EXPRESSION AND EFFECTS OF EPIDERMAL GROWTH FACTOR RELATED PEPTIDES IN HUMAN TERM GESTATIONAL TISSUE

Thesis submitted for the degree of Doctor of Philosophy

by

USULA CHANDINI MANUELPILLAI

Centre for Bioprocessing & Food Technology
Faculty of Engineering & Science
Victoria University of Technology, Victoria, Australia

1999

TABLE OF CONTENTS

Declaration i
Publications ii
Acknowledgments iii
Dedication v
List of Symbols and Abbreviations vi
Summary viii

Chapter 1: Review of the Literature 1

1.1 General introduction 2
1.2 The gestational tissue 3
1.3 Growth factors 6
1.4 The epidermal growth factor (EGF) family 7
1.5 The epidermal growth factor receptor (EGFR) family 11
1.6 EGFR expression in the gestational tissue 13
1.7 Expression of EGFR related receptors in gestational tissue 14
1.8 Expression and effects exerted by EGF in gestational tissue 16
1.9 Expression and effects of EGF related growth factors in gestational tissue 19
1.10 Placental progesterone synthesis 24
1.11 In vitro systems used in the study of placental syntheses and function 29
1.12 Aims 36

Chapter 2: Material and Methods 37

Chapter 3: Expression of EGF /EGFR related growth factor and receptor mRNA in gestational tissue 75

3.1 Introduction 76
3.2 Materials and methods 78
3.3 Results 79
3.4 Discussion 93

Chapter 4: Localisation of Cripto, Heregulin, Heparin-Binding EGF and Amphiregulin mRNA in gestational tissue 100

4.1 Introduction 101
4.2 Materials and methods 103

4.3 Results 104
4.4 Discussion 113

Chapter 5: Transforming growth factor alpha, Amphiregulin protein expression in gestational tissue & cytotrophoblast cells *in vitro* 118

5.1 Introduction 119
5.2 Materials and methods 121
5.3 Results 122
5.4 Discussion 137

Chapter 6: Effects of transforming growth factor α on proliferation, differentiation and progesterone synthesis by cytotrophoblast cells *in vitro* 143

6.1 Introduction 144
6.2 Materials and methods 146
6.3 Results 148
6.4 Discussion 157

Chapter 7: Identification of upregulated mRNA in cytotrophoblast cells treated with transforming growth factor α 162

7.1 Introduction 163
7.2 Materials and methods 165
7.3 Results 166
7.4 Discussion 179

Chapter 8: General discussion and future directions 186

Bibliography 189

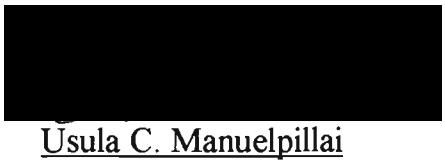
Appendices

Appendix 1 Patient consent form 215

Appendix 2	Multiple sequence alignments of members of the EGF/ EGFR families	217
Appendices 3-11	Nucleotide sequences of the members of the EGF/ EGFR families and the nucleotide sequence traces of the respective RT-PCR generated cDNA	223
Appendix 12	Nucleotide sequence of GAPDH cDNA and nucleotide sequence trace of RT-PCR generated GAPDH cDNA	246
Appendix 13	Nucleotide sequence of 3βHSD cDNA and nucleotide sequence trace of RT-PCR generated 3βHSD cDNA	249
Appendices 14-16	Nucleotide sequence traces of cDNA fragments obtained by representational difference analysis	251

DECLARATION

In accordance with the requirements of Victoria University of Technology for the degree of Doctor of Philosophy, I hereby declare that, to the best of my knowledge and belief, this thesis is my original work, with the various forms of assistance described in the acknowledgments and contains no material previously published elsewhere or extracted whole or in part from a thesis submitted by me for any other degree or diploma at any other University. I consent to this thesis being made available for photocopying and loan, if accepted for the award of the degree.



Usula C. Manuelpillai

PUBLICATIONS

Abstracts

Manuelpillai U, Fairclough R, Bhave M, and Towstoless M. (1995). Expression of Transforming Growth Factor Alpha and Amphiregulin mRNA in human gestational tissue at term. Annual conference of the European Placenta Group, Spa, Belgium.

Manuelpillai U, Fairclough R, Bhave M, and Towstoless M. (1995). Amphiregulin and Transforming Growth Factor alpha mRNA expression in cultured cytotrophoblast cells *in vitro*. Annual conference of the Endocrine Society Australia, Melbourne.

Manuelpillai U, Fairclough R and Towstoless M. (1999). Localisation of Amphiregulin protein in human term gestational tissue. Annual conference of the Perinatal Society of Australia and New Zealand, Melbourne 1999.

Manuelpillai U, Fairclough R and Towstoless M. (1999). Effect of Transforming Growth Factor Alpha on progesterone synthesis by placental cytotrophoblast cells *in vitro*. Annual conference of the European Placenta Group, Schmalding, Austria.

Papers

Expression of Epidermal Growth Factor related peptides and their receptor mRNA in human term gestational tissue. Biology of Reproduction (submitted).

Effects of Transforming Growth Factor Alpha on progesterone synthesis in placental cytotrophoblast cells *in vitro*. Reproduction, Development & Fertility (in preparation).

Amphiregulin and Transforming Growth Factor Alpha protein expression in human term gestational tissue and cytotrophoblast cells *in vitro*. Journal of Reproduction and Fertility (in preparation).

Acknowledgments

First I would like to thank my supervisors Professor Robert Fairclough and Associate Professor Michelle Towstoless for their excellent support, encouragement, suggestions and reviews of manuscripts. A special thank you to Michelle for introducing me to the culture of human trophoblast cells and providing me with the necessary training.

This work required large numbers of human placentae and fetal membranes. I am most grateful to the women who consented to be a part of this study sometimes whilst in labour. To the nursing staff at the Mercy Hospital, Werribee and Western Hospital, Sunshine who liaised between myself and the patients, enabling easy access and answered endless telephone calls regarding progress of their patients in labour and those undergoing elective Caesarean sections. A special thank you to Debbie Watkins who organised the tissue collection at the Mercy Hospital.

This work which is multifaceted would not have been possible if not for the excellent research facilities and management at the Centre for Bioprocessing and Food Technology. I would also like to extend thanks to Associate Professor Debra Middleton and her staff in particular Josie Wilson at the School of Veterinary Sciences, University of Melbourne for enabling me to use their tissue processing facilities and support extended. To Dr. Donna Butkos, at the Howard Florey Institute, University of Melbourne for the thorough training provided on in situ hybridization. A very big thank you to Dr. Sarah Fraser for her assistance in carrying out the Representational Difference Analysis work. This work would have taken much longer if not for the excellent guidance and suggestions provided by Sarah. I would also like to thank Dr. Madol-Serafica for many useful suggestions throughout my studies and for carrying out the multiple sequence alignments. To Dr. Paul Chambers and Dr. Mrinal Bhave, School of Life Sciences, Victoria University for their ideas and general assistance. A special thank you to Nikola Popovik, Buon Sen-Park and Oleg Yuriev for their valuable suggestions on SDS-PAGE and Hisham Al Matubsi for his assistance in conducting the radio immuno assays.

I would also like to fellow students at CBFT in particular Matthew Knight, Ivana Radjovik, Mohideen Mohamed, Lanka Lankaputra, Helen Feng, Anne Wilson, not only for their help but for many of the fun times we had.

A special thank you to Professor Margaret Britz for providing me with a Departmental Scholarship, employing me on a part time basis for oligonucleotide synthesis work and for her support and valuable suggestions.

I am also very thankful for the continuous support extended by my husband Bena and son Niraj. Also to my father and sister for their encouragement and help throughout my studies.

For my parents David and Lalana Soysa

ABBREVIATIONS

GENERAL

AR	Amphiregulin
BTC	Betacellulin
3βHSD	3 beta hydroxy steroid dehydrogenase
c-erb-B2	Epidermal Growth Factor Receptor 2
c-erb-B3	Epidermal Growth Factor Receptor 3
c-erb-B4	Epidermal Growth Factor Receptor 4
cDNA,	complimentary deoxyribonucleic acid
CR	Cripto
CT	cytotrophoblast
DD-RT-PCR	differential display reverse transcription polymerase chain reaction
ds	double stranded
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EVT	extra villous trophoblast
GAPDH	Glyceraldehyde-3-phosphate-dehydrogenase
IGF	Insulin like growth factor
HB-EGF	Heparin Binding-Epidermal-Growth Factor
hCG	human chorionic gonadotrophin
hPL	human placental lactogen
HRG	Heregulin
IgG	immunoglobulin
Iry, IIry	primary, secondary
kDa	kilo Dalton
LH, LHRH	luetinising hormone, LH releasing hormone
mRNA, tRNA, rRNA	messenger, transfer, ribosomal ribonucleic acid
PCR	polymerase chain reaction
PKC, PKA	protein kinase C, A
PMA	phorbol myristate acetate
RDA	representational difference analysis
RIA	radioimmunoassay
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
±SEM	plus or minus standard error of the mean
SDS-PAGE, PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
ST	syncytiotrophoblast
TGFα	Transforming Growth Factor Alpha
VC	villous cotyledon
UV	ultraviolet

MEASUREMENTS

A	absorbance
bp, kb	base pairs, kilobase pairs
°C	degree Celsius
Ci, μCi	Curie/s, microcurie
cm^2	square centimetre
g	gram/s
h	hour/s
l	litre
μl	microlitre
M	molar
μM	micromolar
mg	milligram
μg	microgram

min	minute/s
ml	millilitre
mm	millimetre
mM	millimolar
ng	nanogram/s
nm	nanomolar
pH	$\log_{10}$ H+ ion concentration
rpm	revolutions per minute
sec	second/s
U	units
v/cm	volts per centimetre
v/v	volume per volume
vol	volume
w/v	weight per volume
%	percent
<	less than
>	greater than

CHEMICALS

APS	ammonium persulphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
CO_2	carbon dioxide
dCTP, dNTP	deoxycytosine triosphosphate, deoxynucleotide triosphosphate
ddNTP	dideoxynucleotide triosphosphate
DEPC	diethyl pyro carbonate
DTT	dithioreitol
EDTA	ethylene diamine tetra-acetate
EMEM	Eagles Minimum Essential Medium
EtBr	ethidium bromide
EtOH	ethanol
FCS	fetal calf serum, delipidated, heat inactivated and charcoal stripped
HAC	acetic acid
JMEM	Jokliks Minimum Essential Medium
KCl	potassium chloride
MeOH	methanol
$MgCl_2$	magnesium chloride
MOPS	morpholino propane sulphonic acid
NaCl	sodium chloride
NaOAc	sodium acetate
PBS	phosphate buffered saline
PNK	polynucleotide kinase
SDS	sodium dodecyl sulphate
SSC	standard sodium citrate
TAE	tris acetate ethylene diamine tetra-acetate
TBE	tris borate ethylene diamine tetra-acetate
TBS, TBST	tris buffered saline, TBS with triton X-100
TE	tris ethylene diamine tetra-acetate
Tris	tris(hydroxymethyl)aminomethane

Summary

This thesis was undertaken to determine the expression of several growth factors that belong to the Epidermal Growth Factor (EGF) family in human term gestational tissue. It also examines the effects exerted by Transforming Growth Factor alpha (TGFα), a member of the EGF family, on cultured villous cytotrophoblast (CT) cells isolated from placentae delivered between 38-40 weeks gestation.

The fetus is dependant on the placenta and fetal membranes for the transfer of nutrients, gases and waste products. Therefore the normal growth and development of the placenta and fetal membranes is of vital importance for fetal development. The growth and development of the placenta, fetal membranes and numerous functions performed by their constituent cells is strictly regulated by a number of endogenously produced growth factors and their receptor proteins. Among these the expression of EGF and the EGF receptor (EGFR) in the gestational tissue has been widely studied. Studies *in vitro* using dispersed cells or explants derived from the gestational tissue have shown that EGF regulates the synthesis of hormones, eicosanoids and other bioactive proteins. However the expression of other members of the EGF family such as Heregulin (HRG), Betacellulin (BTC), Cripto (CR), Heparin Binding-Epidermal Growth Factor (HB-EGF) and Amphiregulin (AR) in the gestational tissue is largely unknown. Therefore the first study was undertaken to investigate whether the mRNA of these EGF related growth factors were present in the term gestational tissue. RT-PCR analysis of total RNA prepared from gestational tissue collected from labouring (ie. spontaneous vaginal delivery) and non-labouring patients (ie. Caesarean sections) demonstrated that several of these growth factor mRNA were co-expressed in the villous cotyledons of the placenta, amnion and chorion-adherent decidua.

The receptors c-erb-B2, c-erb-B3 and c-erb-B4 are structurally very similar to the EGFR and are members of the EGFR family. They have been shown to interact with BTC, HRG in cells derived from several carcinomas. As there have been very few studies that have examined the expression of the EGFR related receptors in the gestational tissue, the mRNA expression of c-erb-B2, c-erb-B3 and c-erb-B4 in these tissue was also examined.

Analysis of total RNA revealed that c-erb-B2, c-erb-B3 and c-erb-B4 mRNA's were co-expressed in the villous cotyledons, amnion and chorion-adherent decidua at 38-40 weeks gestation.

In order to examine if these EGF / EGFR related growth factors and receptors may play a role in the growth and differentiation of villous CT cells isolated from term placenta, their mRNA expression in cultured CT and syncytiotrophoblast (ST) cells was also investigated. Using RT-PCR analysis of total RNA prepared from CT cells cultured for 12 and 96h, CR, AR and TGFα mRNA was detected in CT and ST cells whereas HRG and HB-EGF mRNA was detected only in the CT cells. Among the receptors only c-erb-B2 mRNA was detected, and the mRNA was found only in the CT cells.

The relative levels of some of the EGF related growth factor and c-erb-B2, c-erb-B3, c-erb-B4 receptor mRNA expression in the gestational tissue and cultured cells was also examined. Densitometric analysis of RT-PCR generated amplicons showed that similar levels of the growth factor and receptor mRNA were present in the amnion and chorion-adherent decidua.

These findings suggest that a greater number of EGF / EGFR related growth factor and receptor proteins may be synthesised endogenously in the gestational tissues than was previously thought. It also suggests that these growth factors may be regulating the growth and functions performed by the constituent cells of the gestational tissue. In cultured CT cells the findings suggest that there maybe spatial differences in the expression of proteins such as HRG and HB-EGF between CT and ST cells. CT and ST cells are known to express the EGFR and c-erb-B2 receptor proteins implying that there may be a number of autocrine / paracrine regulatory loops being exerted by these growth factors in CT and ST cells.

As HRG, CR, HB-EGF and AR mRNA's were detected in the gestational tissue samples through RT-PCR analysis in the initial study, in situ hybridization studies were carried out to localise their mRNA expression in the placenta and fetal membranes. Using oligonucleotide probes that were directed against the mRNA of each of these growth

factors, CR and HRG mRNA was localised to the amniotic epithelium, decidual cells and to chorionic trophoblast cells. AR and HB-EGF mRNA were detected only in the amniotic epithelium and chorionic trophoblast cell layers in the fetal membranes. These findings suggest that AR, CR, HB-EGF and HRG proteins may be synthesised in these cells and that they may be the sites of autocrine / paracrine regulation by CR, HRG, AR and HB-EGF in the fetal membranes.

In the placental villi, hybridisation signals of variable intensities were detected in the ST cell layer using oligonucleotide probes directed against CR, HRG, AR and HB-EGF, however non-specific signals were also seen in the villous stroma. These findings suggest that the ST cells may be the predominant sites of the synthesis of these EGF related growth factor proteins in the placental villi and that CR, HRG, HB-EGF and AR may be exerting growth and functional regulation on the ST cells of the placental villi.

