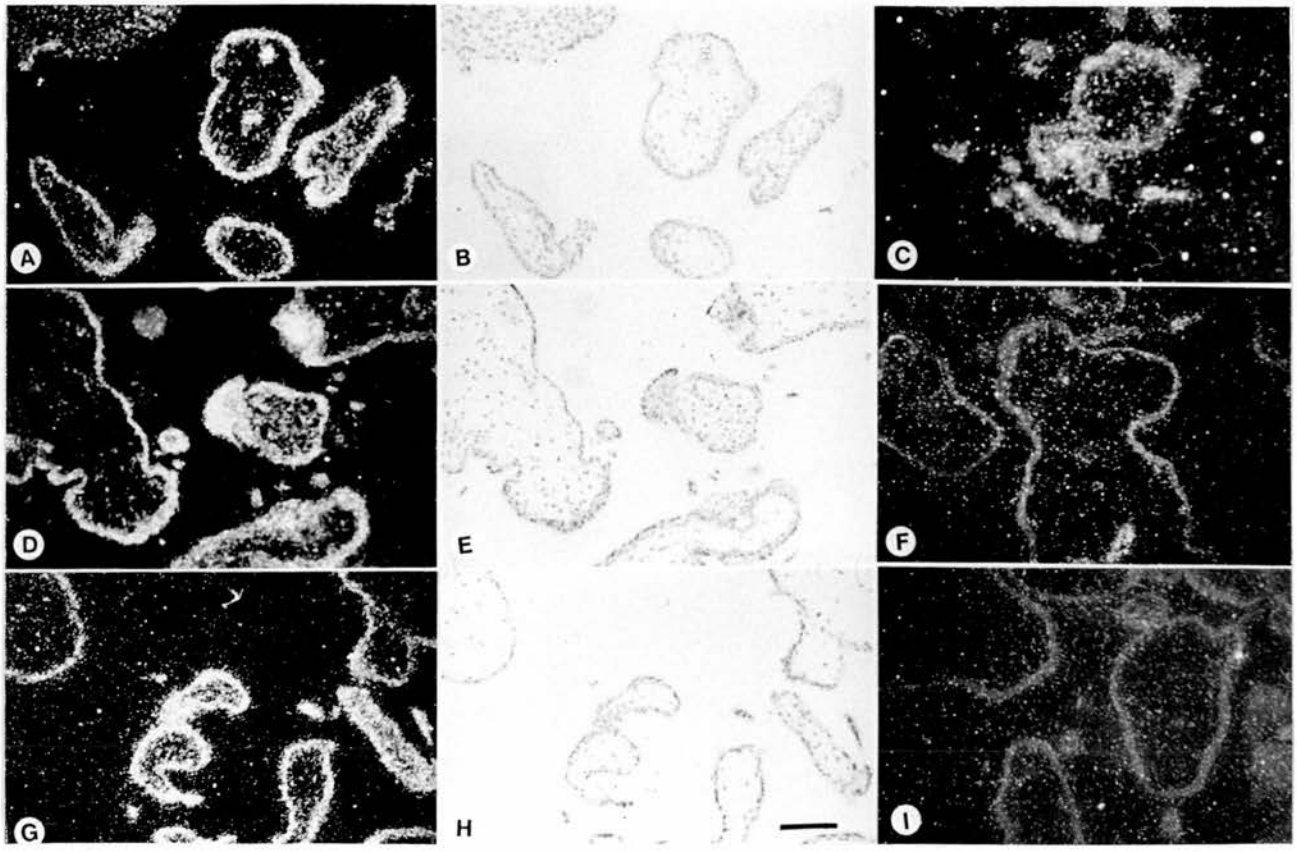
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Plate 8.4. Modulation of POMC gene expression in first trimester placenta

8.4.A &B Dark (A) and light (B) field micrographs of in situ hybridisation of POMC mRNA in first trimester placenta collected after termination of pregnancy with mifepristone and prostaglandins.

8.4.C Negative control. Tissue shown in 8.4.A but using sense probe. Note low level of non specific hybridisation.

8.4.D&E. Dark (D) and light (E) field micrographs of in situ hybridisation of POMC mRNA in first trimester placenta collected after termination of pregnancy by suction curettage without prostaglandins.

8.4.F Negative control. Tissue shown in 8.4.D but using sense probe. Note low level of non specific hybridisation.

8.4.G&H. Dark (G) and light (H) field micrographs of in situ hybridisation of POMC mRNA in first trimester placenta collected after termination of pregnancy by suction curettage pretreated with prostaglandins. Note there is no difference in grain density between the three groups.

8.4.I Negative control. Tissue shown in 8.4.G but using sense probe. Note low level of non specific hybridisation.

The bar indicates 50μm

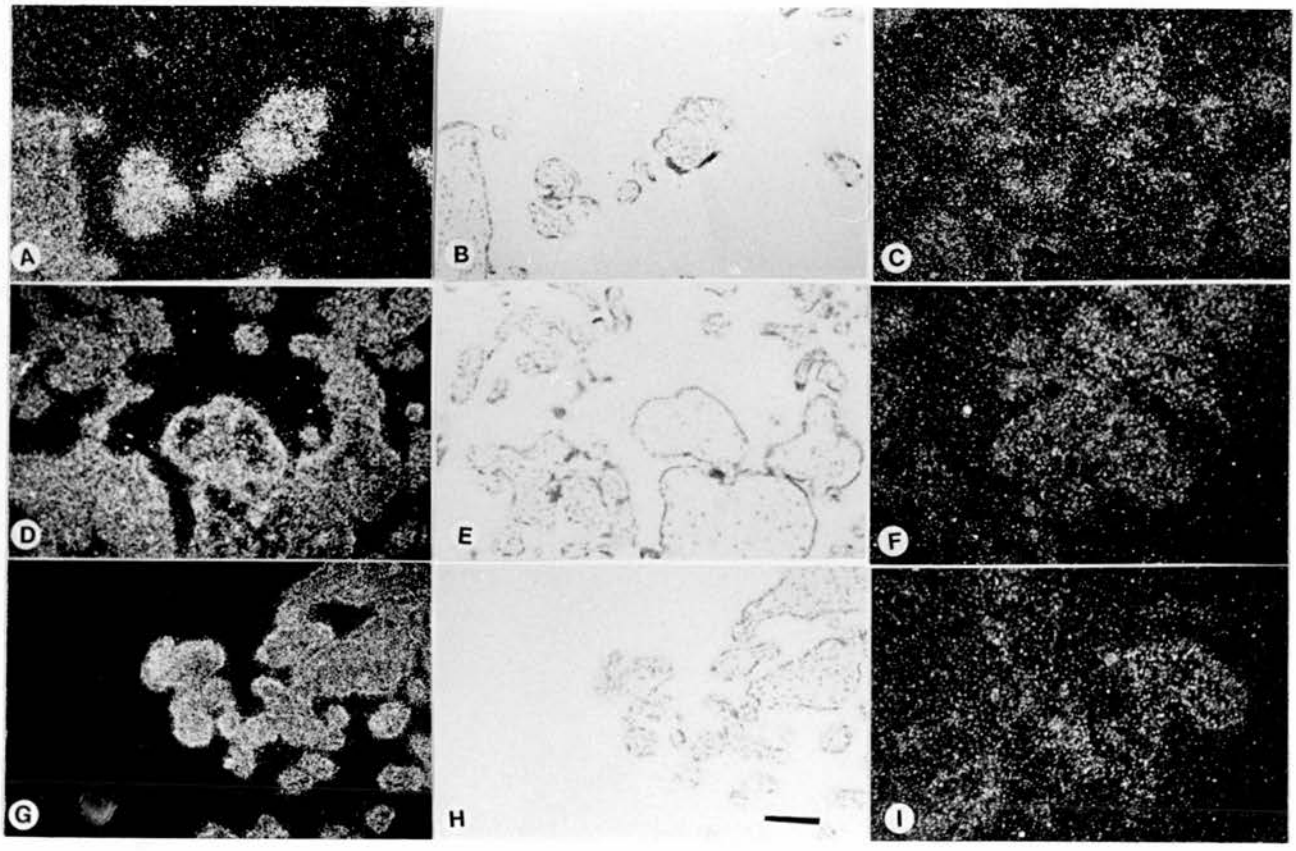
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Plate 8.5. Modulation of POMC gene expression in term placenta

8.5.A & B Dark (A) and light (B) field micrographs of in situ hybridisation of POMC mRNA in term placenta collected after spontaneous delivery.

8.5.C Negative control. Tissue shown in 8.5.A but using sense probe. Note low level of non specific hybridisation.

8.5.D & E Dark (D) and light (E) field micrographs of in situ hybridisation of POMC mRNA in term placenta collected after delivery induced with prostaglandins.

8.5.F Negative control. Tissue shown in 8.5.D but using sense probe. Note low level of non specific hybridisation.

8.5.G & H Dark (G) and light (H) field micrographs of in situ hybridisation of POMC mRNA in term placenta collected after elective caesarean section at term.

8.5.I Negative control. Tissue shown in 8.5.G but using sense probe. Note low level of non specific hybridisation.

The bar indicates 50μm

CHAPTER NINE

GENERAL DISCUSSION

9.1. Localisation of CRH within the Placenta and Fetal Membranes.

It was already well known that both CRH, and CRH mRNA are detectable in placental extracts, in increasing quantities from the first trimester onwards (Shibasaki et al 1982; Sasaki et al 1987 Frim et al 1988; Okamoto et al 1989). Correspondingly there is a rise in maternal plasma CRH concentration during this time and a rapid fall is seen within hours of delivery (Sasaki et al 1984; Goland et al 1986; Campbell et al 1987; Laatikainen et al 1987b; Okamoto et al 1989). All these observations suggested that the placenta both synthesises and releases increasing amounts of CRH throughout pregnancy. CRH localisation has also been described by three groups, but there is some discrepancy between them. These studies set out to provide further evidence of the exact cellular localisation of placental CRH by immunocytochemistry, and to attempt to quantify its production throughout pregnancy by peptide extraction. The role of CRH in the placental-fetal unit is unclear, and it was hoped that information about the precise localisation CRH within particular cell types may provide evidence for the site of action of CRH. In addition, an investigation into the production of CRH with different modes of delivery may provide insight into its potential role in parturition.

Early studies of the localisation of CRH within the placenta suggested that CRH immunoreactivity was located in the cytotrophoblasts (Petraglia et al 1987; Saijonmaa et al 1988). However, as discussed in Chapter 1, this cell type decreases in number during pregnancy reaching relatively small numbers by term (Gosseye and Fox 1984). As mentioned above, it is at this time that the CRH content of the placenta, maternal plasma and amniotic fluid is at its highest (Laatikainen et al 1988; Maser-Gluth et al 1987). More recently CRH has been localised to

the syncytiotrophoblasts as well as the amnion, chorion and decidua (Riley et al 1991). These results are consistent with the observation that syncytiotrophoblasts are the most numerous cells at term. In addition, CRH is released when cells from these tissues are cultured in vitro (Jones et al 1989; Jones and Challis 1989; Jones and Challis 1990a).

The finding of CRH immunostaining in the syncytiotrophoblasts as early as five weeks gestation and in the amnion and chorion from 13 weeks was consistent with the detection of CRH by radioimmunoassay in placental extracts in the first trimester. These results contradicted the findings of Riley et al who could not detect CRH in placenta before the second trimester. However, CRH is detectable in maternal plasma during the first trimester, albeit at low levels, and CRHmRNA has been demonstrated in the placenta by Northern analysis as early as seven weeks gestation (Frim et al 1988). In addition, in vitro studies of cultured placental and amnion cells at 12 weeks gestation have shown a significant output of CRH, data which supports the finding of positive immunostaining in the first trimester (Jones and Challis 1990a). In previous studies positive immunostaining has been reported in cytotrophoblasts (Petraglia et al 1987a; Saijonmaa et al 1988). Saijonmaa et al (1988) used a high concentration of antibody with no preabsorbed control and found immunostaining in the first trimester but not at term. These workers attributed this to the relatively small numbers of cytotrophoblast cells at term but this does not account for the very high levels of CRH found in the placenta at this time. Petraglia et al (1987a) found CRH immunopositive staining in the cytotrophoblasts at term but again this does not account for the high levels of circulating CRH. The findings of the study described in Chapter 5 demonstrating CRH immunostaining in the large numbers of syncytiotrophoblasts at term are consistent with the high maternal plasma and amniotic fluid levels and agrees with the immunohistochemical findings of Riley et al (1991). Furthermore, in vitro studies of cultured placental, amnion, chorion and decidual cells at term have demonstrated significant CRH output at this time. The difference between these studies are difficult to explain but may relate to different methods of tissue fixation and collection, or to the antibodies used.

The finding of CRH immunostaining in the amnion, chorion and decidua is consistent with the results of primary cultures of these tissues (Jones, Brooks and Challis 1989) at term (Jones and Challis 1989) and in the first trimester (Jones and Challis 1990). These studies concluded that the fetal membranes produce CRH both in early and late gestation. In addition, CRHmRNA has been demonstrated in the amnion (Okamoto et al 1990) and decidua (Petraglia et al 1992). The results of the study described in chapter 5 also agree with the findings of Riley et al (1991) who also found positive immunostaining in the epithelium and subepithelial cells of the amnion, in the trophoblast layer, in some cells of the reticular and cellular layers of the chorion and in the stromal cells of the decidua.

9.2. Localisation of ACTH within the Placenta and Fetal Membranes.

In situ hybridisation enables the precise localisation and identification of individual cells which contain a specific nucleic acid sequence. When used with immunocytochemistry, both the mRNA transcribed from the gene itself, and the gene product translated from mRNA can be localised to a particular cell type giving very strong evidence for the site of synthesis. This is the first study to identify POMC gene expression within the placenta by in situ hybridisation. Both the localisation of peptide and its quantification are supported by the localisation and level of expression of POMCmRNA.

Previous studies have found maternal plasma ACTH concentration to rise during pregnancy (Rees et al 1975; Carr et al 1981) and synthesis in vitro of both bioactive and immunoreactive ACTH by human placenta at term and in the first trimester has been reported. In contrast to rising levels of plasma ACTH recorded by some groups, it has also been suggested that placental ACTH levels stay constant during pregnancy (Liotta et al 1977, Rees et al 1975, Odagiri et al 1979), although there is disagreement over the actual levels of peptide present (Genazzini et al 1975). In addition, perifusion of placental fragments produced a release of bioactive ACTH of similar concentration at both early and late gestation. Immunoreactive POMC- derived peptides have been detected

in the human placenta and de novo synthesis of POMC and its derived peptides has been demonstated in cultures of human trophoblasts (Liotta et al 1977, Mulder et al 1986, Waddell et al 1992) Furthermore, POMC mRNA has been detected in human placenta at term (Cheng et al 1986) but levels in the rat were shown to be constant throughout pregnancy. Immunoreactive ACTH has been localised immunocytochemically to the syncytiotrophoblast (Al-Timini & Fox 1986) and showed a similar intensity of staining in first and third trimester placentae, agreeing with those studies that indicated little or no variation in the levels of placental ACTH secretion throughout pregnancy (Liotta et al, 1977, Odagiri et al 1979, Demura et al, 1982) In view of the potential role of ACTH in controlling the HPA axis throughout pregnancy and its possible role in the onset of parturition, this part of the study examined the localisation and content of IR-ACTH in placenta and fetal membranes throughout gestation and the expression of the POMC gene in the same tissues. The results detailed in chapter 6 provide strong evidence for both the site of synthesis and expression of placental POMC peptides. The finding of an increase in the intensity of ACTH immunostaining in cytotrophoblasts seen with gestation is supported strongly by the rise in placental ACTH content and increased POMC gene expression over the same time.

9.3. The modulation of placental CRH.

Both the intensity of CRH immunostaining and placental CRH content increases with increasing gestation. Neither the intensity of the staining nor peptide content changed with spontaneous compared with caesarean delivery suggesting that labour may have no effect on placental CRH production. In vitro studies (Jones et al 1989) of primary cultures of placental and fetal membrane cells showed increased ability to produce CRH in cells obtained after spontaneous delivery on day 2 of culture when compared to tissue from elective caesarean section. This increase, however, was not seen on subsequent days of culture. It was suggested that this represented release of preformed CRH on day 2 whereas later

release was due to both synthesis and secretion. Maternal plasma CRH concentrations have been shown to remain at constant levels during the first, second and third stages of labour (Laatikainen et al 1987b).

The hypothesis that progesterone may have a regulatory effect on placental CRH production was tested using the antiprogestogen, mifepristone. There was no difference in intensity of staining in placentas from patients pretreated with mifepristone compared to those who were not, and in addition to this, there was no significant difference in the CRH content of these placentas compared with those obtained after suction termination. Forty-eight hours of exposure to mifepristone is likely to be a sufficient duration to produce an increase in peptide synthesis, however the anti-glucocorticoid side effects of mifepristone may counteract its anti-progestogenic effects since glucocorticoids have been shown to stimulate CRH release by the placenta and fetal membranes *in vitro* (Jones et al., 1989; Jones & Challis, 1990a). Another explanation for the lack of effect of mifepristone on CRH concentration may relate to the early stage of gestation when these studies were conducted. *In vitro* studies have demonstrated that progesterone inhibits CRH release by the placenta at term (Jones et al., 1989) but has no effect in early pregnancy (Jones & Challis, 1990a). It may be that mifepristone given later in pregnancy would affect output of CRH whereas in the first trimester it does not. Nonetheless, treatment with mifepristone results in a clinical effect producing abortion. It would appear from the data that this effect of mifepristone is not mediated via the induction of CRH synthesis as CRH appeared to be constitutively present in all tissues studied.

9.4 The modulation of placental ACTH.

It is known that the placenta is not merely a storage site for POMC-derived peptides but can actively synthesise them (Liotta and Krieger 1980). There is disagreement over the levels of placental ACTH during pregnancy. Some studies of placental ACTH content have not shown any variation in placental ACTH content throughout pregnancy (Liotta

et al 1977; Rees et al 1975; Odagiri et al 1979) There is also disagreement as to whether ACTH plasma concentrations change with pregnancy (Liotta et al 1977; Rees et al 1975; Genazzani et al 1975) One study showed constant plasma levels during pregnancy with a rise after labour (Laatikainen et al 1987). The study descibed in chapter 6 provided strong evidence of a rise in placental ACTH throughout pregnancy. However ACTH content and POMC gene expression were not affected by labour or the administration of prostaglandins or mifepristone in the first trimester. Previous studies of placental ACTH immunostaining (Al-Timini and Fox 1986) localised ACTH to the syncytiotrophoblast but found no change in the intensity or localisation of the immunostaining throughout gestation. Immunostaining is not a reliable method for the quantification of peptide content and these studies were not supported by separate measurements of ACTH content. The effects of labour, prostaglandins or mifepristone on ACTH immunostaining have also never previously been investigated.

This is the first study to localise and quantify POMC gene expression in the placenta throughout gestation using in situ hybridisation. Previous studies using northern analysis (Cheng et al 1986) have identified POMC mRNA in human placenta but only at term. In rat placenta, the expression of POMC gene appears to be constitutive as the concentration of POMC mRNA does not change during pregnancy (Cheng et al 1986). This study clearly showed that the POMC gene is expressed in the first trimester and that this expression increases in the final trimester prior to labour. Labour itself did not appear to affect POMC gene expression, as there was no significant difference between hybridisation signal in placentas collected after labour and those collected at elective caesarean section. The POMC gene is also expressed in the fetal membranes, consistent with the finding of positive immunostaining and the extraction of ACTH from these tissues. In the first trimester there was no difference in POMC gene expression with method of termination, suggesting that, at least in the short term, prostaglandins and mifepristone have no effect on placental ACTH output.

9.5. ACTH and CRH and parturition

As described in chapter 1, it has been hypothesised that activation of the hypothalamopituitary axis plays a central role in the onset of parturition. This conclusion has been drawn largely from what is known about parturition in sheep. Although there are major differences, human and ovine parturition also share some common mechanisms. In the sheep, activation of the HPA axis controls the onset of parturition. Over the last few weeks of gestation, the fetal hypothalamus releases CRH stimulating the production of ACTH by the pituitary (Brooks and Challis 1988). The resulting increased cortisol production by the fetal adrenal increases the activity of placental 17-hydroxylase which is required to convert progesterone to oestrogen (Flint et al 1975). The relative increase in oestrogen (a myometrial stimulant) compared to progesterone (a myometrial relaxant) results in myometrial excitability, prostaglandin production and uterine sensitivity to oxytocin. As discussed in chapter 1, prostaglandins appear to constitute the final common pathway in women as well as sheep. The main difference between the species is that human placenta lacks 17-hydroxylase and therefore cannot convert progesterone to oestrogen. Indeed, there is no change in the levels of these steroids at the time of labour (Liggins 1983). Although there is little evidence to suggest that the fetal HPA axis influences placental steroids in the human as it does in the sheep, it has been suggested that activation of the axis is involved in the onset of human labour. The object of these studies was to support or refute this claim. The evidence supporting activation of the HPA axis in the initiation of human labour was based on the finding that maternal plasma CRH rose exponentially in the third trimester, peaking at delivery and high levels are found in the umbilical cord plasma and amniotic fluid at term (Goland et al 1988; Sasaki et al 1987: Campbell et al 1987). As discussed earlier this CRH is placentally derived and the study described in chapter 5 showed a gestationally related increase in placental CRH content which supports these findings. In addition, this study located the CRH within the syncytiotrophoblast, a cell type which is found in large numbers at

term, consistent with the increased peptide content found at this time. Plasma ACTH has been reported to rise in the third trimester (Rees et al 1975; Genazzani et al 1975; Carr et al 1981; Okamoto et al 1989; Smith et al 1990), although there is disagreement as to whether placental ACTH content changes with gestation. Concentrations reported by Liotta et al (1977), Rees et al (1975) and Odagiri et al (1979) are essentially similar. Significantly lower levels were reported by Genazzani et al (1975), but there was lack of parallelism in the immunoassay of placental extract with standard ACTH. The findings of the study described in chapter 6 were of an increase in placental ACTH content in parallel with increased POMC gene expression, highly supportive of the hypothesis that placental ACTH increases with gestation. In vitro studies have also suggested that placental CRH may directly stimulate placental ACTH production (Petraglia et al 1987b) consistent with the findings of these studies of a rise in both these peptides.

In vitro studies using monolayer cultures of placenta and fetal membranes have shown increasing output of CRH after spontaneous labour than after elective caesarean section (not in labour) (Jones et al 1989). However, the results of the study described in chapter 7 do not agree with this finding, as there was no significant difference in placental CRH content between these two groups. In addition, similar in vitro work has shown CRH output by cultured placenta is inhibited by progesterone. Again, it might be expected that placental tissue collected after termination of pregnancy with the antiprogestin mifepristone would show an increase in CRH content, but there was no significant difference between tissues collected by simple curettage and those collected after the administration of mifepristone and prostaglandins.