The findings from the two earlier studies showed that both AR and TGFα mRNA were present in gestational tissue collected between 38-40 weeks of pregnancy. In order to investigate whether AR, TGFα proteins were also present in the placenta, amnion and chorion, tissue lysates were initially analysed using Western hybridisation. Immunoreactive TGFα and AR proteins were detected in the lysates prepared from the placenta, amnion and chorion-adherent decidua. Next, sections of fixed tissue was probed with antisera directed against AR, TGFα in order to identify cells containing immunoreactive AR and TGFα proteins.

The findings from the immunohistochemistry studies showed that isolated CT and ST cells in the chorionic villi contained immunoreactive TGFα and AR proteins and that the two growth factor proteins were co-expressed in the majority of tissue sections analysed. In the amnion, isolated epithelial cells were found to express AR and TGFα. The most intense immunostaining for both AR and TGFα was observed in the decidual cells adhering on to the chorionic trophoblast cells. Immunoreactive TGFα protein were also detected on the surfaces of the chorionic trophoblast cells, however immunoreactive AR protein was generally not detected in these cells. These results indicate that immunoreactive AR and TGFα proteins are co-expressed in several cell types and that a

number of autocrine, paracrine regulatory loops may be exerted by TGFα and AR on these cells.

The immunohistochemistry studies performed on sections of placental villi showed that AR and TGFα proteins were present on the CT and ST cells. Therefore an *in vitro* study was carried out to determine whether there were differences in AR and TGFα protein expression between CT and ST cells. Using Western hybridization analysis, AR and TGFα proteins were found to be produced in both CT and ST cells. TGFα protein was found to be produced in consistently higher amounts per μg of total cellular protein in both CT and ST cells compared with AR protein. Furthermore, AR protein was found to be produced in significantly higher amounts in the ST cells compared with CT cells. These findings suggest that TGFα may be involved in regulating growth and functions performed by both CT and ST cells whereas AR may play a more significant autocrine / paracrine role in ST cells.

The next study was undertaken to investigate the effects that TGFα may be exerting on cultured villous CT cells isolated from placenta after 38-40 weeks gestation. The effects of TGFα on mitosis and aggregation of CT cells was investigated. Previous studies have shown that *in vitro,* CT cells aggregate prior to cellular fusion that leads to the formation of ST cells. Doses ranging between 10-50ng of TGFα per ml were found to exert no significant effect on the total number of cells or the aggregation of cultured CT cells isolated from placenta collected during the third trimester.

Progesterone is one of the principal steroid hormones synthesised by the placenta and this hormone is thought to be essential for the establishment and maintenance of pregnancy. TGFα has been found to regulate progesterone synthesis in human granulosa cells, however the effects of TGFα on progesterone synthesis in CT or ST cells is uncertain. A study as therefore conducted to investigate the effects exerted by TGFα on progesterone synthesis in CT cells *in vitro.* It was found that CT cells isolated from placenta collected at 38-40 weeks gestation, dosed with 15ng TGFα per $0.75x10^6$ cells per ml, and treated up to 18h, did not show a significant increase in the amount of progesterone synthesised compared with control cultures. A further study was undertaken to investigate if TGFα

could be exerting transcriptional control over the mRNA expression of enzymes involved in progesterone synthesis in villous CT cells. The expression of 3-beta hydroxysteroid dehydrogenase (3βHSD) mRNA was examined following treatment with 15ng TGFα per $0.75x10^6$ cells per ml. The results showed that within 15min, the relative amount of 3βHSD mRNA declined following treatment with TGFα. Significant differences between treated and control cells were observed after 30min and 2h post treatment. The decline in the amount of 3βHSD mRNA was found to be transient and steady state levels were reached within 6h post treatment. The findings suggest that TGFα inhibits the expression of 3βSHD mRNA in CT cells.

Another study was undertaken to identify specific mRNA species that may be upregulated in CT cells following treatment with TGFα *in vitro*. Up to eight different cDNA fragments were detected in the tester ie. CT cells treated with 15ng TGFα per $0.75x10^6$ cells per ml using representational difference analysis (RDA), that were absent in the untreated control cells. However only three of the unique cDNA detected in the tester was successfully cloned and sequenced. By comparing the nucleotide sequences of the cloned cDNA fragments with known sequence data, two of the fragments were found to contain significant homology to human cDNA or DNA segments that are currently being sequenced. Hence it was not possible to determine the identity of two of the fragments. A third cDNA fragment was identified as being a part of the human gamma globin gene. There have been no previous reports of the expression of gamma globin mRNA or protein in CT cells or of the regulation of gamma globin mRNA expression by TGFα. These findings suggest that the expression of several mRNA species may be upregulated by TGFα in villous CT cells during the latter part of gestation.

CHAPTER 1

Review of the Literature

The literature relevant to topics presented in the thesis is reviewed in this Chapter. These topics includes the mRNA and/or protein expression of the Epidermal Growth Factor (EGF), EGF receptor families in gestational tissue, cultured trophoblast cells, interaction of the growth factors with their receptors and their regulatory effects. It also examines the literature on the synthesis of progesterone in the placenta, factors that influence its synthesis in placental cytotrophoblast (CT), syncytiotrophoblast (ST) cells in vitro and effects exerted by EGF, TGFα on progesterone synthesis. Further the isolation and culture of villous CT, ST cells which forms an important basis of the research is also reviewed in this Chapter.

1.1 General introduction

The pre-eminent function of the placenta is the transfer of nutrients, gases, metabolites and waste products between the mother and the fetus. The placenta also produces a number of hormones, enzymes and other bioactive molecules that are involved in the establishment and maintenance of pregnancy and the regulation of maternal, fetal organs. Feto-maternal exchanges also occur in the amnion and chorion. By encapsulating the fetus in the fluid filled amniotic cavity, the fetal membranes also provide a protective environment for fetal development.

The growth and synthesis of hormones, enzymes and other bioactive molecules in the gestational tissue is strictly regulated. Growth factors and cytokines that are produced endogenously within the gestational tissues are thought to be the main regulatory elements; therefore the altered expression of these molecules is thought to disrupt the growth and functions performed. Previous studies that have compared expression of growth factors in gestational tissues obtained from normal pregnancies and pregnancies with complications have found that altered expression of growth factors can lead to inadequate invasion and placentation which is strongly linked to pregnancy disorders such as pre-eclampsia (Redman 1991), intra uterine growth retardation (El-Roeiy & Myers 1990), and threatened and spontaneous abortion (Hustin *et al.*, 1990). On the other hand over invasion and proliferation of placental trophoblast cells are involved in the aetiology of placenta accreta, hydatiform moles and choriocarcinoma (Bulmer *et al.*, 1992).

Altered expression of growth factors, their receptors and bindings proteins in the gestational tissue may also have a clinical significance. For example the concentration of Interleukin-6 receptor protein has been found to increase in the placenta just prior to the onset and during pre-term labour (Steinborn *et al.*, 1998). Hypoxic conditions in the placenta is an important factor in the pregnancy disorder pre-eclampsia and studies *in vitro* have shown that Vascular Endothelial Growth Factor mRNA levels increase significantly in trophoblast cells grown under hypoxia compared with normoxic conditions (Taylor *et al.*, 1997) and that Tumour Necrosis Factor α, Interleukin-1α and 1β proteins concentrations increase significantly in villous explants grown under hypoxic conditions (Benyo *et al.*, 1997). Elevated levels of Insulin-Like Growth Factor (IGF) Binding

Protein 1 in amniotic fluid at 15-16 weeks gestation correlate well with intra uterine growth retardation (Hakala-Ala-Pietila *et al.*, 1993). Placental derived growth factors such as activins and inhibins have also been found to be markedly elevated in maternal serum of women pre-eclampsia (Muttukrishna *et al.*, 1997).

The expression and effects exerted by the Epidermal Growth Factor (EGF), in gestational tissue has been widely studied. However the expression and effects exerted by other members of the EGF family in gestational tissue of women with or without pregnancy complications is largely uncertain. This thesis investigates the expression of the EGF related growth factors in the gestational tissue collected from women without pregnancy complications and the effects exerted by TGFα *in vitro* on cytotrophoblast (CT) cells to gain an understanding of the roles played by the EGF related growth factors in regulating the growth and functions performed by the gestational tissue.

1.2 The gestational tissue

1.2.1 Placenta

The placenta is believed to arise from the trophoblast or outer cell layer of the blastocyst (Moore 1989). Studies have shown soon after the blastocyst attaches onto the epithelium of the endometrium, the trophoblast forms a layer of CT cells that can be seen beneath the inner cell mass which later gives rise to the embryo (Moore 1989). The CT cells are stem cells and are thought to form the differentiated syncytiotrophoblast (ST) cells by a process of cellular fusion (Boyd & Hamilton 1970). At 2 weeks gestation the ST cells form a network around lacunae that are filled with maternal blood in the endometrium establishing a primitive uteroplacental circulation (Boyd & Hamilton 1970). With gestational progression, branched, chorionic villi that have an outer layer of ST cells, an inner layer CT cells, connective tissue and fetal capillaries which develop from the mesenchymal core can be distinguished by the third week of gestation (Moore 1989). Two types of branched villi are formed; attached and floating. The anchoring villi have been shown to attach the placenta to the uterine wall. Extravillous trophoblast (EVT) cells are believed to migrate from the tips of the anchoring villi to form the

cytotrophoblastic shell which lines the decidua or form invasive interstitial trophoblast cells that are dispersed in the decidua (Graham & Lala 1992). Other EVT cells have been found to remodel the maternal spiral arterioles and thereby lower the resistance to blood flow (Benirschke & Kaufmann 1995).

Blood from the remodelled maternal spiral arterioles is thought to flow into the intervillous spaces between the chorionic villi enabling gaseous exchange and nutrient transfer from the mother (Benirschke & Kaufmann 1995, Fig. 1.1). This type of placental development as seen in the human where the maternal uterine endometrium and vasculature is eroded during placental development is described as an invasive heamochorial placenta (Renfree 1982).

The various trophoblast lineages, the stroma, fetal capillaries, macrophages and fibroblasts within the villi form the fetal component of the placenta (Boyd & Hamilton 1970). The overlying decidua forms the maternal component of the placenta (Graham & Lala 1992). Studies have shown that the placenta continues to increase rapidly in size up to the middle of the second trimester and thereafter the overall size of the placenta increases slowly to term (Renfree 1982). A schematic diagram through a full term placenta is given in Fig. 1.1A.

The main functions of the placenta are gaseous exchange, nutrient transfer to the fetus, removal of fetal waste products thereby acting as a selective barrier between maternal and fetal circulations (Moore 1989). The placenta is also an endocrine organ and synthesises a number of hormones that include human chorionic gonadotrophin (hCG), human placental lactogen (hPL), human chorionic somatotrophin, estrogen and progesterone. These hormones are thought to be essential for the establishment and / or maintenance of pregnancy (Heap & Flint 1988).

1.2.2 The fetal membranes

The amnion and chorion comprises the fetal membranes. It is thought that the cells of the amnion originate from the epiblast which arises from the inner cell mass, whereas the

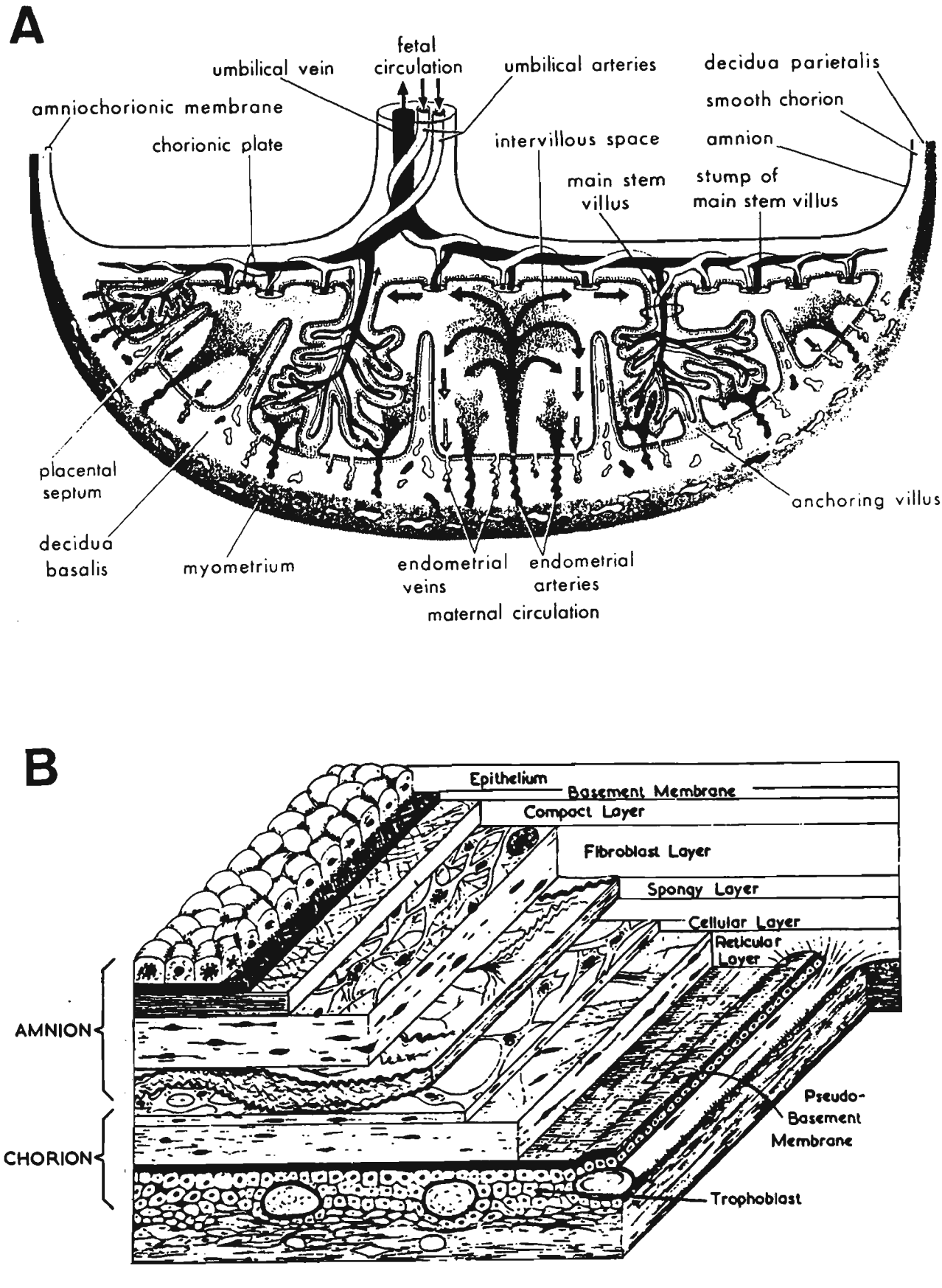


FIG 1.1 Schematic drawing of a section through a villous cotyledon of human term placenta **(A)** and the reflected fetal membranes at term **(B).** A) from Moore 1989; B) from Bourne 1962.

chorion is thought to originate from the extra-embryonic mesoderm and the trophoblast layer (Moore 1989). Studies have shown that due to the continued expansion of the amniotic cavity, the amnion is pushed towards the chorion and the two membranes fuse at about 20 weeks gestation (Moore 1989). Connective tissue is also thought to be deposited in the amnion and chorion as gestation proceeds. At term the compact, spongy and reticulated layers of the fetal membranes are largely composed of connective tissue and contain fibroblast and macrophage cells (Bourne 1962; Fig. 1.1B).

A part of the fetal membranes overlie the placenta while the remainder is known as the reflected membranes (Bourne 1962). Studies have shown that the chorion of the reflected membranes close to the placenta may have a blood supply but amnion is generally believed to be avascular (Bourne 1962).