In vitro cell culture studies have also demonstrated interactions between the activation of the hypothalamo-pituitary-adrenal axis and the control of prostaglandin production. ACTH output is stimulated by the addition of PGE_2 and $PGF_{2\alpha}$ and this effect is blocked by CRH antagonists, indicating that CRH is an intermediary in this process (Petraglia et al 1987b). In addition CRH is able to stimulate PGE_2 and $PGF_{2\alpha}$ production from cultured human placenta and fetal membranes

thus forming a positive feedback loop between prostaglandins, CRH and ACTH. However placental and fetal membrane contents of both CRH and ACTH were not affected by the administation of prostaglandins for the induction of labour at term, nor were placental contents of these peptides affected by the administration of prostaglandins for termination of pregnancy in the first and second trimesters. Consistent with these findings, there was no increase in levels of POMCmRNA in the same tissues, indicating that there was no increase in gene expression.

The results of these studies support previous findings of an increase in placental CRH with advancing gestation and provide conclusive evidence of a rise in not only ACTH content, but POMC gene expression with advancing gestation. This study is the first to localise POMC gene expression in the placenta by in situ hybridisation. Although the results of the studies investigating the modulation of CRH and ACTH secretion by labour or the administration of prostaglandins do not directly support the hypothesis that activation of the hypothalamo-piuitary-adrenal axis is implicated in the onset of human labour, it appears that these hormones are present constitutively in placenta and fetal membranes. This does not preclude more subtle changes in the synthesis or export of these peptides which may be important in the physiological or pharmacological control of uterine contractility. The constitutive presence of ACTH and CRH suggests that they may have a role in the general endocrine milieu of the placenta rather than being simply related to parturition. In addition, it is known that both these peptides enter the fetal circulation and may affect the fetal hypothalamo-pituitary-adrenal axis. In the fetal sheep, activation of the hypothalamo-pituitary-adrenal axis with resultant cortisol production is linked to organ maturation by enhancing enzyme activities (Liggins 1983) and a similar mechanism may exist in humans. Indeed, high levels of CRH have been associated with lung maturation in vivo in humans (Laatikainen et al 1988) and in the developing mouse lung (Keegan et al 1994). This effect may be to facilitate fetal lung maturation by glucocorticoids, either by the stimulation of ACTH

from the pituitary or via a paracrine loop involving ACTH synthesis in the lung itself (Texier et al 1991)

It has been known for a long time that the human placenta secretes large quantities of specific proteins, peptide hormones and steroids, many of which have an unknown function in both maternal and fetal circulations. This has led to suggestions that these hormones are merely waste products of a poorly understood phenomenon (Gordon and Chard 1979). This would seem to be an enormous waste of both energy and amino acids and instead it has been hypothesised that they are present to provide the trophoblast with information about the maternal environment, enabling it to function as a coherent unit (Chard 1993). This may partly account for the presence of ACTH and CRH in the placenta and would not rule out any potential effect on the fetus and a possible role in the onset of parturition.

Conclusions: Placental CRH is located within the syncytiotrophoblasts of the human placenta and within the fetal membranes and decidua. Its concentration rises with advancing gestation and is unaffected by labour, the administration of exogenous prostaglandins or mifepristone. ACTH is located within the cytotrophoblasts in early pregnancy and the syncytiotrophoblasts after the end of the first trimester. Placental content increases with advancing gestation in parallel with POMC gene expression, and these are unaffected by labour, or by exogenous prostaglandins and mifepristone. Thus both CRH and ACTH appear to be constitutively present in the human placenta throughout pregnancy.

Appendix 1. Preparation of TESPA (3, aminopropyltriethoxysilane) coated slides.

Washed slides were cleaned and coated by the following procedures:

1. Baked at 250°C for 6 hours.
2. Immersed in a 2% solution of TESPA in acetone for 30 seconds.
3. Allowed to air dry and then reimmersed in 2% TESPA for 30 seconds.
4. Immersed in acetone for a further 30 seconds.
5. Washed in Still-plus distilled water for 30 seconds.
6. Allowed to air dry.
7. The slides were stored at room temperature.

(Gloves were worn to avoid contamination with RNAse.

Appendix 2. In situ hybridization solution.

A. Solutions.

1. Phosphate buffered saline (PBS), pH 7.2(100 mM),
   The following were added to 800ml of Still-plus water

85.0g	NaCl	(1.3M)
10.7g	Na_2HPO_4	(70mM)
3.9g	NaH_2PO_4	(30mM)

   Made up to 1 litre with Still-plus water.

2. 4% Paraformaldehyde.
   4g of paraformaldehyde was dissolved in 100ml of 10mM PBS by heating to 60°C, with stirring.The solution was allowed to cool and the pH adjusted to 7.4 with concentrated NaOH. The solution was made fresh every 36 hours.

3. 20X Standard Saline Citrate (20XSSC)
175.3g of NaCl and 88.2g of sodium citrate were dissolved in 800mls of Still-plus water, adjusted to pH 7.0 with 1N HCl, and made up to 1L and then autoclaved.

4. 0.2% Glycine.
70mg of glycine were dissolved in 350mls of Still-plus water. This solution was made up fresh every day.

5. Proteinase K
The proteinase K was dissolved in Still-plus water at a stock concentration of 10mg/ml and stored at -20°C.

6. 1N HCl: made up with Still-plus water and autoclaved.

7. 0.1M Tris/HCl
12.1g of Tris base was dissolved in 800mls of Still-plus water and the pH was adjusted to 7.5 with concentrated HCl. The volume of the solution was made up to 1L with Still-plus water.

8. RNAse buffer: 20mM Tris?HCl pH 7.6 containing 1mM EDTA.

B. Hybridization buffer.

1. Deionized Formamide.
50 ml of formamide were mixed with 5g of mixed-bed ion exchange resin (Bio-Rad AG 501-x8, 20-50 mesh). The solution was stirred overnight at 4°C, filtered twice with whatman No.1 filter paper and stored at 20°C.

2. Denhardt's solution (50x).
5mls of Still-plus water were added to each vial of Denhart's (50mg each of BSA, Ficoll and PVP). The solution was aliquoted and stored at -20°C.

3. Salmon testis denatured DNA (10mg/ml)

4. Yeast transfer RNA (10mg/ml)
20mg of yeast transfer RNA was dissolved in 2ml of DEPC treated water and placed on ice for 30 mins, aliquoted and stored at -20°C.

5. 50% (w/v) dextran sulphate.
1g of dextran sulphate was dissolved in 2ml of Still-plus water and stored at 4°C.

6. 1M DTT
5g of DTT was added to 32.4 ml Still-plus water.

C. Hybridization buffer formulae

1. Digoxigenin-labelled Oligonucleotide Hybridisation buffer.

Stock concentration		Final concentration
100%	deionized formamide	43%/37%
20x	SSC	3x
50x (1%w/v)	denhardt's	0.1%
50%(w/v)	dextran sulphate	4%
10mg/ml	tRNA	200µg/ml
10mg/ml	salmon testis DNA	200µg/ml
1M	DTT	10mM

2. ^{35}S -labelled Oligonucleotide hybridisation buffer.

As above

3. ^{35}S-labelled Riboprobe hybridisation buffer.

Stock concentration		Final concentration
100%	deionised formamide	50%
25x	SSPE	5x
50x (1%w/v)	denhardt's	0.1%
10mg/ml	yeast tRNA	200µg/ml
10mg/ml	salmon testis DNA	200µg/ml
10%	SDS	0.1%
1M	DTT	10mM

Appendix 3. Probe labelling.

1. End-terminal oligonucleotide labelling with ^{35}S.

5x Tailing buffer	10µl
(500mM potassium cacodylate,pH 7.2,10mM $CoCl_2$1mM DTT)	
DNA (0.1µM)	4µl
^{35}S dATP (Amersham) (1µM)	4µl
Terminal Transferase (100 units)	5µl
Water	14µl

1. The reaction mixture was incubated for five minutes at 37°C in order to add 10-15 bases.
2. 375µl TE (10mM Tris-HCl, 1mM EDTA ,pH 7.6), 25µl 4M NaCl and 25µg/ml of yeast tRNA were added.
3. 450µl of phenol/chloroform/isoamyl alcohol were added (50:49:1)
4. The top (aqueous) phase was extracted with chloroform/ isoamyl alcohol (49:1)
5. The top (aqueous) phase was mixed with 1ml of ice-cold absolute ethanol and precipitated at -20°C for 1 hour.
6. The sample was spun and the pellet washed twice with ethanol.
7. The pellet was dried in air and then reconstituted in 50µl TE and 1µl 5M DTT.

8. 1µl aliquots were added to 2mls of scintillation fluid and counted in a beta counter.

2. End-terminal oligonucleotide labelling with digoxigenin.

DNA	5µg
dCTP (50nM stock)	1µl
Digoxigenin dUTP(1.25µM)	1.25µl
5x Terminal transferase buffer	10µl
Terminal Transferase	25 units

The 50µl reaction mixture was mixed in a small eppendorf tube and then incubated at 37°C for 2 hours. 2.5µl of 0.5M EDTA was added to stop the reaction. the samples were run on a polyacrylamide gel to analyse the efficiency of the reaction.

16% Polyacrylamide gel

Urea	5.75g
Acrylamide(40%)	5ml
10x TBE	1.25ml
TEMED	10µl
Ammonium Phosphate(10%)	50µl

The samples were run at 150V for 2 hours

3. cDNA Riboprobe labelling with ^{35}S

DNA (1µg)	5µl
5x Buffer	10µl
DTT (1M)	4µl
RNAsin	1µl
NTP mix (water,ATP,CTP,GTP, 1:1:1:1)	8µl
100µM UTP	4.8µl
Water	10µl
^{35}S dCTP	5µl
Polymerase (SP6 or T7)	2µl

1. The reaction mixture was incubated at 40°C for 1 hour , respiked with a further 2µl SP6 and then incubated at 40°C for a further 1 hour
2. The DNA was precipitated with 1/10 volume of sodium acetate pH 6.0 and 2.5 volume of 95% ethanol.
3. The mixture was spun and the pellet washed with 100% and then 70% ethanol.
4. The pellet was dried in air and then resuspended in Stillplus water.
5. 1µl of the cDNA solution was counted in a beta counter.

Appendix 4. Autoradiography.

A. Photographic emulsion

1. 60mls of Kodak emulsion, stored at 4°C, was warmed at 45°C for 45 mins.
2. In the dark , the melted emulsion was carefully poured into a dipping chamber and left for 10 mins for the bubbles to rise to the surface.
3. Two clean, blank slides were dipped in to remove the bubbles.

4. Each slide was dipped and slowly withdrawn from the emulsion. The slides were left overnight standing vertically in a moist chamber in the dark.
5. The slides were transfered into a light-tight box with a vial of silica gel to absorb the mosture.
6. The slides were left at 4°C to expose for 2-6 weeks.

B. Developing the slides.

1. The following solutions were cooled on ice to reduce their temperatures to 15°C.

1. 350mls of Kodak developer.
2. 400mls of distilled water.
3. 350mls Kodak fixer (1in9 dilution)

In the dark, the slides were taken through

2. distilled water for 30 seconds
3. fixer for 10 mins.

The slides were then washed in distilled water before counterstaining with haematoxylin and then dehydrated in clean graded ethanols.

Appendix 5. Preparation of solutions for immunocytochemistry.

1. 0.5M TRIS buffer.

60.5g of Tris(hydroxymethlmethylamine) was dissolved in 800mls of distilled water. The pH was adjusted to 7.6 with concentrated HCl.

2. Tris/NaCl buffer.

0.5M Tris buffer	100mls
NaCl	8.5g

make up to 1L with distilled water.

3. Methanol/Hydrogen Peroxide solution (3%)

Hydrogen Peroxide (30%)	30mls
Methanol	270mls

4. Tris/HCl buffer .(0.05M)

0.5M Tris (pH 7.6)	10mls
distilled water	19mls

5. Trypsin Digestion solution (0.1%)

Trypsin (type II)	300mg
$CaCl_2$ (dihydrate)	300mg
Distilled water	300ml

The pH was adjusted to 7.4 with concentrated sodium hydroxide.

6. Normal Sheep serum/ 5% Bovine serum albumin.

Normal sheep serum	10mls
Bovine serum albumin	2.5g
Tris buffered saline	40mls

7. 0.5% Triton-X-100.

Triton-X-100	1.5mls
Distilled water	300mls

8. Avidin-Biotin complex Peroxidase conjugated. (DAKOPATTS)

Avidin	1 drop
Biotinylated horseradish peroxidase	1 drop
Tris/HCl	5mls

Freshly made up 20 mins before use.

9. Chromogen solutions

a. Diaminobenzidine tetrahydrochloride

Diaminobenzidine tetrahydrochloride	5mg
0.05M Tris	10mls
Hydrogen peroxide 0.01%	10µl

This solution was made up immediately prior to use.

b. NBT

Tris/$MgCl_2$ pH 9.5	10 mls
NBT	45µl
X-Phosphate	35µl
Levimasole	10µl

Appendix 6. Deprotection of oligodeoxynucleotide probes.

The CRH oligonucleotides were synthesised on a 391 DNA Synthesizet (Applied Biosystem, PCR-MATE). The next day, the columns containing the synthesised oligonucleotides was removed and the oligonucleotides deprotected as follows:

1. The two ends of the column was connected to two sterile 10ml syringes, one of which contained 2mls of cold ammonia (stored at 4°C).
2. Ammonia was pushed through the column several times and then left in the column for 30 minutes before transfer to a 15ml sterile tube .
3. The solution, capped and sealed with labfilm, was incubated at 55°C in a waterbath overnight to deprotect the oligo.
4. 1/10 volume of 3M sodium acetate, pH 7.0 and 2x volume of 100% ethanol were added to a 400µl aliquot of the deprotected oligo. After precipitation at -70°C for 15 minutes, the oligo was

pelleted by centrifugation at full speed for 10 minutes in a microcentrifuge.

5. The pellet was washed once with 500µl of 70% ethanol and freeze dried.
6. The pellet was then resuspended in 200µl sterile water. The concentration of oligonucleotide was determined by measuring the O.D.$_{260}$ of 1µl of oligo solution in 1ml of distilled water.

Appendix 7.

Solutions for ACTH radioimmunoassay.

Assay buffer

63mM	Na_2HPO_4
13mM	$Na_2EDTA.2H_2O$
0.02%	NaN_3
0.1%	Triton-X100
250 Kallikrein Inhibitor Units /ml	Aprotinin

All standards, antibody and label made up in ACTH assay buffer.

Aboud, T.K. (1988) Maternal and fetal b-endorphin: effects of pregnancy and labour. Archives of Diseases of Children **63** : 707 - 709

Adelantado, J.M., Humphrey, S.J., Lopez-Bernal, A., Turnbull, A.C. (1985) Is there a topographical distribution of prostaglandin E2 receptors in the pregnant human uterus? In: Jones C., Nathanielsz P.W., eds The Physiological Development of the Fetus and Newborn. London Academic Press 489 - 492

Affolter, H-U and Reisine, T. (1985) Journal of Biological chemistry **260** : 15477 - 15481

Alexandrovo, M., Soloff, M.S. (1980) Oxytocin receptors and parturition. 1. Control of oxytocin receptor concentration in the rat myometrium at term. Endocrinology **106** : 730 - 735

Al-Timimi, A. & Fox, H. (1986) Immunohistochemical localisation of follicle-stimulating hormone, luteinising hormone, adrenocorticotrophic hormone and prolactin in the human placenta. Placenta, **7:** 163-172

Anderson, A.B.M., Turnbull, A.C. (1969) Relationship between length of gestation and cervical dilatation, uterine contractility and other factors during pregnancy. American Journal of Obstetrics and Gynaecology. **105** : 1207 - 1214

Arbiser, J.L., Morton, C.C., Bruns, G.A., Majzoub, J.A. (1988) The human corticotrophin releasing hormone gene is located on the long arm of chromosome 8. Cytogenetics Cell Genetics **47** : 113 - 116

Assali, N.S., and Hammermesz, J. (1954). Adrenocorticotropic substances from human placenta. Endocrinology **55** : 561 - 567.

Baird, A., Wehremberg, B., Bohlen, P. (1985) Immunoreactive and bioactive growth hormone releasing factor in the placenta. Endocrinology **117** : 1598-1693

Barnea, E.R., Lavy, G., Fakih, H., Decherney, A.H. (1986) The role of ACTH in steroidogenesis. Placenta **7** : 307-313

Behan, D.P., Linton, E.A. & Lowry P.J. (1989) Isolation of the human plasma corticotrophin- releasing factor binding protein. Journal of Endocrinology, **122** : 23-31

Benirschke, K. (1956). Adrenals in anencephaly and hydrocephaly Obstetrics and Gynaecology **8** : 412-415

Bilezikjian, L.M. Vale, W. (1982) Glucocorticoids inhibit corticotrophin-releasing factor induced production of adenosine 3,5-monophosphate in cultured anterior pituitary cells. Endocrinology **113** : 657-662

Bolte, E., Mancuso, S., Eriksson, G., Wiqvist, N., Diczfalusy, E. (1964). Studies on the aromatization of neutral steroids in pregnant women. I. Aromatisation of C-19 steroids by placentas perfused in situ. Acta Endocrinologica. **45** : 535-559

Branchdud, C.L., Goodyear, C.G., Lipowski, L.S. (1983) Progesterone and Oestrogen production by placental monolayer cultures; effect of dehydroepiandrosterone and LH-releasing hormone. Journal of Clinical Endocrinology and Metabolism **56** : 761-766

Braunstein, G.D., Rasor, J., Adler, D., Danzer, H., Wade, M.E. (1976) Serum human chorionic gonadotrophin levels throughout normal pregnancy. American Jornal of Obstetrics and Gynaecology **126** : 678-681.

Braunstein, G.D. (1988) Physiological functions of human chorionic gonadotrophin during pregnancy. In Mochizuki M., Hussa R. (eds): Placental Protein Hormones. Amsterdam: Elsevier Science Publishers BV pp33 - 41

Brigati, D.J., Myerson, D., Leary, J.J., Spalholtz, B., Travis, S.Z., Fong, S.Z., Hsiung, G.D., Ward, D.C. (1983). Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin labelled hybridisation probes.

Brooks, A.N., Challis, J.R.G. (1988). Regulation of the hypothalamopituitaryadrenal axis at birth. Canadian Journal of Physiology and Pharmacology. **66** : 1106-1111

Buongiorno-Nardelli, M., and Amaldi. P. (1969) Autoradiographic detection of molecular hybrids between rRNA andDNA in tissue sections. Nature. **225** : 946-7

Burns, J., Graham, A.K., Frank, C., Fleming, A.K., Evans, M.F., McGee, J. O'D. (1987) Detection of low copy human papilloma virus DNA and mRNA in routine paraffin sections of cervix by non-isotopic ISH. Journal of Clinical Pathology. **40**. 858-864

Cabrol, D., Dubois, P., Sedbon, E. (1987). Prostaglandin E2 induced changes in the distribution of glycosaminoglycans in the isolated rat uterine cervix. European Journal of Obstetrics Gynaecology and Reproductive Biology. **26** : 359-365

Calder, A.A. (1980) Pharmacological manangement of the unripe cervix in the human. In: Naftolin F, Stubblefield P.G. eds. Dilatation of the Uterine cervix. New York; Ravens Press. 1980 : 317-333

Caldeyro-Barcia, R. (1959) Uterine contractility in obstetrics. In: Proceedings of the second International Congress of Gynaecology and Obstetrics. Montreal Vol 1. 65-78

Caldeyro-Barcia, R., Sereno, J.A. (1961). The response of the human uterus to oxyocin throughout pregnancy. In: Caldeyro-Barcia R., Heller H. eds Oxytocin. Oxford: Pergammen Press. 177

Campbell, E.A., Linton, E.A., Wolfe, C.D.A., Scraggs, P.R., Jones, M.T. &Lowry, P.J. (1987) Plasma corticotrophin releasing hormone concentrations during pregnancy and parturition. Journal of Clinical Endocrinology and Metabolism, **63** : 1054-1059

Carr, B.R., Parker, C.R., Madden, J.D., MacDonald, P.C., & Porter, J.C. (1981) Maternal plasma corticotrophin and cortisol relationships during human pregnancy. American Journal of Obstetrics and Gynaecology. **139** : 416-422

Carsten, M.E., and Miller, J.D. (1987). A new look at uterine muscle contraction. American Journal of Obstetrics and Gynaecology. **157** : 1303-1315

Challis, J.R.G., & Olsen, D.M. (1988) Parturition. In the physiology of reproduction (Eds) Knobil. E & Neill, J. pp2177-2216. New York: Raven Press.

Challis, J.R.G., Brooks, A.N., Jones, S.A., Mitchell, B.F., Power, S.G.A. (1988) Placental, membrane and uterine interactions in the control of birth. Fetal and Neonatal Development; Perinatology press.

Challis, J.R.G. & Brooks, A.N. (1989) Maturation and activation of the hypothalamopituitary adrenal function in fetal sheep. Endocrine reviews. **10** : 182-204

Challis, J.R.G. & Hooper, S. (1989) Birth : outcome of a positive cascade. Balliere's Clinical Endocrinology and Metabolism. **3** : 781-793

Chan E.C., Smith R. (1992) Beta- Endorphin immunoreactivity during human pregnancy. Journal of Clinical Endocrinology and Metabolism **75** : 1453-1458

Chan, E-C., Smith, R., Lewin, T., Brimsmead, M.W., Zhang, H-P., Cubis, J., Thornton, K., Hurt, D. (1993) Plasma corticotrophin releasing hormone, β-endorphin and cortisol inter-relationships during human pregnancy. Acta Endocrinologica **128** : 339-344

Chard, T. (1993) Placental Radar. Journal of Endocrinology **138** : 177-179

Chen, R., Lewis, K.A., Perrin, M.H., Vale, W. (1993). Expression cloning of a human corticotrophin releasing factor receptor. Proceeding of National Academy of Sciences. **90** : 8967-8971

Cheng, C.-L.-C., Chang, C.-C., Krieger, D.T. & Bardin, C.W. (1986) Expression and regulation of the pro-opiomelanocortin-like gene in the ovary and placenta. Endocrinology. **118** : 2382-2389

Cheung, P.Y.C., Walton, J.C.,Tai. H.-H.., Riley, S.C. & Challis, J.R.G. (1990) Immunocytochemical distribution and localisation of 15-hydroxyprostaglandin dehydrogenase in human fetal membranes, decidua, and placenta. American Journal of Obstetrics and Gynaecology. **163** : 1445-1449

Clark, D., Thody, A.J., Shuster, S., Bowers, H. (1978). Immunoreactive α-MSH in human plasma in pregnancy. Nature. **273** : 163-164

Csapo, A.I. (1961). Defence mechanisms of pregnancy. In: Progesterone and the defence mechanism of pregnancy. Ciba Foundation Study Group No 9. Boston ; Little Brown, 1-27

Cunnah, D., Jessop, D., Besser, G.M., Rees, L.H. (1987) Measurement of circulating corticotrophin-releasing factor in man. Journal of Endocrinology. **113** : 123-131

Danforth, D.N. (1983). The morphology of the human cervix. Clinical Obstetrics and Gynaecology. **26** : 7-13