The amnion forms a fluid filled membranous sac that surrounds the embryo and later the fetus. Most amniotic fluid is thought to be derived from maternal blood and at latter stages of gestation from fetal urine (Perry 1981; Moore 1989). The fluid provides a buoyant medium for symmetrical growth and development of the fetus. The fluid also cushions the fetus against jolts and prevents adherence of the amnion on to the surface of the fetus (Moore 1989). The amniotic epithelium participates in amniotic fluid renewal, secretes hCG and eicosanoids (Perry 1981). The chorionic trophoblast has been shown to produce hCG, pregnancy specific glycoprotein and traffic molecules such as prolactin between the mother and fetus (Plouzek *et al.*, 1993). The fetal membranes are also thought to transport a wide variety of enzymes and other essential molecules between the fetus and mother (Plouzek *et al.*, 1993).

1.3 Growth factors

Peptide molecules that transmit signals involved in intercellular communication between animal cells are collectively known as growth factors (McKay & Leigh 1993). In

contrast growth factors are produced and secreted by non-specialised cells, secreted in a non-directional manner into the extracellular fluid where they generally act as local mediators (Hollenberg 1989). A large number of different peptides currently in excess of 130 have been characterised as growth factors and grouped into families on the basis of their structural homologies. Some of the main growth factor families are the transforming growth factor β, platelet derived growth factor, fibroblast growth factor, interleukins, insulin like growth factor and the epidermal growth factor families (McKay & Leigh 1993).

Growth factors are believed to exert autocrine, intracrine effects on cells that produces the peptides and juxtacrine, paracrine effects on adjacent cells following interaction with specific receptor proteins. Growth factors may also be involved in endocrine stimulation (McKay & Leigh 1993; Fig. 1.2). A number of effects exerted by growth factors have been identified including stimulation, inhibition of cell division, differentiation, migration, stimulation of metabolite transport, chemotaxis, up or down regulation of mRNA and/or protein expression of other growth factors, regulatory substances and themselves. They are may work in a synergistic or antagonistic manner with other growth factors and/or hormones (Sporn & Roberts 1988; McKay & Leigh 1993).

1.4 The Epidermal Growth Factor (EGF) Family

EGF was isolated from the mouse submaxillary gland and was the first polypeptide mitogen identified (Cohen & Elliot 1963). Subsequently a number of other peptides were found to be structural and functional homologues of EGF and were grouped under the EGF family. These growth factors include Transforming Growth Factor Alpha (TGFα), Amphiregulin (AR), Heparin Binding-EGF (HB-EGF), Cripto (CR), Heregulin (HRG), Betacellulin (BTC) and Vaccinia Virus Growth Factor (Sporn & Roberts 1988; McKay & Leigh 1993).

The cDNA of each of the EGF family members have been isolated and found to encode large proteins that have greater molecular masses than the bioactive growth factors. It is

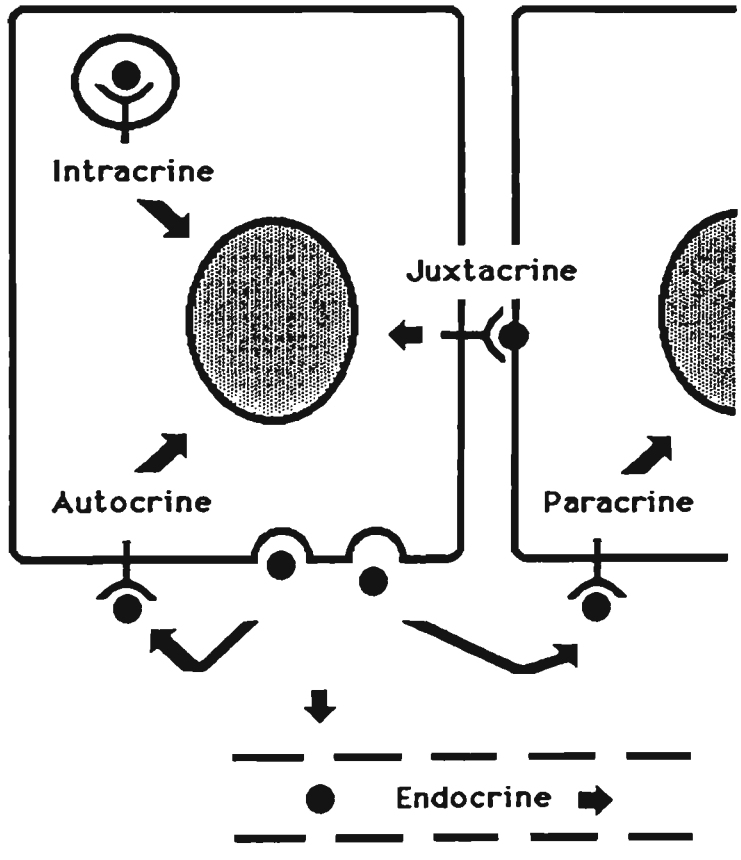


FIG 1.2 Various modes of growth factor activity on target cells. Growth factors are depicted as black circles interacting with their receptor proteins. Arrows pointing to nucleus signify stimulatory or inhibitory effect. Diagram from McKay & Leigh 1993.

thought that the growth factor mRNAs are translated into large precursor proteins known as prohormones or prepro growth factors (Massague & Pandiella 1993). Analysis of the amino acid sequences of the prohormones have shown that they possess features that are typical of membrane spanning proteins having hydrophilic cytosol and extracellular domains plus a hydrophobic region that spans the cell membrane (Massague & Pandiella 1993; Table 1.1 & Fig. 1.3). Therefore the precursors are thought to be transported to cell membranes where they are believed to form integral membrane bound proteins. Smaller, mature, secreted bioactive peptides are thought to be released from the prohormones as a result of proteolytic cleavage/s (Bringman *et al.*, 1987; Adamson 1990;

Evain Brion 1992). The actual proteolytic enzymes however have not been identified with any certainity, but the putative cleavage sites have been identifed using a combination of amino acid sequence analysis of precursors and known protease cleavage sites (Adamson 1990). In the case of CR however the putative cleavage sites have yet to be determined.

Mature secretory forms of EGF, TGFα, AR peptides have also been found to exhibit considerable size heterogeneity although the biological activities appears to be unaffected. For example TGFα peptides ranging in size between 6 and 48kDa have been immunoprecipitated from conditioned media (Bringman *et al.*, 1987; Kohler *et al.*, 1992) and AR proteins ranging between 12-55kDa have been found in media conditioned by breast carcinoma cells (Martinez-Lacaci *et al.*, 1996). It is believed that factors such as the degree and extent of N, O linked glycosylation, partial or complete proteolytic cleavage contribute to the size heterogeneity (Derynck 1988) TGFα has also been found to undergo palmitoylation in addition to glycosylation (Bringman *et al.*, 1987).

TABLE 1.1 Features of the members of the Epidermal Growth Factor Family

growth factor	number of amino acids		relative molecular mass (kDa)	homology of mature growth factor to EGF	interacts with
	precursor	mature growth factor			
EGF	1207	53	6	_	EGFR
AR	252	84	9-55	38	EGFR
BTC	178	50-80	20-30	?	EGFR
CR	188	?	34	?	not known
HB-EGF	204	not known	22	42	EGFR
HRG	640	not known	45	27	c-erb-B2, B3, B4
TGFα	160	50	6-60	40	EGFR

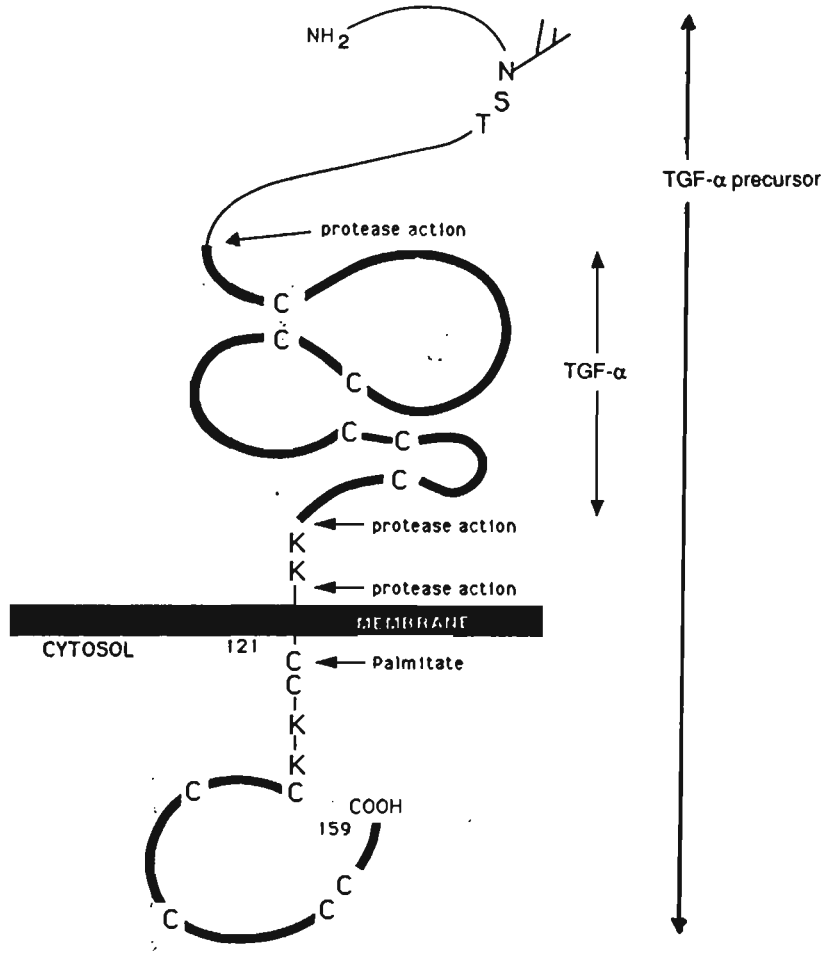


FIG. 1.3 Essential features of the membrane anchored form of members of the EGF family illustrated by TGFα. C=cysteine, N=asparagine, S=serine, T=threonine, K=lysine and Y-shaped projections represent carbohydrate moieties. Diagram modified from Adamson 1990.

Amino acid sequence analysis of the mature secreted growth factors have shown that they possess between 32-45% homology to EGF. The secreted growth factors also possess six cysteine residues in the in the spacing pattern $CX_7CX_{3-5}CX_{10-12}CXCX_5GXRC$ where C=cysteine, G=glycine, R=arginine and X=other amino acids (Fisher & Lakshamanan 1990). The cysteine residues form three disulphide bonds although in CR only two functional disulphide bonds appear to be present (Ciccodicola *et al.*, 1989). In addition to

these features AR and HB-EGF possess very basic amino acids that are located closer to the amino termini which is thought to enable these growth factors to bind heparin (Plowman *et al.*, 1990a; Higashiyama *et al.*, 1993). Amino acid sequences that are thought to enable the translocation of AR to nuclei have also been described (Modrell *et al.*, 1992). Additional features of the members of the EGF family members are also given in Table 1.1.

Numerous studies have demonstrated that the EGF related growth factors are often found to be co-expressed in tumour cells. AR, TGFα and CR proteins have been found to be co-expressed in human breast carcinomas (Panico *et al.*, 1996) and human endometrial carcinomas (Nikura *et al.*, 1996). Similarly HRG, AR and CR proteins have been found to be co-expressed in human breast carcinomas (Normanno *et al.*, 1995). BTC protein is known to be expressed in pancreatic beta tumour cells (Shing *et al.*, 1993). In carcinoma cells the expression of CR, BTC, AR and TGFα proteins and or mRNA has often been found to be elevated compared with non-transformed control tissue; therefore it has been suggested that they may be involved in the malignant transformation of these cells (Prigent & Lemoine 1992b). However the expression of these growth factors is not confined to transformed cells. Immunohistchemical studies have shown that TGFα is expressed in a number of epithelial cells in the digestive, respiratory and reproductive systems (Yasui *et al.*, 1992). Studies have also shown that AR mRNA is expressed in a number of normal tissues that include, breast, placenta and ovaries (Plowman *et al.*, 1990a). HB-EGF mRNA has also been detected in skin, midbrain, lung, heart and kidneys (Vaughan *et al.*, 1992).

1.5 Epidermal Growth Factor Receptor (EGFR) Family

The EGFR familiy consists of four receptors; ie. EGFR, c-erb-B2, c-erb-B3 and c-erb-B4. Analysis of the cDNAs of each of the receptors have shown that they encode proteins that have characteristic features of transmembrane proteins containing a hydophilic extracellular and intracellular domains and a hydrophobic transmembrane region (Adamson 1990; Feige *et al.*, 1992; Fig. 1.4). Each of these receptor proteins have been immunoprecipitated from cell membrane fractions and this finding lends support that they

are located on the cells plasma membrane (Adamson 1990; Feige *et al.*, 1992). However it has been demonstrated that EGF receptors may be found on nuclear fractions as well (Cao *et al.*, 1995). Analysis of the amino acid sequences of the EGFR, c-erb-B2, c-erb-B3 and c-erb-B4 receptor proteins have shown that they exhibit a very high degree of amino acid sequence homology reaching nearly 70% in the extracellular domains (Plowman *et al.*, 1993b; also see Appendix 2b).

Numerous studies have shown that the EGFR interacts with several members of the EGF family such as EGF, AR, TGFα, BTC and HB-EGF (Adamson 1990; Prigent & Lemoine, 1992b), whereas HRG has been found to interact with each of the EGFR related receptor proteins (Beerli *et al.*, 1995; Lewis *et al.*, 1996). BTC is also reported to interact with c-erb-B4 receptor protein in haematopoeitic cell line Ba/F3 (Riese *et al.*, 1996b). In the case of each receptor, the ligand has been shown to bind onto the extracellular domain and this results in receptor dimerisation followed by activation of the tyrosine kinase domain which is located in the intracellular part of the receptor molecule (Ullrich & Schlessinger 1990; Fig. 1.4). The activated tyrosine kinase phoshorylates phospholipase C proteins and activates the protein kinase C signal transduction pathway (Ullrich & Schlessinger 1990).

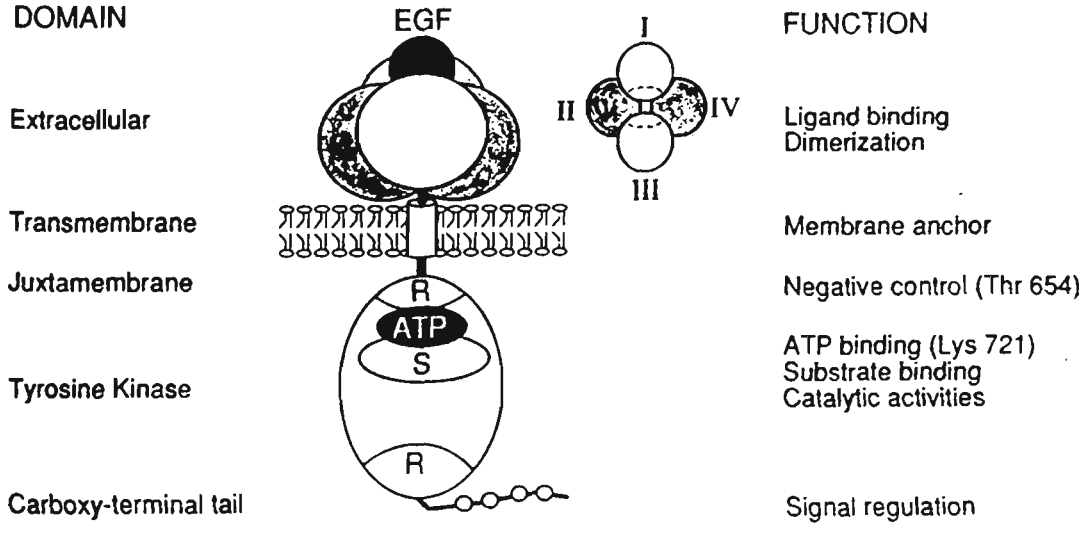


FIG. 1.4 The structural and functional domains of the EGFR. Diagram from Ullrich & Schlessinger 1990.

Numerous studies have shown that the members of the EGFR and c-erb-B3 is widely distributed in normal and transformed tissue (Prigent & Lemoine 1992a, b). The presence of c-erb-B2 and c-erb-B4 mRNA and proteins have been demonstrated in breast and ovarian carcinoma cells (Lewis *et al.*, 1996; Ethier *et al.*, 1996)

1.6 EGFR expression in the gestational tissue

Studies have shown that EGFR mRNA transcripts, 10.5, 5.8 and 1.8kb in size are expressed in the human placenta throughout gestation (Ilekis *et al.*, 1995). The different sized transcripts are thought to be the result of alternate splicing. Further, alternate splicing is considered to be a possible regulatory mechanism as only the 10.5kb transcript is translated into functional protein (Ilekis *et al.*, 1995). Studies *in vitro* using dispersed trophoblast cells have tried to identify factors that may regulate EGFR mRNA expression. Retinoic acid acting via its nuclear receptor has been found to inhibit the expression of the 10.5 and 5.8kb mRNA transcripts by 47% in term placental CT cell cultures (Roulier *et al.*, 1994). Ligands of the EGFR such as EGF and TGFα have not been found to regulate the expression of EGFR mRNA in trophoblast cells *in vitro*. It has been shown however that during the differentiation of CT cells, which were isolated from third trimester placentae, into ST cells, EGFR mRNA levels increased ten fold (Alsat *et al.*, 1993). However the factors that enhance the expression of EGFR mRNA during the differentiation of CT cells into ST cells remain uncertain.

In some studies the EGFR protein has been localised exclusively to the ST cells from 6 weeks to term (Magid *et al.*, 1985; Maruo *et al.*, 1987a) whereas Kawagoe and co-workers (1990), localised EGFR protein to both ST and CT cells from 6 weeks gestation to term. Other studies report that the EGFR protein levels are highest in first trimester tissue and lowest at term (Maruo *et al.*, 1987a). In contrast Brown and co-workers (1987), reported the opposite and also found that EGFR numbers were significantly higher in placenta of male fetuses compared with female fetuses towards term. *In vitro* studies using CT cells isolated from first and third trimesters show that receptor numbers are higher in cells isolated from the third trimester compared with cells from the first

trimester (Carson *et al.*, 1983; Mirlesse *et al.*, 1990), hence lends support to the finding that EGFR numbers do increase with gestational age.

Two types of receptor binding sites, exhibiting either a high or low affinity has also been identified in studies using placental membrane fractions throughout gestation (Carson *et al.*, 1983). Other studies have shown that high affinity and low affinity receptor numbers on placental membranes are down regulated with increasing concentrations of EGF (Lai & Guyda 1984).

Although the receptors of most growth factors are found embedded on the cells plasma membrane, some studies show that the EGFR protein may not be localised only to the plasma membranes since functional EGFR receptors have been found on the intracellular organelles such as lysosomes, rough and smooth endoplasmic reticulum (Ramani *et al.*, 1986), and nuclear membranes of the human placenta (Cao *et al.*, 1995).

In the case of the human fetal membranes, EGFR receptor proteins have been found using binding studies with radioactively labelled EGF and localised to the chorionic trophoblasts and amniotic epithelium (Rao *et al.*, 1984). The receptors have also been found in the decidua throughout gestation (Chegini *et al.*, 1985).

1.7 Expression of EGFR related receptors in gestational tissue

There have been a few studies that have investigated the expression of the EGFR related receptors c-erb-B2, c-erb-B3 and c-erb-B4 in gestational tissues. The expression of c-erb-B2 mRNA has been studied in gravid mice. Murine uterine cells were found to express the c-erb-B2 receptor mRNA, protein for several days after implantation with the highest levels coinciding with implantation (Lim *et al.*, 1997). In the study by Lim (1997), it was suggested that the receptor may be involved in feto-maternal signalling during implantation and embryonic stages, however the expression of the c-erb-B2 ligands in the embryo was not examined. Mulhauser and co-workers (1993), localised c-erb-B2 mRNA to all trophoblast populations in the human placenta but found that the protein was present only in the apical membranes of the syncytium and distal EVT cells facing the

decidua in first and third trimester placenta. As the c-erb-B2 protein was found only in the differentiated cells they suggested that the c-erb-B2 receptor may play a role in differentiation of trophoblast cells. The findings related to c-erb-B2 protein expression in human early trimester placenta by Jokhi and colleagues (1994), were in agreement with the findings of Mulhauser (1993). In addition Jokhi and colleagues (1994), found that the c-erb-B2 protein was present in the placental giant bed cells which is yet another differentiated trophoblast cell type. King and co-workers (1995) also found c-erb-B2 protein in EVT cells but not villous CT cells and found that EVT cells could be separated from villous CT cells by fluorescence activated cell sorting based on c-erb-B2 expression. Investigations have also been carried out to determine whether the c-erb-B2 protein could be used as a marker for persistent a gestational disease such as choriocarcinoma, however latter studies found that the presence of c-erb-B2 protein was non specific (Cameron *et al.*, 1994. C-erb-B2 protein is thought to be secreted into the maternal serum by the placenta and studies by Meden and co-workers (1997), showed that significantly higher amounts of c-erb-B2 protein is present in women with pre-eclampsia whereas significantly lower levels of the protein were found in cases with intra uterine growth retardation.

In vitro studies have shown that interferon may be involved in regulating c-erb-B2 protein expression in invasive trophoblast cells isolated from first trimester placenta, since the addition of 100IU interferon -α/β per ml of culture medium decreased the expression of c-erb-B2 protein after 24, 48, 72h in culture (Aboagye-Mathiesen *et al.*, 1997).

C-erb-B3 mRNA transcripts 6.2kb in size have been detected in human term placenta (Kraus *et al.*, 1989), but the cellular sites of synthesis have not been determined nor have there been any reports of c-erb-B3 protein expression in the placenta at any stage of gestation.

The expression of c-erb-B4 receptor mRNA, protein has not been described previously in the placenta. It is also uncertain whether c-erb-B2, c-erb-B3 or c-erb-B4 receptor mRNA or protein is expressed in the fetal membranes.

1.8 Expression and effects exerted by EGF in gestational tissue

1.8.1 EGF mRNA and protein expression in the placenta

Studies have shown that EGF mRNA transcripts, 5.2kb in size are expressed in the placenta throughout gestation (Bissonnette *et al.*, 1991). EGF protein has also been localised to different trophoblast sub populations throughout gestation but there is a lack of consistency in the literature. In the study of Ladines-Llave (1991), it was reported that EGF was present exclusively in the CT cells at 4-5 weeks whereas Hofmann and colleagues (1992), reported that EGF staining was light or absent in the CT, light to moderate in the intermediate trophoblasts and intense in the ST cells of placenta collected at 4-6 gestation. In latter stages of pregnancy EGF was reported to be present mainly in the ST and the intensity of staining in both CT, ST cells found to decline with gestational progression (Hofmann *et al.*, 1992). In contrast Ladines-Llave and co-workers (1991), reported that EGF was mainly found in the CT and not ST cells during the second and third trimesters. Although there is no consensus of the cellular localisation of EGF protein in the placenta, it is generally believed that EGF protein is produced endogenously by placental cells. For example by assaying placental tissue lysates for EGF, Bissonnette and co-workers (1991), detected between 3-9pg EGF/mg of protein in samples collected from each of the trimesters. The differences in EGF localisation may also be due to detection of receptor mediated uptake of EGF.

1.8.2 Effects exerted by EGF on placental cells *in vitro*

1.8.2.1 Proliferation and differentiation

The addition of EGF to villous CT cells isolated from the first and second trimesters has been shown to increase the rate of proliferation with cells isolated at 16 weeks showing the greatest response (Filla *et al.*, 1993). In another study normal CT cells isolated from the first trimester and passaged 10-20 times also showed increased mitogenic activity in the presence of EGF (Li & Zhuang 1997).

EGF has also been found to be a stimulator of trophoblast differentiation. Bass and colleagues (1994), reported that the invasiveness of trophoblast cells isolated from the first trimester increased in response to EGF. The peptide may only influence the

invasiveness of the EVT cells in the first trimester as Barnea and colleagues (1990), reported that EGF does not influence the differentiation of CT cells isolated from the first trimester into ST cells. However, CT cells isolated from the third trimester have been found to differentiate more rapidly and more fully into ST cells in the presence of 0.1-1ng.ml EGF (Morrish *et al.*, 1987; Morrish *et al.*, 1997). Further, the differentiation of CT cells from the third trimester into ST cells was reported to occur more rapidly when cells were treated with EGF and IGF-1 when compared to the addition of each individual growth factor (Bhaumick *et al.*, 1992).

1.8.2.2 Effects on placental hormone synthesis

The effects of EGF on hCG has been widely studied at various stages of gestation with different findings being reported. Huot and co-workers (1981), found that EGF did not exert any effect on hCG production in placental organ cultures established after 7-10 weeks gestation. However other studies using placental explants and dispersed placental CT cells isolated from a gestational age between 6-10 weeks showed that hCG concentrations increase in response to EGF (Barnea *et al.*, 1990; Ameniya *et al.*, 1994). Very early in gestation the effects on placental hCG synthesis may depend on the gestational age. Studies have shown that EGF exerts no effect on hCG production in placental explants established at 4-5 weeks gestation, whereas hCG production increases in placental explants established at 6-12 weeks gestation (Maruo *et al.*, 1992).

In organ cultures established from term placenta EGF was shown to enhance hCG production (Maruo *et al.*, 1987b) while other findings indicated that EGF exerts no effect on hCG synthesis (Wilson *et al.*, 1984). Studies have also shown that in choriocarcinoma cell lines JEG-3, JAR hCG synthesis does increase in response to EGF and that in JAR cells the arachidonic acid lipoxygenase system and PMA induced signal trandsduction pathways may play a role in the stimulation of hCG synthesis (Benveniste *et al.*, 1978; Ilekis & Benveniste 1985).

EGF is also thought to stimulate hPL production and hPl stimulation by EGF has been demonstrated in first trimester explants established after 6 weeks gestation and explants

from term tissue (Maruo *et al.*, 1987b; Maruo *et al.*, 1992). In CT cell cultures established from murine placenta at mid gestation, EGF was also found to stimulate hPL-1 but inhibit hPL-2 synthesis (Yamaguchi *et al.*, 1992).

Although the effects of EGF on steroid hormone synthesis in normal placental cells have not been described, it has been shown to stimulate progesterone synthesis in the choriocarcinoma cell line JEG-3 (Bahn *et al.*, 1980). EGF has also been shown to stimulate 17βHSD mRNA and protein synthesis in JEG-3 cells (Lewintre *et al.*, 1994). Furthermore, 17βHSD mRNA and protein expression was further enhanced by PMA; hence EGF is believed to regulate placental estrogen production (Lewintre *et al.*, 1994).

1.8.2.3 Other effects

In term villous CT cells EGF has been found to stimulate the synthesis of the EGFR protein (De Palo & Das 1988), and enhance the down regulation of the IGF-1 receptor protein induced by IGF-1 (Bhaumick & Bala 1991). EGF has also been shown to increase the secretion of the growth factor inhibin which is a member of the TGFβ family (Qu *et al.*, 1992).

1.8.3 Expression and effects of EGF in the fetal membranes

Low copy numbers of EGF mRNA transcripts have been found in the decidua using RT-PCR analysis (Haining *et al.*, 1991). EGF protein has been localised to decidual cells throughout gestation by immunohistochemical studies (Hofmann *et al.*, 1991). However, the effects exerted by EGF in the decidua remains uncertain. It is not known whether EGF mRNA or protein is expressed in the chorion but studies *in vitro* have shown that chorionic trophoblasts synthesise greater quantities of prostaglandin E2 in the presence of EGF (Lundin-Schiller & Mitchell 1991).

It is uncertain if EGF mRNA and protein is expressed in the amnion, however studies *in vitro* have shown that EGF mRNA is expressed in amniotic epithelial cells and that EGF increases the mitosis and prostaglandin E2 synthesis in these cells (Tahara et al., 1995).

Therefore it has been suggested that EGF may play a role in parturition *in vivo* through prostaglandin E2 induced myometrial contractions (Tahara *et al.*, 1995). The effects exerted by EGF on the growth and metabolite synthesis in the fetal membranes however remains largely uncertain.

1.9 Expression and effects of EGF related growth factors in gestational tissue

1.9.1 Transforming Growth Factor α

The expression of TGFα in the gestational tissue has also been examined. Several studies have examined the expression of TGFα in early embryonic stages in order to see whether the growth factor may be involved in implantation and/or early development of the placenta. TGFα mRNA has been found in human, porcine, ovine and murine pre-implantation embryos (Rappolee *et al.*, 1988; Hofmann *et al.*, 1993; Chia *et al.*, 1995). TGFα protein has been localised to the murine trophectoderm (Dardik *et al.*, 1992) and human trophoblast cells during the first trimester (Horowitz *et al.*, 1993). In the two studies the growth factor protein was found in cells that are involved in the process of encystment and placentation, therefore it was proposed that TGFα may play roles in both activities interacting with the EGFR that have been found on the endometrial, trophoblast and decidual cells. The peptide may also exert other effects in embryonic stages and in a recent study it was found that murine blastocysts treated with 250pM TGFα enhanced the expression of HEPG2 mRNA and decreased expression of fibronectin mRNA (Babalola & Schultz 1995).

During 10-40 weeks gestation, the 4.5kb TGFα mRNA transcripts appears to be expressed in the placenta and the expression of TGFα mRNA appears to be higher in all trimesters compared with EGF mRNA (Bissonnette *et al.*, 1991). In the rat however the spatial and temporal mRNA expression during pregnancy appears to be different to that of the human. TGFα transcripts were reported to be absent in the rat placenta throughout gestation, but the mRNA was found to be strongly expressed in the maternal decidua peaking at days 8 post conception in studies where expression was examined by Northern and in situ hybridization (Han *et al.*, 1987). On the other hand Wilcox & Derynck (1988),

reported that TGFα mRNA expression was confined to the mouse placenta and embryo on days 9-10 of gestation.

A few studies have shown that TGFα protein in present on human villous CT, ST, EVT cells of the placenta in samples removed from each trimester (Lysiak *et al.*, 1993; Filla *et al.*, 1993; Horowitz *et al.*, 1993). There is some disagreement among these studies however, about the intensity of immunostaining and cells expressing TGFα particularly in the villous cotyledons removed from third trimester placenta. Using RIA between 90-190ng of immunoreactive TGFα protein was found per mg of human placental tissue compared with 8-9pg EGF per mg of placental tissue (Bissonnette *et al.*, 1991). The significance of the large amounts of TGFα synthesised compared with EGF and effects exerted by TGFα throughout gestation in the placenta however remains largely uncertain.

Lysiak and co-workers (1993), found that between 0.05-6ng of TGFα protein added per ml to EVT cells from the first trimester placenta increased their rate of proliferation, but they also reported that the cells did not aggregate more rapidly in the presence of exogenous TGFα. In this study the cells were characterised as EVT cells using a number of EVT specific antigenic markers, therefore is likely that they would aggregate and differentiate into placental bed giant cells. Li & Zhuang (1997), reported that in a normal placental trophoblast cell line established from first trimester placenta, the cells proliferated more rapidly following treatment with TGFα, but the effects on differentiation could not be investigated as the cell line did not undergo spontaneous differentiation. Filla and co-workers (1993), found that TGFα also exerted mitogenic effects on villous CT cells from the first, second trimesters and that the increased rate of proliferation was comparable to the mitogenic effect exerted by EGF on these cells. In villous CT cells isolated from the third trimester placenta TGFα has been found to increase inhibin synthesis in the presence of hPL unlike EGF which can increase inhibin production in the absence of hPL (Qu & Thomas, 1993). TGFα and EGF may therefore be local regulators of inhibin synthesis in the placenta. In the mouse TGFα has been found to decreases hPL secretion in cultured murine placental cells wheras EGF has been shown to increase the hPL production in murine placental cells (Yamaguchi *et al.*, 1995).