De Souza, E., Perrin, M., Rivier, J., Vale, W.W., Kuhar, M.J. (1984) Corticotrophin-releasing factor receptors in rat pituitary gland; autogtraphic localisation. Brain Research **296** : 202-209

De Souza, E.B. (1985) Beta-2 adrenergic receptors in the pituitary. Neuroendocrinology. **41** : 289-293

De Souza, E.B., Whitehouse, P.J., Kuhar, M., Price, D.L., Vale, W.W. (1986) Reciprocal changes in CRF-like immunoreactivity with CRF receptors in the cerebral cortex in alzheimers disease. Nature. 319 :593-595

Demey-Ponsart, E., Foidart, J.M., Sulon, J., Sodoyez, T. (1982) Serum CBG, free and total cortisol and circadian patterns of adrenal function in normal pregnancy. Journal of Steroid Biochemistry. **16** : 165-169

Dewied, D., Van Ree, J.M., Greven, H.M. (1980) Neuroleptic-like activity of peptides related to [des-Tyr]γ-endorphin: structure activity studies. Life Sciences **26** : 1575-1579

Divers, W.A., Wilkers, M.M., Babaknia, A., Yen, S.S.C. (1981). An increase in catecholamines in the amniotic fluid compartment from middle to late gestation. American Journal of Obstetrics and Gynaecology **139** : 483-489

Dupouy, J.P., Chatelain, A., & Allaume, P. (1980). Absence of transplacental passage of ACTH in the rat: direct experimental proof. Biology of the neonate **37** : 96-102

Economides, D., Linton, E., Nicolaides, K., Rodeck, C.H., Lowry, P.J. & Chard, T. (1987) Relationship between maternal and fetal corticotrophin releasing hormone-41 and ACTH levels in human mid-trimester pregnancy. Journal of Endocrinology. **114** : 497-501

Ellis, M.J., Livesey, J.H., Donald, R.A. (1988) Circulating Corticotrophin-releasing factor like immunoreactivity. Journal of Endocrinology **117** : 299-307

Flint, A.F.P., Anderson, A.B.M., Steel, P.A., Turnbull, A.C. (1975). The mechanism by which fetal cortisol controls the onset of parturition in the sheep. Biochemical Society **3** : 1189

Fraioli, F., Genazzini, A.R. (1980) Human placenta β-Endorphin. Gynaecological and Obstetrical Investigation **11** : 37-41

Frankenne, F., Closset, J., Gomez, F., Scippo, ML., Smal, J., Hennen, G. (1988) The physiology of growth hormones in pregnant women and partial characterisation of the placental growth hormone variant. Journal of Clinincal Endocrinology and Metabolism **66** : 1171-1180

Freemark, M., Handwerger, S. (1989) The role of placental lactogen in the regulation of fetal metabolism and growth. Journal of Paediatric Gastroenterology and Nutrition. **8** : 281-287

Frim, D.M., Emmanuel, R.L., Robinson, B.G., Smas, C.M., Adler, G.K. & Majzoub, J.A. (1988) Characterisation and gestational regulation of corticotrophin releasing hormone messenger RNA in human placenta. Journal of Clinical Investigation. **82** : 287-292

Fuchs, A.R., Fuchs, F., Husslein, P., Soloff, M.S. Fernstrom, M.J. (1982). Oxytocin receptors and human parturition: a dual role for oxytocin in the initiation of labour. Science (New York) **215** : 1396-1399

Fuchs, A.R. (1983) The role of oxytocin in parturition. Current topics in Experimental Endocrinology. **4** : 231-265

Furuhashi, N., Takahashi, T., Jibim, G. (1986) Plasma β-endorphin levels in pregnancy induced hypertension patients. Clin Exp Hypertens [B] Hypertens Pregnancy. **B5** 1-8

Gall, G. and Pardue, M.L. (1969). Formation and detection of DNA-RNA hybrid molecules in cytological preparations. Proceedings of the National Acadamy of sciences. **63** : 378-381

Garfield, R.E., Sims, S., Daniel, E.E. (1977) Gap junctions: their presence and necessity in myometrium during gestation. Science (New York) **198** : 958-960

Garfield, R.E., Kannan, M.S., Daniel, E.E. (1980a) Gap junction formation in myometrium: control by oestrogens, progesterone and prostaglandins. American Journal of Physiology. **238** : c81-89

Garfield, R.E., Merrett, D., Grover, A.K. (1980b). Gap junction formation and regulation in myometrium. American Journal of Physiology. **239** : C217-228

Garfield, R.E., Hayashi, R.H. (1981) Appearance of gap junctions in the myometrium of women during labour. American Journal of Obsterics and Gynaecology. **140** : 254-260

Garfield, R.E., Baulieu, E.E. (1987) The antiprogesterone steroid RU486: a short pharmacological and clinical review with emphasis on the interruption of pregnancy. Bailliere's Clinical Endocrinology and Metabolism, **1** : 207-221

Genazzani, A.R., Fraioli, F., Hurlimann., J., Fioretti, P., Felber, J.P. (1975) Immunoreactive ACTH and cortisol plasma levels during pregnancy. Detection and partial purification of corticotrophin-like placental hormone: the human chorionic corticotrophin (HCC) Clinical Endocrinology. **4** : 1-14

Gibbons, J.M., Mitnick, M., Chieffo, V. (1975) In vitro biosynthesis of TSH and LH releasing factors by the human placenta. American Journal of Obstetrics and Gynaecology. **121** : 127-131

Goland, R.S., Wardlaw, S.L., Stark, R.I., Frantz, A.G. (1981) Human plasma b-endorphin during pregnancy, labour and delivery. Journal of Clinical Endocrinology and Metabolism. **52** : 74-78

Goland, R.S., Wardlaw, S.L., Stark, R.I., Brown, L.S. & Frantz, A.G. (1986) High levels of corticotrophin releasing hormone immunoreactivity in maternal and fetal plasma during pregnancy. Journal of Clinical Endocrinology and Metabolism. **63** : 1199-1203

Goland, R.S., Wardlaw, S.L., Blum, M., Tropper, P.J., & Stark, R.I. (1988) Biologically active corticotrophin releasing hormone in maternal and fetal plasma during pregnancy. American Journal of Obstetrics and Gynaecology. **159** : 884-890

Goland, R.S., Jozak, S., Warren, W.B., Conwell, I.M., Stark, R.I., Tropper, P.J. (1993). Elevated levels of Umbilical Cord Plasma Corticotrophin Releasing Hormone in Growth-Retarded Fetuses. Journal of Clinical Endocrinology and Metabolism. **77** : 1174-1179

Golander, A., Hurley, T., Barrett, J., Hizi, A., Handwerger, S. (1978) Prolactin synthesis by human chorio-decidual tissue: a possible source of amniotic fluid prolactin. Science **202** : 311-313

Golander, A., Zakuth, V., Shechter, Y. Spirer, Z. (1984) Supression of lymphocyte activity in vitro by a soluble factor secreted by explants of human decidua. European Journal of Immunology. **11** : 849-851

Gordon A.J., Calder A.A. (1977). Oestradiol applied locally to ripen the unfavourable cervix. Lancet ii : 1319

Gordon, Y.B. Chard, T. (1979) In placental proteins Eds A. Klopper & T. Chard Springer-Verlag Berlin pp1-21

Gosseye, S. & Fox, H. (1984) An immunohistochemical comparison of the secretory capacity of villous and extravillous trophoblast in the human placenta. Placenta. **5** : 329-348

Greer, I.A., Calder, A.A. (1989). Preinduction cervical ripening with extra-amniotic and cervical prostaglandin E_2. Journal of Obstetrics and Gynaecology. **10** : 18-22

Grino, M., Chrousos, G.P. & Marjioris, A.N. (1987) The corticotrophin releasing hormone gene is expressed in human placenta. Biochemical and Biophysical research communications. **148** : 1208-1214

Grossman, A., Kruseman, A.C.H., Perry, L., Tomlin, S., Besser, G.M. (1982) A new hypothalamic hormone corticotrophin-releasing factor, specifically stimulates the release of adrenocorticotrophic hormone and cortisol in man. Lancet. **2** : 921-925

Grosso, D.S., MacDonald, C.P., Thomasson, J.E., Christian, C.D. (1980) Relationship of newborn serum prolactin levels to the respiratory distress syndrome and maternal hypertension. American Journal of Obstetrics and Gynaecology. **137** : 569-575

Grumbach, MM., Kaplan, S.L. (1964). On the placental origin and purification of chorionic 'growth hormome prolactin' and its immunoassay in pregnancy. Trans NY Acad Sci. **27** : 167-188

Grumbach, M.M., Kaplan, S.L, Sciarra, J.J., Burr, I.M. (1968) Chorionic growth hormone production (CGP): secretion, disposition, biological activity in man, and postulated function as the 'growth hormone" of the second half of pregnancy. Annals of the New York Academy of Sciences. **148** : 501-531

Hafen, E., Levine, M., Garber, R.L. and Gehring, W.J (1983), An improved in situ hybridisation method for the detection of cellular RNAs in Drosophila tissue sections. EMBO Journal **2(4) :** 617-623

Hillhouse, E.W., Grammatopoulos, D., Milton, N.G.N., Quatero, H.W.P. (1993) The identification of a myometrial corticotrophin releasing hormone receptor that increases in affinity during pregnancy. Journal of Clinical Endocrinology and Metabolism. **76** : 736-741

Hillier, K., Calder, A.A., Embrey, M. (1974) Concentrations of prostaglandin F2a in amniotic fluid and plasma in spontaneous and induced labours. Journal of Obstetrics and Gynaecology of the British Commonwealth. **81** : 257-261

Hodgen, G.D., Guylas, B.J. and Tullner, W.W. (1975) Role of primate placenta in cortisol secretion by the maternal adrenals. Steroids. **26** : 233-240

Hofler, H., Childers, H., Montminy, M.R., Lechan, R.M., Goodman. R.H., Wolfe, H.J. (1986). In situ hybridisation methods for the detection of somatostatin mRNA in tissue sections using antisense RNA probes, Journal of Histochemistry. **18** : 597-604

Jaffe, R.B. Seron- Ferre, M., Crickard, K., Kortnik, D., Mitchell, B.F., Huhtaniemi, I.T. (1981) Regulation and function of the fetal primate adrenal gland and gonad. Recent progress in hormonal research. **37** : 41-103

Jailer, J.W., and Knowlton, S. (1950). Adrenal-like activity in a pregnant Addisonian. J. Clin Invest. **29** : 1430-1436

Jingami, H., Mizuno, N., Takahashi, H., Shibahara, S., Furutani, Y., Imura, H., Numa, S. (1985) Cloning and sequence analysis of cDNA for rat corticotrophin-relreasing factor precursor. FEBS letters. **191** : 63-65

John, H.L., Birnstiel, M.L., Jones, K.W. (1969) RNA-DNA hybrids at the cytological level. Nature. **223** : 912-913

Johnston, T.A., Greer, I.A., Kelly, R.W., Gallacher, A., Calder, A.A. (1990). Plasma prostaglandin metabolites in human labour. In: 7th International; Conference on Prostaglandins and Related Compounds, Florence. Milan: Fondazionne Giovanni **165.**

Jones, S.A., & Challis, J.R.G. (1989) Local stimulation of prostaglandin production by corticotrophin releasing hormone in human fetal membranes and placenta. Biochemical and Biophysical research communications. **159** : 192-199

Jones, S.A., & Challis, J.R.G. (1990a) Steroid, corticotrophin-releasing hormone, ACTH and prostaglandin interactions in the amnion and placenta of early pregnancy in man. Journal of Endocrinology. **125** : 153-159