Using the choriocarcinoma cell lines JAR, JEG-3 and BeWo, 17βHSD protein concentrations were found to increase significantly in JAR and JEG-3 cells treated with 100ng of TGFα per ml hence it is thought to play a significant role in placental estrogen synthesis (Lewintre *et al.*, 1994). Whether an increase in 17βHSD protein concentrations actually occurs in normal villous CT cells in response to TGFα however remains uncertain. There may be differences in the effects exerted by TGFα between normal and transformed CT cells as it has been shown that JAR, JEG-3 cells fail to increase in number in response to TGFα unlike normal villous CT cells isolated from first trimester placenta (Filla & Kaul 1997).

In the case of the fetal membranes TGFα protein has been immunolocalised to the chorionic trophoblasts, decidua and amniotic epithelium (Lysiak *et al.*, 1993; Filla *et al.*, 1993; Horowitz *et al.*, 1993). Very few studies however have been carried out to determine the effects of TGFα on cells isolated from the fetal membranes. One line of thought is that it may play an indirect role in human parturition in a very similar manner to that described for EGF. Studies *in vitro* have shown that TGFα can enhance the production of prostaglandin E2 in cultured amnion cells and that the enhanced production is similar to amounts produced in the presence of EGF (Tahara *et al.*, 1995). It has also been shown that prostaglandin E2 produced in the amnion can be transported across the amnio-chorion junction without loss of activity which supports the view that prostaglandins produced by the fetal membranes may induce myometrial contractions at the end of gestation (Bennett *et al.*, 1990). Other studies that have examined the intensity of immunostaining of eicosanoid enzymes in myometrium indicate that there are significantly lower levels of TGFα and eicosaniod enzymes in tissues with failed labour compared to tissue following normal labour, suggesting that TGFα also plays a role in prostaglandin synthesis in the human myometrium (Faber *et al.*, 1996).

1.9.2 Amphiregulin

Previous studies in mice have shown that AR mRNA in induced in response to progesterone at day 4 in uterine epithelial cells (implantation stage), that the effect can be

abrogated by the progesterone receptor antagonist RU-486 suggesting a role for AR in implantation (Das *et al.*, 1995). There have been no reports of AR expression during implantation in the human, the mRNA was found to be highly expressed in the human placenta by Plowman and colleagues (1990a), although the gestational age of placental tissue is not mentioned in this report. Lysiak and co-workers (1995), subsequently localised AR protein to the nuclei and cytoplasm of ST cells up to 18 weeks gestation but not villous CT, EVT or decidual cells throughout pregnancy Similar findings to that of Lysiak (1995), were reported in the placenta using the same antisera described in the earlier study (Watanabe *et al.*, 1997).

The only known effect exerted by AR in placental cells is that 1-100ng/ml of AR increases the rate of proliferation of EVT cells from the first trimester in a dose dependant manner (Lysiak *et al.*, 1995).

Whether AR is expressed in the amnion and chorion remains uncertain. In tissue adjacent to the chorion and placenta such as the gravid endometrium AR mRNA has been identified using RT-PCR in the pig (Kennedy *et al.*, 1994). However whether AR protein is expressed in the gravid human endometrium is uncertain.

1.9.3 Cripto

By screening cDNA libraries from fertilised murine eggs, 2 and 8 cell blastocysts Johnson and colleagues (1994), found that CR mRNA is not a carryover maternal message unlike TGFα and EGF, but is expressed specifically at the blastocyst stage. In this study it was also reported that CR message is transiently expressed in the extraembryonic tissue at the early neural plate presomite stage but absent in extraembryonic structures with the gestational progression. Studies have also shown that in mice CR plays a role in the formation of the embryonic heart (Dono et al 1993; SE Johnson *et al.*, 1994).

In the human, a study carried out by Watnabe and colleagues (1997), found that CR protein was present in the ST cell layer of human placental chorionic villi throughout

gestation with found that CR protein expression was highest in the third trimester placenta (Watanabe *et al.*, 1997). It is uncertain if CR mRNA/protein expression occurs in the human fetal membranes. The effects exerted by CR in cells of the gestational tissues have not been elucidated.

1.9.4 Heparin Binding-Epidermal Growth Factor

Although HB-EGF mRNA or protein expression has not been investigated in the placenta or fetal membranes its expression has been studied during encystment of the embryo. In the rodent luminal epithelial cells at the sites of apposition in the uterus have been found to express HB-EGF mRNA and protein after day four of mating (Das *et al.*, 1994). Using ovariectomized rats it was shown that HB-EGF mRNA and protein could be upregulated in the uterine luminal epithelium in response to estradiol or estriol plus progesterone, hence HB-EGF expression at encystment is probably regulated by these hormones (Wang *et al.*, 1994). However Zhang and co-workers (1994a), reported that progesterone repressed HB-EGF mRNA expression in the rat uterine epithelial cells but enhanced expression in uterine stromal cells. In addition it was shown that the antiprogestin ZK 98.299 was able to abolish HB-EGF mRNA expression in rat uterine stromal cells, supporting the belief of the stimulative effects of progesterone on HB-EGF expression (Zhang *et al.*, 1994b). Conflicting reports of HB-EGF mRNA expression is also described in the gravid porcine endometrium. In one study HB-EGF mRNA was not detected in the porcine endometrium by RT-PCR analysis (Kennedy *et al.*, 1994) while another found transcripts in endometrial tissue, localised the protein to the luminal epithelium and found 13.5, 17kDa immunoreactive HB-EGF proteins in uterine luminal flushings (Kim *et al.*, 1995). In vitro studies have shown that murine blastocysts adhered to 32D cells which express membrane bound form of HB-EGF in co-culture showing that the peptide may be an important attachment factor in the murine uterus (Raab *et al.*, 1996).

1.9.5 Heregulin and Betacellulin

In a study that examined HRG expression in a number of tissues by Northern hybridization the mRNA was reported to be absent in the human placenta (Holmes *et al.*, 1992). In this study however the gestational age of the placenta was not mentioned. The expression and effects of HRG in the fetal membranes and BTC in the gestational tissue remains uncertain.

In summary the expression and effects that may be exerted by the EGF related growth factors in the gestational tissue remains largely uncertain.

1.10 Placental progesterone synthesis

Progesterone is regarded as one of the principal steroid hormones produced by the primate placenta; the other being estrogen (Simpson & MacDonald 1981). In women progesterone is produced by the nascent placenta as early as two weeks post conception (Csapo & Pulkkinen 1978). These investigators also found that between 2-7 weeks of gestation progesterone synthesis by the corpus luteum declined while placental synthesis increased and surpassed secretion by the corpus luteum by about 8 weeks gestation. Placental progesterone has been found to enter both maternal and fetal circulations with the fetal circulation having 2-3 times greater concentrations than the maternal circulation (Albrecht & Pepe 1990). Measurements of progesterone concentrations in maternal serum has shown that the hormone levels increases gradually from about 18ng to 200ng / ml between 8 weeks gestation and term respectively (Tulchinsky *et al.*, 1972), and that from 8 weeks onwards placental synthesis alone is thought to be sufficient to support pregnancy (Csapo & Pulkkinen 1978).

1.10.1 Effects exerted by progesterone during human pregnancy in vivo

In early pregnancy progesterone is thought to suppress ovulation by inhibiting pituitary luteinising hormone release (Jones 1989). Previous studies have also shown that progesterone enhances endometrial cell proliferation and differentiation, decidualization

and due to its anti-inflammatory and immunosupressive properties is believed to facilitate the implantation of the blastocyst in the early stages of gestation (Albrecht & Pepe 1990). During the latter stages of pregnancy, progesterone is thought to increase the mucification of the vagina, the thickness of the myometrial cells increasing the overall size of the uterus, maintaining a quiescent state of contractile myometrial cells and causing the growth of the alveoli, ducts of the mammary glands (Heap & Flint 1988). Progesterone may also be involved in maternal weight gain although the mechanism is not clear. In mice, maternal weight gain after progesterone treatment is thought to be due to increased water retention and stimulation of the appetite. In pregnant women progesterone treatment has been shown to increase salt excretion although this effect can be counterbalanced by estrogen, hPL and prolactin (Heap & Flint 1988). Estrogen is also known to modulate the effects of progesterone. It has been found that when progesterone concentrations are higher by three orders of magnitude compared with estrogen, the two hormones act synergistic manner; when the differences are lower the two hormones act in an antagonistic manner (Heap & Flint 1988). Although in women the effects of progesterone on parturition remains uncertain, in sheep a fall in placental progesterone production prior to birth is thought to induce prostaglandin release which in turn increases myometrial contractility (Challis & Olson, 1988).

1.10.2 Biosynthetic pathways of placental progesterone synthesis

The pathways of progesterone synthesis in the human placenta have been elucidated largely *in vitro* using organ, explant and dispersed cell cultures. These studies have identified two possible biosynthetic pathways of progesterone synthesis ie. the *de novo* and LDL pathway (Albrecht & Pepe 1990).

1.10.2.1 The de novo pathway

Progesterone is known to be synthesised from cholesterol and in the *de novo* pathway, cholesterol produced endogenously by the placenta is thought to be converted to

progesterone (Albrecht & Pepe 1990). However studies using radioactively labelled acetate have shown that conversion of acetate into cholesterol in the perfused placenta was negligible (Telgedy *et al.*, 1970). In choriocarcinoma cells Simpson and co-workers (1978), reported that the activity of enzyme HMG CoA reductase which converts acetate into cholesterol was very low and estimated that only 1-2% of cholesterol required for placental progesterone synthesis would be produced *de novo*. On the other hand Moise and colleagues (1986), found that between 30-50% of progesterone was synthesised *de novo* cholesterol in the choriocarcinoma cell line JEG-3.

1.10.2.2 LDL pathway

In this pathway cholesterol bound to low density lipoproteins (LDL) that are present in the maternal serum is believed to be the substrate for progesterone synthesis. Simpson and co-workers (1979), found that CT cells take up and degrade LDL and to a lesser extent high density lipoproteins (HDL) and that the process involves lysosomal degradation. LDL is thought to enter the CT and ST cells via LDL receptors while pulse chase experiments have shown a complex pathway of internalisation involving coated pits, endosomes and multivesicular bodies (Malassine *et al.*, 1987). LDL has been shown to be converted to pregnenolone by the enzyme cholesterol side chain cleavage cytochrome (P450scc) found on the inner mitochondrial membranes of the CT and ST cells (Hall 1986). Pregnenolone has been shown to be converted to progesterone by the enzyme 3β-hydroxysteroid dehydrogenase /Δ^5-Δ^4 isomerase (3βHSD) that is found in both placental mitochondrial and microsomal fractions (Koide & Torres 1965). Progesterone may also be converted to intermediates of estrogen synthesis such as 17α-hydroxyprogesterone or 16α-hydroxyandrostenedione in placental cells (Albrecht & Pepe 1990).

1.10.3 Factors affecting placental progesterone synthesis *in vitro*

The factors affecting placental progesterone synthesis have been investigated from different perspectives. For example the effects of the substrates, other placental hormones, intermediates of estrogen, testosterone synthesis and inhibition or enhancement

of the activities of P450scc, 3βHSD enzymes have been investigated. On the other hand there are very few reports on the effects exerted by growth factors on placental progesterone synthesis or their influence on P450scc or 3βHSD mRNA and protein expression.

Previous studies have shown that placental CT and ST cells from term placenta grown in the presence of 35-420μg LDL protein/ml, showed an increase in the progesterone secretion that was reflected with increased LDL concentration whereas the same concentrations of HDL did not exert any effect (Winkel *et al.*, 1980). Maslar and colleagues (1990), found that addition of labelled pregnenolone to media containing placental explant cultures established from placentae after 8-12 weeks gestation, resulted in an increase in progesterone synthesis and that 66-100% of radioactively labelled pregnenolone was converted into progesterone.

Thyroid hormone at a concentration of 100μM has been shown to increase progesterone synthesis by explant cultures established from placentae between 7-8 weeks gestation (Maruo *et al.*, 1992). Cells isolated from term placentae and cultured up to four days with 2mM cAMP analog 8-Br-cAMP, 50μM forskolin or 20ng cholera toxin was also found to increase progesterone secretion and Rodway and co-workers (1988), suggested that this may be due to enhancement of β-adrenergic receptor stimulation. An earlier study using explants from term placenta showed that calcium ions could also stimulate progesterone synthesis presumably through calcium induced β-adrenergic receptor stimulation (Kasugai *et al.*, 1987).

Inhibition of progesterone synthesis has been demonstrated in placental organ cultures established from second and third trimester tissue treated with 10-40μg luteinising hormone releasing hormone (LHRH) and analysed after 72-96h in culture (Wilson & Jawad 1980). In their study, Wilson & Jawad (1980), suggested that the hormone exerted a direct inhibitory effect on steroid hormone synthesis. Branchaud and co-workers (1983), however found that inhibition of progesterone synthesis by LHRH occurs only in the presence of 10^{-5}M dehydroepiandosterone (DHA) or DHA sulphate (DHAS) in monolayer cultures established from term placental CT cells cultured up to 5 days.

Other studies have shown that the enzyme P450scc isolated from 8-12 week and term placenta is inhibited by cholesterol side chain cleavage products such as 20α-hydroxycholesterol (Rabe *et al.*, 1985). In this study and another by Das and co-workers (1985), it was reported that estrogen and progesterone inhibited the activity of 3βHSD enzyme. Rabe (1985), also found that pregnenolone, several intermediates of estrogen synthesis pathway, cortisol and cortisone also exerted inhibitory effects on 3βHSD and suggested that placental progesterone synthesis is regulated by autonomous factors and not by external endocrine factors.

It has also been shown that phorbol esters such as TPA which are inducers of signal transduction pathways can also stimulate progesterone synthesis. EGF has been reported to stimulate progesterone secretion by JEG-3 choriocarcinoma cells even when relatively small doses of 2-3ng/ml were incorporated while 10ng/ml EGF was found to increase the incorporation of radioactively labelled acetate into cholesterol (Bahn *et al.*, 1980). It is possible that EGF may exert it effects via the TPA signal transduction pathway. To our knowledge the only other growth factor that has been shown to regulate placental progesterone synthesis is IGF-1. Nestler & Williams (1987), found that CT cells isolated from term placenta treated with 20ng/ml IGF-1 resulted in a 19-36% increase in the progesterone concentrations. In this study the cultures were treated with 25-hydoxycholesterol, therefore IGF-1 was believed to enhance the activity of P450scc in its conversion of the hydroxycholesterol into pregnenolone.

Although the EGF related peptide, TGFα, has not been reported to regulate progesterone synthesis in the CT or ST cells, this growth factor has been found to regulate the hormone in other types of cells.

1.10.3.1 Regulation of progesterone synthesis by TGFα in vitro

Previous studies have shown that in rat corpora luteal cells incubated with 0.5-50ng TGFα/ml progesterone synthesis increased between days 3-5 when the media was changed daily (Tekpetey *et al.*, 1995a). Human granulosa lutein cells cultured in the

presence of 50ng/ml TGFα were shown to stimulate progesterone production four fold (Tekpetey *et al.*, 1995b). In the rat ovarian granulosa cells progesterone production was found to increase when cells were treated with TGFα, and incorporation of follicle stimulating hormone into media containing TGFα produced a synergistic elevation of progesterone over the culture period of ten days (Yeh *et al.*, 1993). In contrast, Adashi & Resnick (1986), found that TGFα inhibits progesterone synthesis by reducing aromatisation in cultured rat ovarian granulosa cells.

It therefore appears that TGFα is either a positive or negative regulator of progesterone synthesis in non-gestational tissues.