Jones, S.A., & Challis, J.R.G. (1990b) Effects of corticotrophin releasing hormone and adrenocorticotrophin on prostaglandin output by hu,man fetal membranes and placenta. Gynaecological and Obstetric Investigation. **290** : 165-168

Jones, S.A., Brooks, A.N., Challis, J.R.G. (1989) Steroids modulate corticotrophin releasing factor production in human fetal membranes and placenta. Journal of Clinical Endocrinology and Metabolism. **68** : 825-830

Junquiera, L.C.U., Zugaib, M., Montes, G.S., Toledo, D.M.S., Krisztian, R.M., Shigihara, K.M. (1980). Morphological and histochemical evidence for the occurence of collagenolysis and for the role of neutrophilic polymorphonuclear leucocytes during cervival dilatation. American Journal of Obstetrics and Gynaecology. **138** : 273-281

Kearns, M. & Lala, P.K. (1983). Life history of decidual cells; a review. American Journal of Reproductive Immunological Microbiology. **3** : 78-82

Keegan, C.E., Herman, J.P., Karolyi, I.J., O'Shea, S., Camper, S.A., Seasholtz, A.F. (1994) Differential Expression of Corticotrophin Releasing Hormone in Developing Mouse Embryos and Adult Brain. Endocrinology. **134** : 2547-2555

Kelly, A.C., Rodgers, A., Dong, Ke-Wen. (1991) Gonadotrophin releasing hormone and Human Chorionic Gonadotrophin gene expression in human placental development. DNA cell Biology. **10** : 411-421

Kessler, C. (1990) The digoxigenin system: principle and applications of the novel nonradioactive DNA labelling and detection system. Biotechnology International. 183-194

Khoder, G.S., Siler-Khoder, T.M. (1978) The effect of luteinising hormone releasing factor (LRF) on HCG secretion. Fertility and Sterility. **30** : 301306

Khoder, G.S., Siler-Khoder, T.M. (1980) Placental LH releasing factor and its synthesis. Science. **207** : 315-318

Klausner, D.A., & Ryan, K.J. (1964). Estriol secretion by the human term placenta. Journal of Clinical Endocrinology and Metabolism. **24** : 101-104

Kierse M.J.N.C. (1979). Endogenous prostaglandins in humAn parturition. In: Kierse, M.J.NC., Anderson, A.B.M., Gravenhorst, J. eds. Human Parturition. The Hague: Leiden University Press 101-141

Kishimoto, Y., Tominaga, T., Aso, T., Kinoshita, M., and Mori, T. (1987). Human trophoblast and endometrial interactions in vitro. Acta Obstetrica Gynaecologica Japan. **39** : 463-470

Knobil, E., & Briggs, F.M. (1955). Fetal- maternal endocrine interrelationships; The hypophyseal-adrenal system. Endocrinology. **57** : 147-152

Krieger, D.T., Liotta, A.S., Brownstein, M.J., Zimmerman, E.A. (1980). ACTH, b-lipotropin and related peptides in brain, pituitary and blood. Recent progress in Hormonal Research. **36** : 272-344

Kurki, T., Laatikainen, T., Salminen-Lappalainen, K., Ylikorkala, O. (1991). Maternal plasma corticotrophin releasing hormone- elevated in preterm labour but unaffected by indomethacin or nylidrin. **98** : 685-691.

Laatikainen, T.J., Saijonmaa, O., Salminen, K., Wahlstrom, T. (1987a) Localization and concentration of b-endorphin and b-lipotrophin to human placenta. Placenta **8** : 381-387

Laatikainen, T.J., Virtanen, T., Raisenen, I., Salminen, K. (1987b) Immunoreactive corticotrophin releasing factor and corticotrophin in plasma during pregnancy, labour and the puerperium. Neuropeptides. **10** : 343-353

Laatikainen, T.J., Raisenen, I.J., Salminen, K., (1988) Corticotrophin releasing hormone in amniotic fluid during gestation and labour and in relation to fetal lung maturation.European journal of Obstetrics and Gynaecology Reproductive Biology. **29** : 23-29

Laatikainen, T.J., Virtainen, T., Kaaja, R., Salminen, K., Lappalainen, K. (1991) Corticotrophin releasing hormone in maternal and cord plasma in pre-eclampsia. European journal of Obstetrics and Gynaecology Reproductive Biology. **39** : 19-25

Ledger, W.L., Webster, M., Harrison, L.P., Anderson, A.B.M., Turnbull, A.C. (1985). Increase in cervical extensibility during labour induced after isolation of the cervix from the uterus in the pregnant sheep. American Journal of Obstetrics and Gynaecology. **151** : 397-402

Lee, J.N., Seppala, M., Chard, T. (1981) Characterization of placental LH-releasing factor like material. Acta Endocrinologica. **96** : 394-399.

Lee, J.N., Wu, P., Chard, T. (1982). Identification of somatostatin in human placenta. Acta Endocrinologica, **99** : 601-604

Liggins, G.C. (1983) Initiation of spontaneous labour. Clinical Obstetrics and Gynaecology. **26** : 47-55

Linton, E.A. & Lowry, P.J. (1986) A large molecular carrier of CRH-41 in human plasma. Journal of Endocrinology. **111**(supp): Abstract 150

Linton, E.A., Mclean, C., Niewenhuysen Krusman, A.C., Tilders, F.J., Van der Veen, E.A., Lowry, P.J. (1987) Direct measurement of human plasma CRH by a 'two-site' immunoradiometric assay. Journal of Clinical Endocrinology and Metabolism. **64** : 1047-1053

Linton, E.A., Wolfe, C.D.A., Behan, D.P., Lowry P.J. (1988) A specific carrier substance for human corticotrophin releasing factor in the late gestational maternal plasma which could mask the ACTH-releasing activity. Clinical Endocrinology. **28** : 315-324

Linton, E.A., Behan, D.P., Saphier, P.W., Lowry, P.J. (1990) Corticotrophin releasing hormone (CRH) binding protein: reduction in the adrenocorticotrophin- releasing activity of placental but not hypothalamic CRH. Journal of Clinical Endocrinlogy and Metabolism. **70** : 1574-1580

Linton, E.A., Perkins, A., Woods, R. (1992) Measurement of CRH-BP in normal human pregnancy and early post partum. Journal of Endocrinology. **132**(suppl): 39

Linton, E.A.., Perkins, A.V., Woods, R.J., Eben, F., Wolfe, C.D.A., Behan, D.P., Potter, E., Vale, W.W., Lowry, P.J. (1993) Corticotrophin-releasing hormone binding protein (CRH-BP): Plasma levels decrease during the third trimester of normal human pregnancy. Journal of Clinical Endocrinology and Metabolism. **76** : 260-262

Liotta, A.S., Osathanondh, R., Ryan, K.J. & Krieger, D.T.(1977) Presence of corticotrophin in human placenta: demonstration of in vitro synthesis. Endocrinology. **101** : 1 552-1558

Liotta, A.S. Krieger, D.T. (1980) In vitro biosynthesis and comparative post translational processing of immunoreactive precursor corticotrophin / b-endorphin by human placenta and pituitary cells Endocrinology. **106** : 1504-1510

Mackenzie, L.W., Garfield, R.E. (1985) Hormonal control of gap junctions in the myometrium. American Journal of Physiology. **248 :** C296-308

Maclennan, A.H., Green, R.C., Grant, P., Nicolson, R. (1986) Ripening of the human cervix and induction of labour with intracervical purified porcine relaxin. Obstetrics and Gynaecology. **68** : 598-601

Margioris, A.N., Grino, M., Protos, M., Gold, P.W. & Chrousos, G.P. (1988a) Corticotrophin releasing hormone and oxytocin stimulate the release of placental proopiomelanocortin peptides. Journal of Clinical Endocrinology and Metabolism. **66** : 922-926

Margioris, A.N., Grino, M., Rabin, D., Chrousos, G.P. (1988b) Human placenta and the hypothalamopituitaryadrenal axis. Advances in Experimental Medicine and Biology. **245** : 389-398

Marsten, J.B., and Smith, C.W.J. (1985), The thin filaments of smooth muscles. Journal of Muscle Research and Cell Motility. **6** : 669-708

Maser-Gluth, C., Lorenz, U. & Vecsei, P. (1987) In pregnancy, corticotrophin releasing factor in maternal blood and amniotic fluid correlates with gestational age. Hormone and Metabolic Research. **16**:suppl 42-44

Masuhiro, K., Matsusaki, N., Nishino, E., Taniguchi, T., Kameda, T., Li, Y., Saji, F., Tanizawa, O. (1991) Trophoblast-derived interleukin-1 stimulates the release of human chorionic gonadotrophin by activating IL-6 and IL-6 receptor systems in first trimester human trophoblasts. Journal of Clinical Endocrinology and Metabolism. **72** : 594-601

McLachlan, R.L., Healy, T.L. Robertson, D.M. (1986) The human placenta, a novel source of inhibin. Biochemical Biophysical Research Communications. **140** : 483-490

Meinkoth, J. and Wahl, G. (1984). Review: Hybridisation of nucleic acids immobilised on solid supports. Analytical Biochemistry. **138** : 267-284

Merz, W.E., Erlewein, C., Licht, P., Harbarth, P. (1991). The secretion of human chorionic gonadotrophin as well as the a and b messenger RNA levels are stimulated by exogenous gonadoliberin pulses applied to first trimester placenta in a superfusion culture system. Journal of Clinical Endocrinology and Metabolism. **73** : 84-92

Midgely, A.R., Pierce, G.B., Deneau, G.A., Gosling, J.R.S. (1963) Morphogenesis of syncytiotrophoblast in vivo: an autoradiographic demonstration. Science. **141** : 349-353

Miyakava, I., Ikeda, I., Maeyama, M. (1974) Exogenous ACTH does not cross placental barriers. Journal of Clinical Endocrinology and Metabolism. **39** : 440-442

Miyake, A., Sakimoto R., Anno, T. (1982) Changes in LH releasing hormone in human placenta throughout pregnancy. Obstetrics and Gynaecology. **60** : 144-150

Nagashima, K., Yagi, H., Yunoki, H., Noji, T., Kuroume, T. (1987) Cord blood levels of corticotrophin-releasing factor. Biology of the Neonate. **51**: 1-4

Nakai, Y., Nakao, K., Oki, S., Immura, H. (1984) Presence of immunoreactive b-lipoprotein and b-endorphin in human placenta. Life Sciences. **23** : 2013-2018

Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A.C.Y., Cohen, S.M., Numa, S. (1979). Nucleotide sequence of cloned cDNA for bovine corticotrophin-b-lipotrophin precursor. Nature. **278** : 423-427

Namba, Y., Smith, J.B., Fox, G.S., Challis, J.R.G. (1980) Plasma cortisol levels during caesarean sections. British Journal of Anaesthetics. **52** : 1027-1031

Newnham, J.P., Tomlin, S., Ratter, S.J. (1983) Endogenous opiod peptides in pregnancy. British Journal of Obstetrics and Gynaecology. **90** : 535-538

Nicolson, W.E., Decherney, S.G., Jackson, R.V., DeBold, R.C., Uderman, H., Alexander, N.A., Rivier, J., Vale, W., Orth, D.N. (1993) Plasma distribution, disappearance, half time, metabolic clearance and degradation of synthetic ovine CRH in man. Journal of Clinical Endocrinology and Metabolism. **57** : 1263-1269

Nolten, W.E., Lindheimer, M.D., Rueckert, P.A., Oparil, S., Ehrlich, E.N. (1980) Diurnal patterns and regulation of cortisol secretion in pregnancy. American Journal of Obstetrics and Gynaecology. **139** : 492-498