1.11 In vitro systems used in the study of placental syntheses and function

Studies involving placental syntheses and functions in vivo, particularly those using human subjects are hindered due to ethical, moral and practical reasons. These difficulties have been largely overcome by developing and refining in vitro systems for the study of placental syntheses and function. Currently the most widely used system is that of dispersed cell cultures, however others such as placental perfusion, organ and explant cultures are some of the other important systems used to study placental growth, structure and function (Ringler & Strauss 1990).

1.11.1 Placental perfusion, organ & explant cultures - advantages & disadvantages

Studies involving human placental perfusion were first reported nearly 50 years ago. For sometime thereafter the whole, intact placenta was perfused via cannula inserted into blood vessels, the two perfusates were collected separately and analysed (Lamb *et al.*, 1967). Studies involving perfusion of the whole placenta are thought to have played a significant role in elucidating the metabolism of radioactively labelled steroids in the placenta (Varangot *et al.*, 1965; Cedard *et al.*, 1970). More recent investigators however favoured the use of a single lobule or cotyledon instead of the whole placenta since the

perfusion circuits could be more easily monitored (Abramovich *et al.*, 1987). Eaton and co-workers (1982), used this approach to investigate the uptake and metabolism of EGF by perfused lobules from third trimester human placenta. They found that within 30min over 60% of the perfused EGF entered the lobules and that after 2h about 13% of degraded EGF re-entered the maternal circulation.

The main advantages of using the whole placenta or lobule perfusion systems are that substances are presented to the placenta by the normal vascular route that it would take *in vivo*, secretion into maternal, fetal compartments can be monitored simultaneously and that the normal morphological, physiological relationships between placental cells are maintained (Panigel, 1972). However other studies have identified several disadvantages including extensive ischaemia due to the generation of free radicals, tissue oedema, degenerative changes in the ST layer, hyperplasia, hypertrophy of the CT cells and damage to intracellular organelles (Contractor *et al.*, 1984; Ringler & Strauss 1990). Another disadvantage described is that as the tissue appears to degenerate fairly rapidly therefore the period of study is usually limited to less than a day (Panigel 1972).

Organ cultures have been established by partially submerging smaller pieces of chorionic villi generally 1-1.5cm^3 pieces in nutrient media (Huot *et al.*, 1979). The advantages and disadvantages of organ culture have been found to be very similar to that described earlier for placental perfusion, but organ cultures can be maintained for longer periods *in vitro* (Ringler & Strauss 1990). Studies have also shown that nutrient flow and gas exchange present difficulties. This is believed to be the result of partial contact of the tissue with the nutrient media and the fact that submerged tissue is not exposed to the gas phase (Ringler & Strauss 1990). Organ cultures have been used to examine placental hCG and progesterone synthesis (Ahmed *et al.*, 1988; Barnea *et al.*, 1990). Maruo and colleagues (1987b), reported that 100ng/ml EGF added to placental organ cultures established from term tissue increased hCGα, total hCG and hPL synthesis.

Placental explant cultures are very similar to organ cultures in the manner in which they are established but differ from organ cultures in that smaller pieces of tissue, generally between 1-2mm^3 are used and that the tissues are immersed and not partially submerged

in the culture media (Chung *et al.*, 1969). As a result of immersion, hypoxic conditions are established which brings about that the proliferation and outward migration of trophoblast cells from the explants (Patillo *et al.*, 1968) while inhibiting the growth of mesenchymal cells (Ringler & Strauss 1990). Placental explants established from regions containing CT cell columns have been reported to yield a significantly higher proportion of trophoblast outgrowths compared with randomly selected chorionic villi (Patillo *et al.*, 1968). In more recent studies however chorionic villi freed of maternal decidua and minced finely have been shown to successfully produce outgrowths (Genbacev *et al.*, 1992). In this study Genbacev and colleagues found that outgrowth from villi were facilitated when explants adhered onto a matrix such as type 1 rat tail collagen with 10% decidual extracts or Matrigel (which is composed of type IV collagen, laminin, heparin sulphate proteoglycans and enactin). Other investigators have reported that chorionic villi also adhere onto culture vessels, produce outgrowths and migrant cells (Irving *et al.*, 1995).

Immunohistochemical studies have shown that the majority of cells migrating from placental explants cells react with HLA-A, B, C, Ki76 (Genbacev *et al.*, 1992) and integrin α1, 3, 5, v, β1, NDOG-5, PCNA, IGF-II antisera (Irving *et al.*, 1995). These antigens have been described as being characteristic of EVT cells, hence this system is used to isolate EVT rather than villous CT cells (Irving *et al.*, 1995, Genbacev *et al.*, 1992).

An advantage of the explant culture system is that trophoblast cells can be obtained with relative ease without resorting to lengthy, time consuming multi-step procedures. Contamination by fibroblasts have been reported as a difficulty that is encountered with explant cultures (Ringler & Strauss 1990). Irving and co-workers (1995), found that 61% of migratory populations established from chrionic villi were a mixture of trophoblasts and fibroblasts. Another disadvantage is that explant cultures can be established only from chorionic villi up to about 20 weeks gestation (Genbacev *et al.*, 1992). Therefore the system cannot be used for studies involving placenta from the third trimester. It is believed that chorionic villi from the third trimester are unable to form

outgrowths due to the inherent lack of invasiveness and degradive activity by CT cells during the latter stages of gestation (Loke & Burland 1988; Bloxam *et al.*, 1997a).

1.11.2 Dispersed cell cultures

Dispersed placental cells can be obtained by digesting chorionic villi with proteolytic enzymes such as trypsin, dispase or protease XV (Bloxam *et al.*, 1997a, b), in combination with collagenase and/or DNase (Ringler & Strauss 1990). In earlier studies the digested mixtures were centrifuged to remove undigested material and cells cultured without further purification. Detailed microscopic examination of these cultures however showed that both CT cells and non-trophoblastic elements were present in varying proportions (Loke & Whyte 1983). Visual examination also showed that ST cells were rarely present immediately after dispersal and a plausible explanation was that the ST cells were broken down during the proteolytic digestion (Ringler & Strauss 1990). In order to reduce contamination by non trophoblastic elements Kliman and co-workers (1986), introduced a density gradient centrifugation step through a discontinuous Percoll gradient. They found that CT cells sedimented between 1.048-1.062g/l and could be separated from monocytes, connective tissue that sedimented at different densities. Although Kliman's report (1986), did not specify the percentage of CT cells in the isolates other workers demonstrated that between 90-95% of the isolated cells were CT cells (Douglas & King 1989). In order to reduce contamination further, simple steps such as filtration through nylon mesh filter of known pore size prior to density gradient centrifugation were introduced and shown to reduce the incidence of macrophages and fibroblasts (Yeger *et al.*, 1989). Using a different approach Douglas & King (1989), reacted the primary isolates with HLA-A, B, C and HLA-DR histocompatibility antisera. They reported that the antisera bound to cells from the villous mesenchyme. Further they removed the contaminating cell rossettes with IgG coated onto magnetic microspheres and a magnetic particle concentrator. Using this modification the investigators found that pure CT cells could be obtained. Instead of microspheres Schmon and co-workers (1991), used HLA-A, B, C antibodies coated onto plastic dishes to remove contaminating cells. The antigen CD9 has also been used in a similar manner to enrich for CT cells by negative selection (Yui *et al.*, 1994). CD9 is a

cell surface antigen expressed by fibroblasts. Yui and colleagues (1994), reacted the CD9 antisera with primary isolates and eluted the isolates through columns filled with glass beads coated with IgG. In the study of Yui and co-workers (1994), it was found that the percentage of CD9 positive cells could be reduced from greater than 3.3 to 0.1%.

In order to assess the purity and cytotrophoblastic nature of the dispersed cells, the morphological appearance, surface and cytoskeletal antigens are some of the important criteria that have been used. CT cells appear as rounded, mono-nucleated cells that are between 10-30μm in diameter with the majority of cells 10μm in diameter (Kliman *et al.*, 1986). Antisera that reacts with all trophoblast populations such as placental alkaline phosphatase have been utilised for immunocytochemical characterisation, but staining with antibodies that recognise intracellular antigens such as cytokeratins or steriodogenic enzymes P450scc, P450 aromatase, 17βHSD present in CT cells are reported to be superior to surface antigens that may leach out during immunostaining (Ringler & Strauss 1990). Antisera against hCG, hPL, SP1 that are predominantly expressed by ST cells (Kliman *et al.*, 1986), and vimentin produced by mesenchymal cells are widely used as negative control antisera (Douglas & King 1990).

The main advantage of cell dispersion is that essentially pure, homogenous cultures of CT cells can be isolated from placenta obtained from different stages of pregnancy. Experimental data also shows that *in vitro* the CT cells elaborate enzymes and hormones synthesised *in vivo*, hence appear to behave in a similar manner to cells *in vivo* (Ringler & Strauss 1990). The purity of the preparations has allowed a number of studies to be undertaken including growth factor, receptor mRNA, protein expression, ligand-receptor interaction, intermediates of signal transduction and autocrine, paracrine, juxtacrine regulatory effects of growth factors on CT cells. The purity is also an important factor for RT-PCR and subtractive hybridization analyses where misleading results can be obtained due to the presence of a few contaminating cells. In appropriate media the cells can be maintained *in vitro* for up to 3 weeks (Yeger *et al.*, 1989), enabling temporal studies. The main disadvantage is that the CT cells are removed from their normal cellular and physiological environment (Ringler & Strauss 1990).

1.11.3 Differentiation of villous cytotrophoblast cells in vitro

Another important aspect of the CT cell culture system is that the cells can be induced to undergo differentiation in vitro. CT cells isolated from the first trimester have been reported to differentiate into EVT or ST cells. The culture media and extracellular matrix on which the cells are grown appears to be critical factors that influences the differentiation into EVT or ST by cells isolated from the first trimester placenta. Loke & Burland (1988), obtained 80-90% EVT cells when culture dishes were coated with an extracellular matrix (supplied by Denley, UK) and modified Ham's F10 medium with EGF, 20% FCS after 2 days in culture. On the other hand, CT grown on fibronectin coated plates and cultured in serum free defined media supplemented with D-valine and EGF have been reported to form ST cells within 3-4 days (Daniels-McQueen *et al.*, 1986).

CT cells isolated from the third trimester are believed to differentiate into ST cells by a process of aggregation and fusion which can be observed within 24h in culture (Kliman *et al.*, 1986). Evidence of syncytial formation by fusion of CT cells has also been demonstrated by observing the re-distribution of cytoskeletal elements such as desmoplakin and through electron microscopic studies. Douglas & King (1990), found that desmoplakin was dispersed in the cytoplasm of CT cells but that it became re-arranged into punctate figures at points of cell contact during aggregation that was consistent with formation of desmosomes during transition into syncytia. Electron microscopic studies also reveals that desmosomes appear between aggregated CT cells, that the intervening plasma membranes breaks down and that a large number of nuclei become clustered together (Bax *et al.*, 1989).

Factors that influence syncytial formation in vitro have also been extensively investigated. When villi are digested in media containing calcium and magnesium salts are formed syncytia more quickly and this may be due to the fact that calcium is required for desmosome formation (Douglas & King 1990). Another suggestion that has been put forward is that calcium dependant adhesion molecules such as E-cadherin are involved in aggregation (Ringler & Strauss 1990; Bloxam *et al.*, 1997b). The density of the initial plating is also thought to be a contributory factor (Bloxam *et al.*, 1997b). Other studies

have shown that the culture media influences syncytial formation. For example CT cells grown in DMEM, EMEM, RPMI-1640 enabled syncytial formation (Bloxam *et al.*, 1997b), while one study reported that complete keratinocyte growth medium was superior to DMEM in inducing ST cell formation (Douglas & King 1990). Serum is thought to be one of the most important factors that influences the formation of ST cells in vitro. Between 5-30% fetal calf, newborn calf or maternal serum in the culture medium have been found to induce ST cell formation in vitro (Richards *et al.*, 1994; Bloxam *et al.*, 1997b). It is thought that serum provides the mobility factors that enables movement, cell to cell contact and aggregation (Bloxam *et al.*, 1997b). It has also been suggested that the nature of the extracellular matrix protein is another important factor for syncytial formation, particularly if the CT cells are grown in the absence of serum. Matrix proteins such as fibronectin, collagen types 1, IV, Matrigel have been found to facilitate ST cell formation (Bloxam *et al.*, 1997b).

The syncytial nature of the cells has been most commonly assessed by immunological and or biochemical characterisation. The most widely used antisera includes cytokeratin, hCG, hPL, SP1 (Kliman *et al.*, 1986; Yeger *et al.*, 1989). Measurements of secreted hCG or hPL are among the most widely used biochemical tests that have been applied to assess the degree of syncytial formation (Bloxam *et al.*, 1997b). There have been reports however that CT cells may also secrete hCG after a number of days in culture without apparent syncytial formation (Kao *et al.*, 1988).

1.11.4 Dispersed cell cultures of transformed placental cells

Although normal EVT cell lines have been successfully produced (Irving *et al.*, 1995), transformed cell lines such as JAR, BeWo and JEG-3 established from choriocarcinomas are widely used to study CT syntheses and function. They are reported to have many biochemical features that are similar to dispersed villous CT cells however the cells are aneuploid and are unable to differentiate into ST cells therefore imposing limitations on the types of studies that can be conducted (Ringler & Strauss 1990).

In summary, the dispersed CT cell culture system appears to be one of the most suitable *in vitro* systems that is presently available to study the expression and functions exerted by growth factors in the placenta during the third trimester.

1.12 Aims

The major aims of this thesis are:

- to determine whether the EGF related growth factors HRG, AR, TGFα, CR, BTC, HB-EGF and c-erb-B2, c-erb-B3 and c-erb-B4 mRNA are expressed in the placenta, amnion and chorion between 38 and 40 weeks gestation

- to determine if the EGF / EGFR related growth factor, receptor mRNAs are expressed in CT and ST cells under *in vitro* conditions

- to localise the expression of some the EGF related growth factor mRNAs (that were detected in the initial studies) in the chorionic villi of the placenta and fetal membranes collected between 38-40 weeks gestation

- to localise AR and TGF proteins in the chorionic villi and fetal membranes obtained after 38 weeks gestation.

- to investigate AR and TGF protein expression in CT and ST cells *in vitro*

- to determine the effects exerted by TGF on proliferation and aggregation of villous CT cells *in vitro* and

- to identify mRNA species that were being upregulated in villous CT cells following treatment with TGF *in vitro.*

CHAPTER 2

Materials and Methods

This chapter gives details of the materials utilised and experimental procedures that were conducted to obtain the results described in Chapters 3 to 7.

2.1 Gestational tissue

Human placenta and fetal membranes were retrieved with prior informed consent of women undergoing elective Caesarean sections or spontaneous vaginal deliveries after 38-40 weeks of gestation post conception. Patients selected for the study were non-smokers, had consumed no or low levels of alcohol during gestation and did not suffer from pregnancy related diabetes or hypertension. Placenta were delivered at the Western General Hospital, Sunshine and the Mercy Hospital for Women, Werribee. Ethical approval for collection and experimentation involving human tissue was obtained from the Human Ethics Committees of the Western General Hospital, Mercy Hospital for Women and the Victoria University of Technology, (see consent form Appendix 1).

Specimens were collected only from placenta that were free of calcification, normal in size and appearance for the gestational age (Fig 2.1). The amnion and smooth chorion were peeled apart and 3-5g of tissue was cut out from each of the membranes. Specimens weighing between 5-7g and/or 50-70g were dissected out from the villous cotyledons taking care to avoid the villous chorionic membrane. Handling and retrieval were carried out under aseptic conditions and specimens collected within 30-40min of placental delivery.

The villous cotyledons (50-70g) were used for the isolation of cytotrophoblast cells (section 2.2.1) while samples from amnion, chorion-adherent decidua and villous cotyledons (5-7g) were used for the following studies:

- expression of EGF/EGFR growth factor and receptor mRNA by RT-PCR analysis (section 2.4 & Chapter 3)

- localisation of growth factor mRNA expression by in situ hybridization (section 2.6 & Chapter 4)

- analysis of TGFα and AR protein expression by Western hybridization (section 2.7) and localisation of protein expression by immunohistochemistry (section 2.8 & Chapter 5).

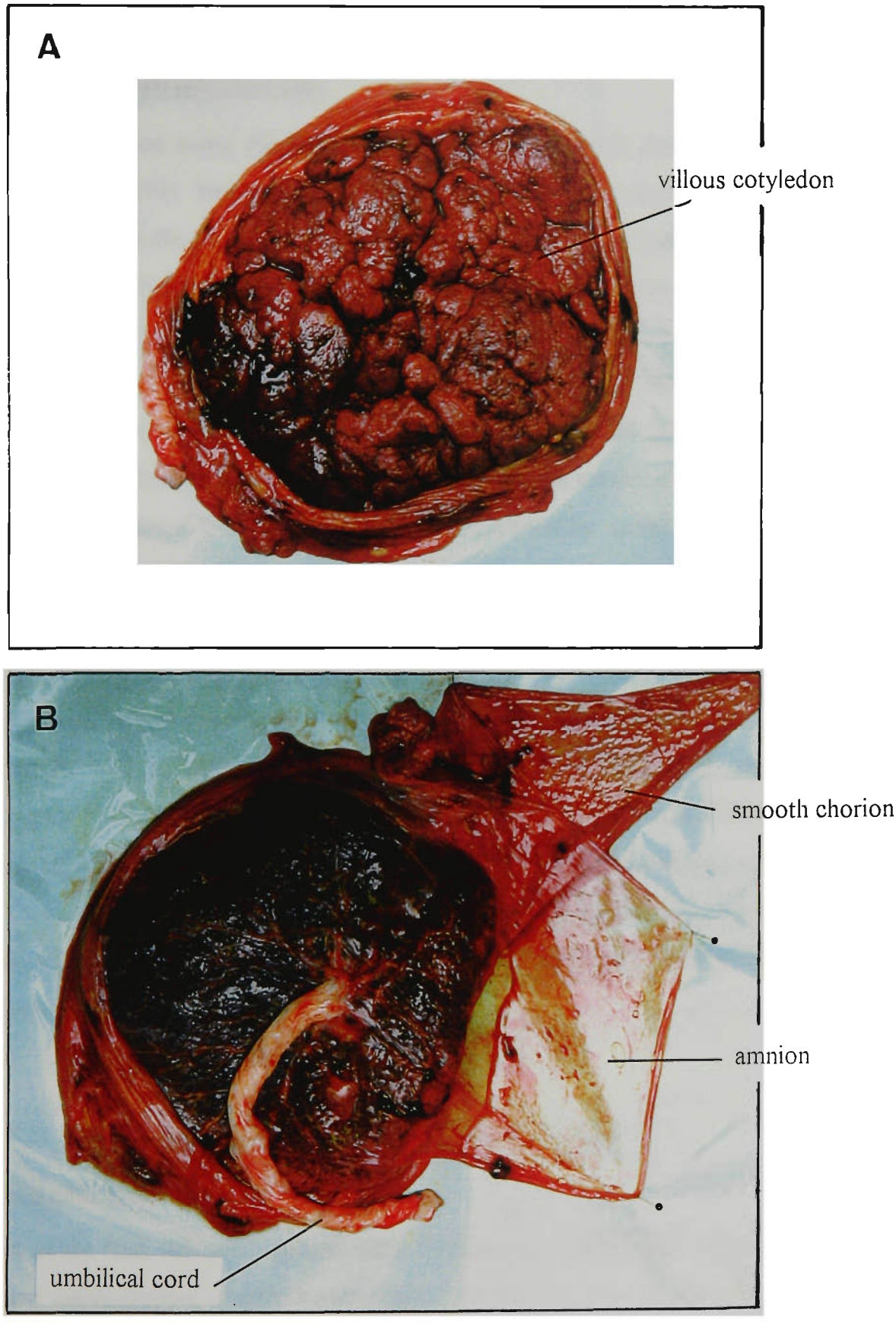


FIG. 2.1 Human placenta delivered by Caesarean section after 40 weeks gestation. A) maternal surface. B) fetal surface. Part of the amnion and smooth chorion have been peeled apart. Magnification = approximately 0.4x.

2.2 Isolation, characterisation and culture of cytotrophoblast cells

2.2.1 Isolation of cytotrophoblast cells

The cells were isolated using modified versions of the protocols described by Kliman (1986), and Jones (1989). Between 50-70g of villous cotyledonary tissue was rinsed in 4-5 changes of Hanks Buffered Saline Solution (ICN) containing 1mg/l gentamycin (Sigma). The soft villous material was freed of blood vessels, connective tissue and finely minced using a scalpel. The minced tissue was transferred into 50ml Jokliks Minimum Essential Medium (JMEM, ICN) with 250mg trypsin (Sigma), 2.5mg DNase1 (Boehringer Mannheim) and digested with agitation at 37°C for 40min. The cell suspension was filtered through a 0.18mm pore size nylon mesh filter and mixed with 10% charcoal stripped fetal calf serum (FCS, Sigma). Cells were pelleted by centrifuging at 2100rpm for 10min at 20°C, washed free of trypsin in two changes of Eagles Minimum Essential Medium (EMEM, ICN) and once in EMEM with 10% FCS. Note that during washing the cells were generally resuspended in 30-35ml of the appropriate media, pelleted by centrifuging at 2100rpm for 10min at 20°C and supernatant discarded. After the final wash in EMEM with 10% FCS the cells were resuspended in 7.5ml JMEM, layered onto a discontinuous Percoll (Sigma) gradient consisting of 5, 10, 20, 40 and 60% Percoll in JMEM and centrifuged at 2200rpm for 15min at 15°C. Cells sedimenting at a density between 1.04-1.06g/l (ie. between the 40-60% Percoll interface) were collected, washed free of Percoll in EMEM with 10% FCS followed by two changes in EMEM alone. The final cell pellet was resuspended in 20-30ml EMEM.

2.2.2 Total and viable cell counts

An aliquot of the final cell pellet suspended in EMEM was stained with trypan blue and the total and viable number of cells were estimated. Generally 20µl of the cell suspension was diluted with 0.48ml EMEM and 0.5ml trypan blue (0.4% w/v in 0.8% NaCl solution). Cells were stained for 5min and counted on a haemocytometer. The total number of cells/ml was calculated using the following formula:

$$\text{average number of cells in ten 1mm squares} \times \text{dilution factor} \times 10^4 = \text{cells/ml}$$

The percentage of viable cells was assessed by trypan blue exclusion and calculated using the following formula:

$$\frac{\text{total number of cells / ml with unstained nuclei}}{\text{total number of cells / ml with stained and unstained nuclei}} \times 100\% = \% \text{ viable cells}$$

2.2.3 Characterisation of freshly isolated cytotrophoblast cells

The nature and purity of the isolated cells were assessed by immunocytochemical staining against the intracellular antigens cytokeratins 8 and 18. The cells were diluted to $1x10^6$ viable cells/ml in EMEM and 50µl of the cell suspension placed on poly-L-lysine (Sigma) coated coverslips. The cells were allowed to adhere for 30min at room temperature and washed thrice in 1x PBS solution (10mM phosphate pH7.4, 0.8% NaCl). Cells were fixed in ice cold MeOH at -20°C. Non-specific binding blocked in 10% non-immune goat serum in 1x PBS and stained with mouse monoclonal antibodies against human cytokeratins 8 and 18 (Sigma) diluted 1:200 and 1:800 respectively. Biotinylated goat anti-mouse IgG (Histostain SP kit, Zymed) served as the IIry antibodies. The cell fixation and immunostaining procedures are described in detail in sections 2.8.2 & 2.8.3 respectively.

2.2.4 Cytotrophoblast cell culture

Cells were diluted to the required density in EMEM with 10% FCS and plated in multiwell dishes (ICN or Iwaki) pre-coated with 2µg collagen type I from rat tail (Boehringer Mannheim) / cm^2 plate surface area. The seeding densities varied and the densities used in each of the different experiments are described in the appropriate sections that follow.

Cultures were incubated in a humidified 5% CO_2 : 95% air atmosphere at 37°C in a water jacketed CO_2 incubator (TS Autoflow, Nuaire). The cells were observed under an

Olympus CK-2 inverted microscope and photographed with an Olympus OM4Ti SLR camera using Kodak ASA 400 film.

2.2.5 Syncytiotrophoblast cell culture

CT cells were diluted to a density of $0.75x10^6$ cells/ml in EMEM with 10% FCS and 2-3ml of the cell suspension was plated in 35mm diameter wells and incubated as described earlier. After 48h the spent media was removed and replaced with 2-3ml EMEM containing 15% FCS. The cells were cultured up to 96h. Cultures were observed and photographed as described in section 2.2.4.

2.2.6 Characterisation of cultured cytotrophoblast & syncytiotrophoblast cells

The nature and purity of cultured cells were also assessed by immunocytochemical staining against cytokeratins 8 and 18. The differentiation of cytotrophoblasts into syncytia was monitored by microscopic examination and by measuring the amounts of hCG secreted into the culture media.

2.2.6.1 Immunocytochemical staining

CT cells were diluted to $0.5x10^5$ cells/ml in EMEM with 10% FCS, a 50µl drop of the cell suspension was placed on poly-L-lysine coated coverslips and allowed to adhere for 30min at room temperature. Two coverslips were placed in 35mm diameter wells containing 2ml EMEM with 10% FCS. Cells were cultured for 12 or 96h as described in section 2.2.4. For 96h cultures, media was replaced with EMEM containing 15% FCS after 48h. The fixation and immunocytochemical staining for cytokeratins 8 and 18 were carried out as described in section 2.2.3.

2.2.6.2 Measurement of hCG concentration in cell culture media

CT cells were seeded at a density of $0.75x10^6$ cells/ml/well in EMEM with 10% FCS and cultured. Four wells were seeded with cells from each patient. Media from 2 wells was collected after 12h and pooled. Fresh media (1ml EMEM with 15% FCS per well) was added after 48h and 84h to each of the two remaining wells. Cells were grown for a further 12h, the media collected and pooled. Cells and debris present in the media were removed by centrifuging at 15,000rpm for 5min. Supernatants were collected and stored at -20°C. The concentration of hCG/ml in the media was determined using a chemiluminescent assay specific for human hCGα and hCGβ (Chiron Diagnostics). The assays were conducted at the Dept. of Biochemistry, Western Hospital, Footscray. The data was expressed as the mean, ±SEM of hCG in the culture media collected after 12 and 96h in culture.

2.2.7 Experimentation involving cell cultures

CT cells cultured for 12 or 96h were used for studies on:

- expression of EGF/EGFR related growth factor and receptor mRNA by RT-PCR analysis (section 2.4 & Chapter 3)
- expression of TGFα and AR proteins by Western hybridization and immunocytochemistry (sections 2.7, 2.8 & Chapter 5).

CT cells were also treated with TGFα to study its effects on:

- total cell numbers (section 2.9.1 & Chapter 6)
- formation of multicellular aggregates (section 2.9.2 & Chapter 6)
- progesterone synthesis (section 2.10 & Chapter 6)
- expression of 3βHSD mRNA (section 2.10.3 & Chapter 6) and
- to identify mRNA species that were being upregulated or transcribed as a result of treatment using Representational Difference Analysis (section 2.11 & Chapter 7).

2.3 Preparation & electrophoresis of total cellular RNA

2.3.1 Gestational tissue

The RNA was extracted according to a modified procedure of Chomczynski & Sachhi (1987). Specimens from the amnion, smooth chorion-adherent decidua and villous cotyledons that had been rinsed in ice cold 0.8% NaCl solution, snap frozen in liquid nitrogen and stored at -80°C were used for the extractions.

Between 200-250mg of tissue was homogenised in 5ml denaturing solution using a Polytron homogeniser (speed setting 5-6, 20sec bursts 5-8x). The denaturing solution consisted of 4M guanidium isothiocyanate (Sigma), 25mM sodium citrate pH7, 0.1M 2β-mercaptoethanol and 0.5% N-lauroylsarcosine. The RNA was partitioned into the aqueous phase, DNA, proteins into the organic phase by adding 0.5ml 2M NaOAc pH4, 5ml water saturated phenol pH3.7-4.0 and 1ml 49:1 chloroform:isoamyl alcohol. The mixture was vortexed thoroughly and incubated on ice for 15min with intermittent shaking. The aqueous and organic phases were separated by centrifuging at 5500rpm for 15min at 4°C in a Beckmann J2-HS centrifuge. The aqueous phase was collected and RNA precipitated by adding an equal volume of isopropanol. RNA was pelleted by centrifuging at 10,000rpm for 10min at 4°C. The pellet was re-dissolved in 5ml denaturing solution at 65°C to remove any residual contamination by the organic phase. RNA was re-precipitated with 0.5ml 3M NaOAc pH6, 12.5ml absolute ethanol and pelleted by centrifuging at 10,000rpm for 10min at 4°C. The pellet was washed with 5ml, 70% EtOH (in sterile DEPC treated water) and dissolved in 100-200µl DEPC treated water at 65°C.

A 10µl aliquot was diluted to 1ml in DEPC treated water and purity assessed by calculating the ratio of A_{260}:A_{280}. The concentration was determined using the formula:

$$\frac{A_{260} \times 100 \times 40}{1000} = \mu g/ml$$

The balance RNA solution was stored under EtOH at -80°C after adding 0.1vol 3M NaOAc pH6 and 2.5vol absolute EtOH.

2.3.2 Cytotrophoblast cells

RNA was extracted from fresh and cultured CT and ST cells by two methods. Freshly isolated cells were pelleted by centrifuging at 2500rpm, for 5min at 4°C. The supernatant was discarded, cells snap frozen in liquid nitrogen and stored at -80°C. The media was aspirated from cultured cells and cells processed immediately. Freshly isolated and cultured cells were homogenised using a Polytron homogeniser (4-6 bursts, 20sec/burst, speed setting 5).

2.3.2.1 Spin column procedure

Silicon columns (RNeasy minipreps, Qiagen) were used to prepare total RNA from $\leq 50x10^6$ cells or cells grown on ≤ 2 culture plates (surface area/plate=60cm^2). The protocol supplied by the manufacturer was followed. Briefly cells were homogenised in 1.2ml lysis buffer provided and applied onto a column. The DNA and proteins were washed off the column and RNA eluted in 30-40µl DEPC treated water.

2.3.2.2 Single step procedure

The reagent Trizol (Gibco) was used to prepare RNA from $\geq 50x10^6$ cells or cells grown on ≥ 3 culture plates (area/plate=60cm^2). Cells were homogenised in Trizol reagent (0.5 ml/$50x10^6$ cells, 6ml/60cm^2 plate surface area). Lysates were incubated for 5min at room temperature and mixed with 0.2ml chloroform for every ml of Trizol used. The lysate was shaken vigorously and centrifuged at 10,000rpm for 15min at 2°C. The aqueous phase was carefully removed and an equal volume of isopropanol was added. Samples

were left overnight at -20°C to precipitate the RNA and thereafter pelleted by centrifuging at 12,000rpm for 15min at 2°C. The pellet was washed in 3ml, 75% EtOH in DEPC H_2O. The EtOH was evaporated and the RNA pellet dissolved in 50-80µl DEPC treated H_2O.

In both cases purity, concentrations were determined and RNA preparations stored as described in section 2.3.1.

2.3.3 Electrophoresis of RNA on formaldehyde agarose gels

Total RNA from tissues, cells were electrophoresed on formaldehyde agarose gels to assess the integrity of the RNA and to prepare samples for Northern transfer (section 2.5.1). Gels containing between 0.8 to 1.0% agarose (Progen) and 0.5M formaldehyde (Sigma) were cast in 1x MOPS buffer. MOPS buffer (1x) consisted of 20mM MOPS pH7, 5mM NaOAC, 1mM EDTA pH8.