Nolten, W.E., and Rueckert, P.A. (1981) Elevated cortisol index in pregnancy; possible regulator mechanisms. American Journal of Obstetrics and Gynaecology. **139** : 492-498

Odagiri, E., Scherrell, B.J., Mount, C.D., Nicolson, W.E., Orth, D.N. (1979) Human placental immunoreactive corticoptrophin, lipotrophin and beta endorphin; evidence for a common precursor. Proceedings of the National Academy of Sciences. USA. **76** : 2027-2031

Okamoto, E., Takagi, T., Sata, H., Iwata, I., Nishino, E., Mitsuda, N., Sugita, N., Otsuki, Y., & Tanizawa, O. (1989) Immunoreactive corticotrophin releasing factor, adrenocorticotrophin and cortisol in human plasma during pregnancy, delivery and postpartum. Hormone and Metabolic Research. **21** : 566-572

Okamoto, E., Takagi, T., Azuma, C., Kimura, T., Tokugawa, Y., Mitsuda, N., Saji, F., Tanizawa, O. (1990). Expression of Corticotrophin-Releasing Hormone (CRH) gene in Human Placenta and Amniotic membrane. Hormonal and Metabolic Research. **22** : 394-397

Olsen, D.M., Skinner, K., Challis, J.R.G. (1983) Estradiol 17-b and 2-hydroxysterol 17-b-induced differerential production of prostaglandins by cells dispersed from human interuterine tissues at parturition. Prostaglandins. **25** : 639-674

Olubadewo, J.O., Rama Sastri, B.V. (1977). Human placental cholinergic system ; stimulation-secretion coupling for release of acetylcholine from isolated placental villus. Journal of Pharmacology and Experimental Therapeutics. **204** : 433-436

Orth, D.N. & Mount, C.D. (1987) Specific high affinity binding protein for corticotrophin releasing hormone in normal human plasma. Biochemical and Biophysical Research Communications. **143** : 411-417

Osathanondh, R., Tulchinsky, D. (1980). Placental polypeptide hormones. In Tulchinsky D, Ryan K.J. eds. Maternal-Fetal endocrinology Philadelphia W.B Saunders 1980 17-42

Patrick, J., Challis, J., Campbell, K., Carmichael, L., Renato, N., Richardson, B. (1980) Circadian rythmns in maternal plasma cortisol and oestriol concentrations at 30 to 31, 34 to 35 and 38 to 39 weeks gestational age. American Journal of Obstetrics and Gynaecology **136** : 325-334

Pelletier, G., Desy, L., Cote, J., Vaudry, H., (1983). Immunocyochemical localization of corticotrophin releasing factor-like immunoreactivity in the human hypothalamus. Neuroscience Letters **41** 259-262

Pepe, G.J., Waddell, B.J., & Albrecht, E.D. (1988). The effects of adrenocorticotrophin and prolactin on adrenal dehydroepiandrosterone secretion in the baboon fetus. Endocrinology. **122** : 646-650

Pepe, G.J. & Albrecht, E.D.(1990) Regulation of the primate fetal adrenal cortex. Endocrine Reviews. **22** : 151-176

Perkins, A.V., Eben, F., Wolfe, C.D.A., Schulte, H.M., Linton, E.A. (1992). Plasma measurements of corticotrophin-releasing hormone binding protein in normal and abnormal pregnancy. Journal of Endocrinology. **138** : 149-157

Perrin, M.H., Donaldson, C.J., Chen, R., Lewis, K.A., Vale, W.W. (1993). Cloning and Functional Expression of a Rat Brain Corticotrophin Releasing Factor receptor. Endocrinology. **133** : 3058-3061

Petraglia, F., Lim, A.T.W, Vale, W. (1987a) Adenosine 3,5 monophosphate, prostaglandins and epinephrine stimulate the secretion of immunoreactive gonadotrophin releasing hormone from cultured human placental cells. Journal of Clinical Endocrinology and Metabolism. **65** : 1021-1025

Petraglia, F., Sawchenko, P.E., Rivier, J. & Vale, W. (1987b) Evidence for local stimulation of ACTH secretion by corticotrophin releasing factor in human placenta. Nature, **328** : 717-719.

Petraglia, F., Vaughan, I., Vale, W. (1989a) Inhibin and Activin modulate the release of Gonadotrophin releasing hormone, human chorionic gonadotrophin and progesterone from cultured human placental cells. Proceedings of the National Academy of Sciences USA. **86** : 5114-5117

Petraglia, F., Sutton, S., & Vale, W. (1989b) Neurotransmitters and peptides modulate the release of immunoreactive CRH from cultured human placental cells. American Journal of Obstetrics and Gynaecology. **160** : 247-251

Petraglia, F., Giardino, L., Coukos, G., Calza, L., Vale, W., Genzzani, A.R. (1990a) Corticotrophin-releasing factor and parturition: Plasma and amniotic fluid levels and placental binding sites. Obstetrics and Gynaecology. **75** : 784-789

Petraglia, F., Vaughan, J., Vale, W. (1990b) Steroid hormones modulate the release of immunoreactive Gonadotrophin releasing hormone from cultured human placental cells Journal of Clinical Endocrinology and Metabolism. **70** : 1173-1178

Petraglia, F., Volpe, A., Genazzani, AR., Rivier, J., Sawchenko, P.E., Vale, W. (1990c). Neuroendocrinology of the human placenta. Frontiers in Neuroendocrinology **11**:

Petraglia, F., Tabanelli, S., Galassi, C., Garuti, G.C., Mancini, A.C., Genazzani, A.R., Gurpide, E. (1992) Human decidua and in vitro decidualised endometrial stromal cells at term contain immunoreactive corticotrophin releasing factor (CRH) and CRH messenger ribonucleic acid. Journal of Clinical Endocrinology and Metabolism. **74** : 1427-1431

Petraglia, F., Potter, E., Cameron, V.A., Sutton, S., Behan, D.P., Woods, R.J., Sawchenko, P.E., Lowry, P.J., Vale, W. (1993). Corticotrophin releasing factor Binding protein is produced by human placenta and intrauterine tissues. Journal of Clinical Endocrinology and Metabolism. **77** : 919-924

Potestio, F.A., Zakar, T., Olsen, D.M. (1988) Glucocorticoids stimulate prostaglandin synthesis in human amnion cells by a receptor mediated mechanism. Journal of Cinical Endocrinology and Metabolism. **67** : 1205-1210

Potter, E., Behan, D.P., Fischer, W.H., Linton, E.A., Lowry, P.J., Vale, W.W. (1991) Cloning and characterisation of the cDNAs for human and rat corticotrophin releasing factor binding proteins. Nature. **349** : 423-426

Potter, E., Behan, D.P., Linton, E.A., Lowry, P.J., Sawchenko, P.E., Vale, W. (1992). The central distribution of a corticotrophin releasing factor (CRH) binding protein predicts multiple sites and modes of interaction with CRH. Proceedings of the National Academy of Sciences USA. **89** : 4192-4196

Quatero, H.W.P. & Fry, C.H. (1989) Placental corticotrophin releasing factor may modulate human parturition . Placenta. **10** : 439-440

Quatero, H.W.P. Srivatsa G., Gillham B. (1992) Role for cyclic adenosine monophosphate in the synergistic interaction between oxytocin and corticotrophin releasing factor in isolated human gestational myometrium. Clinical Endocrinology. **36** : 141-145

Quatero, H.W.P., Noort, W., Fry, C., Keirse, M. (1991) Role of prostaglandins and leukotrienes in the synergistic interaction between oxytocin and corticotrophin releasing hormone on the contraction force in isolated human gestational myometrium. Prostaglandins. **42** : 137-150

Raisenen, I. (1987) Plasma levels and diurnal variation of b-endorphin, b-lipotrophin and corticotrophin during pregnancy and early puerperium. European Journal of Obstetrics and Gynaecology and Reproductive Biology. **27** : 13-20

Rees, L.H.., Burke, C.W., Chard, T., Evans, S.W., Letchworth, S. (1975) Possible placental origin of ACTH in normal human pregnancy. Nature. **254** : 620-621

Riley, S.C., Walton, J.C., Herlick, J.M. & Challis, J.R.G. (1991) The localisation and distribution of corticotrophin releasing hormone in the human placenta and fetal membranes throughout gestation. Journal of Clinical Endocrinology and Metabolism. **72** : 1001-1007

Riley, S.C., & Challis, J.R.G. (1991) Corticotrophin-releasing hormone production by the placenta and fetal membranes. Prostaglandins. **12** : 105-119

Riddick, D.H., Luciano, A.A., Kusmik, W.F., Maslar, I.A. (1978) De novo synthesis of prolactin by human decidua. Life Sciences. **23** : 1913-1920

Rivier, J., Speiss, J., & Vale, W. (1983) Characterisation of rat hypothalamic corticotrophin releasing hormone. Proceedings of the National Academy of Science USA. **80** : 4851-4855

Rivier C.L., Plotsky, P.M. (1986) Mediation by corticotrophin releasing factor (CRH) of adenohypophysial hormone secretion. Annual Review of Physiology. **48** : 475-480

Robinson, B.G., Emmanuel, R.L., Frim, D.M. & Majzoub, J.A. (1988) Glucocorticoid stimulates expression of corticotrophin releasing hormone gene in human placenta. Proceedings of the National Academy of Sciences USA. **85** : 5244-5248

Rodger, M.W., Baird, D.T. (1987) Induction of therapeutic abortion in pregnancy with mifepristone in combination with a prostaglandin pessary. Lancet **ii**: 1415-1418

Rodger, M.W., Logan, A.F., Baird, D.T. (1988) Induction of early abortion with mifepristone (RU486) and two different doses of prostaglandin pessary (gemeprost). Contraception. **39** : 497-502

Rosenberg, S.M., Maslar, I.A., Riddick, D.H. (1980) Decidual production of prolactin in late gestation: further evidence for a decidual source of amniotic fluid prolactin. American Journal of Obstetrics and Gynaecology. **138** : 681-685

Sacerdote, P., Panieri, A.E. (1989). Analysis of the beta-endorphin struture-related activity on human monocyte chemotaxis: importance of the N and C-terminal. Peptides. **10** : 565-569

Saijonmaa, O., Laatikainen, T. & Wahlstrom, T (1988) Corticotrophin releasing factor in human placenta: localisation, concentration and release in vitro. Placenta. **9** : 373-385

Saphier, P.W., Faria, M., Grossman, A., Coy, D.H., Besser, G.M., Hodson, B., Parkes, M., Linton, E.A., Lowry, P.J. (1992) A comparison of the clearance of ovine and human corticotrophin-releasing hormone (CRH in man and sheep: a possible role for CRH-binding protein. Journal of Endocrinology. **133** : 487-495

Sapolsky, R., Rivier, C., Yamamoto, G., Plotsky, P., Vale, W. (1987). Interleukin-1 stimulates the secretion of hypothalamic corticotrophin-releasing factor. Nature **238** : 552-557

Sasaki, A., Liotta, A.S., Luckey, M.M., Marjioris, A.N., Suda, T. & Krieger, D.T. (1984) Immunoreactive corticotrophin releasing factor is present in human plasma during the third trimester of pregnancy. Journal of Clinical Endocrinology and Metabolism. **59** : 812-814

Sasaki, A., Sinkawa, O., Margioris. A.N., Liotta, A.S., Sato, S., Murakami, O., Go, M., Shimuzi, Y., Hanew, K., Yoshinaga, K. (1987) Immunoreactive corticotrophin releasing factor in human plasma during pregnancy, labour and delivery. Journal of Clinical Endocrinology and Metabolism. **64** : 224-229

Sasaki, A., Tempst, P., Liotta, A.S., Margioris, A.N., Hood, L.E., Kent, S.B.H.., Sato, S., Shinkawa, O., Yoshinaga, K. & Krieger, D.T. (1988) Isolation and characterisation of corticotrophin releasing hormone like peptide from human placenta. Journal of Clinical Endocrinology and Metabolism. **67** : 768-773

Sasaki, A., Shinkawa, O., & Yoshinaga, K. (1989) Immunoreactive corticotrophin releasing factor in amniotic fluid. American Journal of Obstetrics and Gynaecology. **162** : 194-198

Sasaki, A., Shinkawa, O., Yoshinaga, K. (1990). Immunoreactive corticotrophin-releasing hormone in amniotic fluid. American Journal of Obstetrics and Gynaecology. **162** : 194-198

Schulte, H.M., Chrousos, G.P., Booth, J.D., Oldfield, E.H., Gold, P.W., Cutler, G.B.J., Loriaux, D.L. (1984) CRH pharmacokinetics in man. Journal of Clinical Endocrinology and Metabolism. **58** :192-196

Schulte, H.M. & Healy, D.L. (1987) Corticotrophin releasing hormone and adrenocorticotrophin like immunoreactivity in human placenta, peripheral and uterine vein plasma. Hormone and Metabolic Research. **16:** suppl 44-46

Schultz, D.M., Giordano, D.A., Schultz, D.H. (1962). Weights of organs of fetuses and infants. Archives of Pathology. **74** : 244-250

Schurmeyer, T.H., Argerinos, P.C., Gold, P.W., Galluci, W.T., Tomai, T.P., Cutler, G.B.J.,Chrousos, G.P. (1984). Human CRH in man: pharmacokinetic properties and dose response of plasma adrenocorticotrophin and cortisol secretion. Journal of Clinical Endocrinology and Metabolism. **59** : 1103-1108

Shibahara, S., Morimoto, Y., Furitani, Y., Notake, M., Takahashi., Shimuzu, S., Horikawa,S & Numa, S (1983) Isolation and sequence analysis of the human corticotrophin releasing factor precursor gene. EMBO Journal. **2** : 775-779

Shibasaki, T., Odagiri, E., Shizume, K., & Ling, N. (1982) Corticotrophin releasing factor like activity in human placental extracts. Journal of Clinical Endocrinology and Metabolism. **55** :384-386

Simpson, E.R., Macdonald, P.C. (1981) Endocrine physiology of the placenta. Annual Review of physiology. **43** : 163-188

Siler-Khoder, T.M. (1983) Hypothalamic releasing hormones of the placenta. Clinical Perinatology. **3** : 553-566

Siler-Khoder, TM., Khoder, GS. (1989) Dose response analysis of GnRH stimulation of HCG release from human term placenta. Biol Reproduction. **25** : 353-358

Silman, F.G., and Bergman, F.G. (1953). ACTH activity of placental extracts J. Obstetrics and Gynaecology of the British Empire. **60** : 123-125

Silman, R.E., Chard, T., Rees, L.H., Smith, I., Young, I.M. (1975). Observations on melanocyte stimulating hormone-like peptides in human maternal plasmaduring late pregnancy. Journal of Endocrinology. **65** : 45p-47p

Singer, R.H., Lawrence, J.B., Rashtichian, T. (1987). Chapter 4. Towards a rapid and sensitive in situ hybridisation methodology using isotopic and non-isotopic probes. In Valentino, K.L., Eberwine, J.H., and Barchas, J.D. (eds), In situ Hybridisation. Oxford University Press, Oxford pp70-94

Sitteri, P.K., and Macdonald, P.C. (1963) The utilization of circulating dehydroepiandrosterone sulphate for oestrogen synthesis during human pregnancy. Steroids. **2** : 713-730

Sitteri, P.K., and Macdonald, P.C. (1966) Placental oestrogen biosynthesis during human pregnancy. Journal of Clinical Endocrinology and Metabolism. **26** : 751-761

Smith, R., Cubis, J., Brinsmead, M. (1990) Mood changes, obstetric experience and alterations in plasma cortisol, beta-endorphin and corticotrophin-releasing hormone during pregnancy and the puerperium. Journal of Psychosomatic Research. **34** : 53-69

Soloff, M.S. (1985) Oxytocin receptors and mechanisms of oxytocin action. In: Amico J.A. Robinson A.G. eds. Oxytocin: Clinical and Laboratory Studies. Amsterdam: Elsevier. 1985 : 259-276

Stark, R.I., Frantz, A.G. (1983) ACTH-β-endorphin in pregnancy. Clinical Perinatal Endocrinology. **10** :653-667

Suda, T., Tomori, N., Yajima, F., Sumitomo, T ., Nakagami, Y., Ushiyama, T., Demura, H., Shizume, K. (1985) Immunoreactive corticotrophin releasing factor in human plasma. Journal of Clinical Investigation. **76** : 2026-2029

Suda, T., Iwashita, M., Tozawa, F., Ushiyama, T., Tomori, N., Sumitomo, T., Nakagami, Y., Demura, H., & Shizume, K. (1988) Characterisation of corticotrophin releasing hormone binding protein in human plasma by chemical cross-linking and its binding during pregnancy. Journal of Clinical Endocrinology and Metabolism. **67** : 1278-1283

Suda, T., Iwashita, M., Ushiyama, T., Tozawa, F., Sumitomo, T., Nakagami, Y., Demura, H., Shizume, K, (1989) Responses to corticotrophin-releasing hormone and its bound and free forms in pregnant and nonpregnant women. Journal of Clinical Endocrinology and Metabolism. **69** : 38-42

Szalay, S., Husslein, P., Grunberger, W. (1981) Local application of prostaglandin E_2 and its influence on collagenolytic activity of cervical tissue. Singapore Journal of Obstetrics and Gynaecology. **12** : 15-19.

Takahashi, K., Mouri, T., Yamamoto, T., Itoi, K., Murakami, O., Yoshinaga, K., Sasano, N. (1989) Corticotrophin-releasing hormone in the human hypothalamus. Free-floating immunostaining method. Endocrinology Japan. **36** : 275-279

Texier, P-L., De Keyzer, Y., Lacave, R., Vieau, D., Lenne, F., Rojasmiranda, A., Bertagna, X. (1991) Proopiomelanocortin gene expression in normal and tumoral human lung. Journal of Clinical Endocrinology and Metabolism. **73** : 414-420

Thomson, M., Chan, E-C., Falconer, J., Marsden, G., Smith, R. (1988) Secretion of corticotrophin-releasing hormone by superfused human placental fragments. Gynaecological Endocrinology. **2** : 87-100

Thornton, S., Davison, J.M., Baylis, P.M. (1988). The progress of labour is not dependant on changes in plasma oxytocin. In Husslein P., Egarter C. eds. 1st European Congress on Prostaglandins in Reproduction. Vienna; Facultas. 127

Tropper, P.J., Goland, R.S., Wardlaw, S.L., Fox, H.E. & Frantz, A.G. (1987) Effects of betamethasone on maternal plasma corticotrophin releasing factor, ACTH and cortisol during pregnancy. Journal of Perinatal Medicine. **15** : 221-225

Tulchinsky, D., and Hobel, C.J. (1973). Plasma human chorionic gonadotrophin, estrone, estradiol, estriol, progesterone and 17a-hydroxyprogesterone in human pregnancy.III. Early normal pregnancy. American Journal of Obstetrics and Gynaecology. **117** : 884-893

Tuimala, R., Kauppila, A., Haapalahti, J. (1976) ACTH levels in amniotic fluid during pregnancy. British Journal of Obstetrics and Gynaecology. **83** : 853-856

Tyson, J.E., Mowat, G.S., McCoshen, J.A. (1984) Simulation of a probable biological action of decidual prolactin on fetal membranes. American Journal of Obstetrics and Gynaecology. **148** : 296-300

Tyson, J.E., McCoshen, J.A., and Dublin, N.H. (1985) Inhibition of fetal membrane prostaglandin production by prolactin; relative importance in the initiation of labour. American Journal of Obstetrics and Gynaecology. **151** : 1032-1038

Uldjberg, N., Ekman, G., Herltoft, P., Malmstrom, A., Ulmstem, U., Wingerup, L. (1983a). Human cervical connective tissue and its reaction to prostaglandin E2. Acta Obstetrica et Gynaecologica Scandinavica Supplement **113** : 163-166

Uldjberg, N., Ulmstem, U Ekman, G. (1983b). The ripening of the human uterine cervix in terms of connective tissue biochemistry. Clinical Obstetrics and Gynaecology. **26** : 14-26

Uldjberg, N., Ekman, G., Malstrom, A., Olssen, K., Ulmstem, U. (1983c). Ripening of the human uterine cervix related to changes in collagen, glycosaminoglycans and collagenolytic activity. Clinivcal Journal of Obstetrics and Gynaecology. **147** : 662-666

Vale, W., Speiss, J., Rivier, C. & Rivier J. (1981) Characterisation of a 41-residue ovine hypothalamic peptide that stimulates secretion of a corticotrophin and beta-endorphin. Science. **213** : 1394-1397

Vale, W., Rivier, C., Brown, M.R., Speiss, J., Koob, G., Swanson, L., Bilezikjian, L., Bloom, F., Rivier, J. (1983). Chemical and biological characterization of corticotrophin-releasing factor. Recent Progress in Hormone Research. **39 :** 245-250

Van der Rest, M. (1980) Collagen and its metabolism. In: Naftolin F, Stubbefield P. (eds) Dilatation of the cervix. Raven, New York. pp61-78

Vicovac, L.J., Genbacev, O. (1988) Coincubation- an experimental approach to the study of decidual-trophoblastic interaction. Placenta. **9** : 109-115

Von Maillot, K., Stuhlsatz, H.W., Mohanaradhkrishnan, V., Greiling, H. (1979). Changes in the glycosaminoglycan distribution pattern in the human uterine cervix during pregnancy and labour. American Journal of Obstetrics and Gynaecology. **135** : 503-506

Waddell, B.J. Burton, P.J. (1993) Release of bioactive ACTH by perifused human placenta at early and late gestation. Journal of Endocrinology **136** : 345-353

Warren, W.B., Goland, R.S., Wardlaw, S.L., Stark, R.I., Fox, H.E., Conwell, I.M. (1990). Elevated maternal plasma corticotrophin releasing hormone levels in twin gestation. Journal of Perinatal Medicine. **18** : 39-42

Winters, A.J., Oliver, C., Colston, C., MacDonald, P., Porter, J. (1974) Plasma ACTH levels in the human fetus and neonate as related to age and parturition. Journal of Clinical Endocrinology and Metabolism. **39** : 269-273

Wolfe, C.D.A., Patel, S.P., Campbell, E.A., Linton, E.A., Anderson, J., Lowry, P.J., Jones, M.T. (1988a) Plasma corticotrophin releasing factor in normal pregnancy. British Journal of Obstetrics and Gynaecology. **95** : 997-1002

Wolfe, C.D.A., Patel, S.P., Linton, E.A., Campbell, E.A., Anderson, J., Dornhorst, A., Lowry, P.J. Jones, M.T. (1988b) Plasma corticotrophin releasing factor in abnormal pregnancy. British Journal of Obstetrics and Gynaecology. **95** : 1003-1006

Yagel, S., Lala, P.K., Powell, W.A, Casper, R.F. (1989) Interleukin-1 stimulates human chorionic gonadotrophin secretion by first trimester human trophoblast. Journal of Clinical Endocrinology and Metabolism. **68** : 992-995

Yuen, B.H., Moon, Y.S., Shin, D.H. (1986) Inhibition of human chorionic gonadotrophin production by prolactin from term human trophoblast. American Journal of Obstetrics and Gynaecology. **154** : 336-340