RNA was recovered from EtOH storage by withdrawing the required volume and pelleted by centrifuging at 15,000rpm for 10min at 4°C. Generally for quality assessment between 1-3µg was resolved and 15-30µg resolved for Northern transfer. The pellets were dissolved in RNA loading buffer (1µl/µg RNA). The loading buffer consisted of 50% v/v deionised formamide, 6.25% v/v formaldehyde pH>3.7 in 1x MOPS buffer. The solution was heated at 65°C for 10min, immediately cooled on ice and mixed with 0.1µg EtBr/µg RNA. The total volume was noted and 1/5 vol RNA loading dye (0.1mg/ml bromophenol blue, 50% v/v glycerol) was incorporated. Samples were loaded onto the gel and electrophoresed in 1x MOPS buffer at 2.3-3.5 V/cm for 4-5h. Between 2-3µg of a RNA size marker (Promega) was electrophoresed alongside the RNA samples. The resolved RNA was visualised by placing the gel on an UV transilluminator (304 nm) and photographed with a Polaroid MP-4 Land camera using type 665 or 667 film.

2.4 RT-PCR analysis of growth factor & receptor mRNA expression

The expression of EGF/EGFR related growth factor and receptor mRNA in gestational tissue (n=8, CS=5, SD=3) and CT cells cultured for 12 and 96h (n=4) was examined. The CT cells were seeded at $0.75x10^6$ cells/ml in EMEM with 10% FCS and cultured as described in sections 2.2.4 & 2.2.5.

2.4.1 Design, synthesis & purification of primers

Since the members of the EGF/EGFR families contain regions that have significant homology, the RT and PCR primers were designed from regions of low homology. Such regions were initially identified by aligning the amino acid sequences using the Pretty Box Multiple Sequence Alignment software program version 3 (sequence alignments are given in Appendices 2a, b). Next, the cDNA sequences were accessed from the Australian National Genomic Information Service (ANGISS) and nucleotides that corresponded to the regions of low amino acid homology were identified. Stretches of nucleotides 20-24 bases in length with a GC content of approximately 50% were selected for constructing the primers. Additionally the RT/reverse and forward primers were generated from separate exons except for BTC, HRG and c-erb-B4 where such data was unavailable. The nucleotide sequences of the primers are given in Table 2.1; the cDNA sequences of each of the growth factors, receptors and regions used for primer construction are given in Appendices 3-11.

The primers were synthesised on an automated DNA synthesiser (PCR Mate 391, Perkin Elmer). Crude oligonucleotides were cleaved, deprotected at 65°C in 35% NH_4OH solution and purified by NaOAc, EtOH precipitation. The primers were dissolved in 100-150µl H_2O and stored at -20°C. A 10µl aliquot was diluted, absorbance at 260nm was measured by UV spectrophotometry and concentrations determined using the following formula:

$$\frac{A_{260} \times \text{dilution factor} \times 33}{1000} = \mu g/\mu l$$

TABLE 2.1 Nucleotide sequences of the RT, reverse and PCR primers

Specific for	Primer sequence 5'→3'; RT=reverse transcription only, R=RT and reverse PCR primer, F=forward PCR primer
BTC	R- GATAGGAGTTATATCTTTACCCAG F- GAGGAAAACTGTGCAGCTACCACC
CR	RT-GCTTTGTATAGAAAGGCAGATGCC R- GGTAGAAATGCCTGAGGAAAGCAG F- CAGAGATGACAGCATTTGGCCCCA
HRG	RT-GGCTGACACATACCTTTCACTATG R- GGCATGCCTGAGGAAGCTGTTACA F- CCAGAAGAGAGTGCTGACCATAAC
HB-EGF	R- GATGACCAGCAGACAGACAGATGA F- CAAGAGGCAGATCTGGACCTTTTG
AR	R- CTGACATTTGCATGTTACTGCTTC F- GTTACCTCAAGAAGTGAGATGTCT
TGFα	RT-AGTGGCAGCAAGCGGTTCTTCCCT R- TGTATCAGCACACATGTG F- TGCCCAGATTCCCACACT
c-erbB-2	R- TTCCATCCTCTGCTGTCACCTCTT F- GACATCTTCCACAAGAACAACCAG
c-erbB 3	R- TGCACCATACCATGTTCCTCAAGG F- ACTGTGCACAAAGGAGTGTGGATC
c-erbB-4	R- GTCCCCATGAATACCAGTGACTAG F- AGATGCTACGGACCTTACGTCAGT
GAPDH*	R- AGGTCCACCACCCTGTTGCTGTA F- TCCTGCACCACCAACTGCTTAGC

*R &F primers described by Daneau *et al.*, 1994; cDNA sequence given in Appendix 12.

2.4.2 RT-PCR

Between 0.5-1.0μg of total RNA from tissues or CT cells cultured for 12, 96h was mixed with 0.75μM RT or reverse primer, 5mM $MgCl_2$, 1mM of each dNTP, 1U RNAse inhibitor, 2.5U Murine Leukemia Virus reverse transcriptase in 1x PCR buffer in a total volume of 20μl. Reagents from the RNA PCR core kit (Perkin-Elmer) were used for RT and PCR. The RNA was reverse transcribed into first strand cDNA at 42°C for 60min. Next the reverse transcriptase was inactivated at 95°C for 5min. The first strand cDNA was diluted to 100μl in a mix containing 0.15μM each of forward, reverse primer, 2mM $MgCl_2$, 0.2mM of each dNTP, 2.5U Taq polymerase in 1x PCR buffer. Samples were denatured for 2min at 95°C, followed by 30-40 cycles of PCR. The PCR cycling

parameters were as follows: denaturation at 95°C for 30sec, anneal-extend at 60°C for 90sec (CR, HRG, HB-EGF, GAPDH), anneal-extend at 62°C for 90sec (c-erb-B2, c-erb-B3, c-erb-B4), anneal at 52°C for 30sec, extend at 62°C for 45sec (AR, TGFα, BTC). In all cases complete strand extension was carried out at 72°C for 7min. Negative control reactions consisted of reaction without reverse transcriptase, with 7ng genomic DNA from human placenta and 0.5μg yeast tRNA. RT and PCR cycling were carried out in a Perkin Elmer 480 or Peltier PTC-200 thermal cycler. The reaction products were resolved by agarose gel electrophoresis (section 2.4.4).

Note before RT-PCR using BTC, HRG or c-erb-B4 specific primers (where information on exons was unavailable), the RNA was treated with DNAse I to remove residual genomic DNA to prevent ampification of the DNA. Ten μg of RNA was treated with 2U RNase free DNAse1(Gibco) in 10mM Tris.HCL pH7.5, 1mM DTT, 50mM NaCl, 10mM $MgCl_2$ buffer with 80U RNase inhibitor for 30min at 37°C. Following digestion the RNA was extracted with 49:1 phenol:chloroform and precipitated with NaOAC, EtOH.

2.4.3 Semi quantititative RT-PCR

The relative levels of AR, BTC, HB-EGF and receptor mRNA present in gestational tissue and CT cells cultured for 12 and 96h was studied by semi quantitiative RT-PCR using GAPDH mRNA as an internal standard (n=3). Preliminary optimisation experiments were carried out varying the quantity of RNA (0.5, 1.0 and 1.5μg) and the number of PCR cycles (20, 25, 30) to determine the optimum conditions. Thereafter 1μg of total RNA was reverse transcribed and cycled 20 or 25x for receptors and growth factors respectively. The compositions of the RT-PCR mixtures and cycling parameters were the same as outlined in section 2.4.2. GAPDH mRNA in each sample was also reverse transcribed and amplified by PCR by setting up identical reaction mixtures except that GAPDH specific primers were used instead of the growth factor or receptor specific primers (Table 2.1). Reaction products were resolved by agarose gel electrophoresis and quantitated by laser denistometry (section 2.4.5).

2.4.4 Agarose gel electrophoresis

Gels containing between 1.3-1.5% agarose and 0.3 μg/ml EtBr were cast in 1x TAE buffer (40mM Tris-acetate, 1mM EDTA pH 7.5-7.9). Samples were prepared for loading by withdrawing 1/10th volume of the RT-PCR or semi-quantitative RT-PCR reaction mix and adding 1μl DNA loading dye (100mM EDTA, 1% w/v SDS, 0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol, 50% v/v glycerol). Samples were loaded onto the gel and electrophoresed in 1x TAE buffer at 4.0-6.3 V/cm for 1-1.5h. The DNA was visualised by placing the gel over a UV transilluminator (304nm) and photographed using a Polaroid MP-4 Land camera and Polaroid type 665 or 667 film. The size of the DNA was estimated against a DNA size marker (Promega or Gibco) electrophoresed alongside the samples.

2.4.5 Laser densitometry

The negative photographic images of EtBr stained cDNA (generated by semi-quantitative RT-PCR resolved on agarose gels) were scanned and quantitated as described by Smith & Klugman 1997. Scans were carried out on a Pharmacia Ultrascan XL laser denistometer and quantitated with the Gel Scan XL version 2.1 software program. Data was expressed as the mean and ±SEM (n=3) relative to GAPDH.

2.4.6 DNA sequencing

RT-PCR amplified cDNA was purified before sequencing using spin columns (Wizard, Promega). Briefly the RT-PCR mix was applied onto the spin column, washed with 2ml of 80% isopropanol and cDNA eluted in 50μl sterile water. The concentration of the eluted cDNA was estimated by electrophoresing 5μl sample on an agarose gel and comparing the band against a known concentration of a DNA size marker (100bp DNA ladder, Promega).

The nucleotide sequence of the cDNA was determined using a protocol based on the dideoxy chain termination method of Sanger *et al.*, 1977. Chain termination reactions were set up using 25-30ng cDNA, 3.2pmol of the forward or reverse PCR primer (Table 2.1) and 8µl of a prepared cocktail mix containing deoxynucleotides, flourescent dye labelled dideoxy nucleotides and AmpliTaq DNA polymerase, FS (ABI Prism, Perkin Elmer). The mixture was cycled 25 times in a Peltier PTC 200 thermal cycler. The PCR cycling profile was as follows: 30sec at 96°C; 15sec at 50 °C and 4min at 60 °C. The dye terminated products were resolved on an automated DNA sequencer (Applied Biosystems Model 373 Stretch). The sequence data was compiled using the gel scanning software program ABI Prism DNA Sequencing Analysis Software Program Version 3.1. The resolution and nucleotide compilation were carried out by staff, Sequencing Unit, Department of Microbiology, Monash University.

2.5 Analysis of mRNA expression by Northern hybridization

2.5.1 Northern transfer

Immediately after electrophoresis formaldehyde agarose gels (section 2.3.3) were equilibrated in 500ml of 10x SSC buffer (1.5M NaCl, 150mM sodium citrate pH7) for 15min with gentle agitation. The gels were recovered and a Northern blot was set up. The RNA was transferred overnight onto a positively charged nylon membrane (Boehringer Mannheim) by capillary action in 500ml 10x SSC buffer. The membranes were recovered, washed in 4x SSC and air dried. The transferred RNA was cross linked onto the membrane by a 3-5min exposure to UV light (254 nm).

The lane containing the transferred RNA marker (Promega) was cut out from the nylon membrane and stained in 0.04% w/v methylene blue in 0.5M NaOAc, pH5.2. The marker was destained in several changes of tap water and air dried. The stained marker and blots were stored between sheets of blotting paper at room temperature.

2.5.2 Preparation of hybridization probes

Oligonucleotides and cDNA fragments generated by RT-PCR were used as hybridization probes. The oligonucleotides were designed, synthesised, purified and quantitated as described in section 2.4.1. They ranged between 36-39mer in length and their nucleotide sequences are given in Table 2.2. The oligomers were labelled at the 5' end with γ-^{32}P by mixing 100ng of the oligomer with 1µl [γ-^{32}P]ATP (3000 Ci/mmol, 10µCi/µl, Bresatec) and 10-15U T4 polynucleotide kinase (PNK, Progen) in 1x forward reaction buffer at 37°C for 45min. The reaction was terminated by heating at 65°C for 10min. The unincorporated label was removed by column chromatography using oligonucleotide binding spin columns (Qiaquick, Qiagen) and buffers supplied by the manufacturer.

cDNA (25ng heat denatured) was labelled with 4µl High Prime solution (4U Klenow polymerase, 2.5µM of each cold dNTP, random oligonucleotides) and 30µCi [α-^{32}P]dCTP (3000Ci/mmol, 10mCi/ml, Bresatec) at 37°C for 10min. The reaction was terminated by heating to 65°C for 10min. The unincorporated label was removed by Sephadex G-50 spin columns in TE buffer (10mM Tris.HCl, 1mM EDTA), essentially as described by Sambrook *et al.*, 1989. The labelled cDNA probes were denatured at 94°C for 10min immediately before incorporation into the hybridization buffer.

2.5.3 Hybridization and washing conditions

Northern blots were prehybridized for 6-8h at 39-42°C (for oligonucleotide probes) and 42-45°C (for cDNA probes) in prehybridization buffer. The prehybidization buffer (1L) consisted of 35g NaCl, 1.9g EDTA, 0.2g each of ficoll, bovine serum albumin (BSA), polyvinylpyrrolidone, 2.6g sodium dihydrogen orthophosphate, 4.7g anhydrous disodium hydrogen orthophosphate, deionised formamide (40% v/v for oligonucleotide probes, 50% v/v for cDNA probes) and 10 µg/ml heat denatured salmon sperm DNA (Gibco) added just prior to prehybridization. Following prehybridization the labelled probe was

TABLE 2.2 Nucleotide sequences of oligonucleotides used as hybridization probes

Specific for	Anti-sense probe 5'→3'	Sense probe 5'→3'
AR	CTG ACA TTT GCA TGT TAC TGC TTC CAG GTG CTC	GTT ACC TCA AGA AGT GAG ATG TCT TCA
CR	GAG GTG CTC ATC CAT CAC AAG GCC ATC ACA GCC GGG TAG	GTC TTT GAA CTG GGA TTA GTT GCC GGG CTG
HB-EGF	CCG GCA GAT GAG GGC CCG GCA CCA CTC ACA GTG TTT TC	ATT GTG TTG GCT GCG TGC CAG GCC TTG GAG
HRG	GGC TGA CAC ATA CCT TTC ACT ATG AGG AGA	AAG AGA GTG CTG ACC ATA ACC GGC ATC TGC

incorporated directly into the prehybridization solution and blots hybridized overnight at the temperatures stated earlier.

Blots were washed twice in 2x SSC, 0.1% SDS for 5min at room temperature followed by 2 stringency washes of 20min each in 0.1x SSC, 0.1% SDS at the hybridization temperatures for cDNA probes and 5°C higher than the calculated melting temperature (Tm) for oligonucleotide probes.

Hybridization was detected by autoradiography on X-ray film (Hyperfilm, Amersham). Blots were exposed to X-ray film between 1-5 days at -80°C. X-ray films were developed by conventional methods. The size of the hybridizing transcript was estimated against the dye stained RNA size marker (section 2.3.3).

2.6 Localisation of mRNA expression by in situ hybridization

Cellular sources of AR, CR, HRG and TGFα mRNA in villous cotyledons, amnion and chorio-adherent decidua (n=5 delivered by CS) was investigated by in situ hybridization.

2.6.1 Fixation and embedding of tissue

Approximately 2-3mm thick pieces from villous cotyledons, 0.5cm^2 pieces from the amnion and chorion-adherent decidua were dissected out avoiding major blood vessels. Tissues were rinsed separately in ice cold sterile 0.8% NaCl solution to remove surface blood contamination. Initially tissues were fixed for 4, 6, 12 and 24h in 3 fixatives (Bouins, neutral buffered formalin and 4% w/v paraformaldehyde (BDH) in 0.1M phosphate buffer pH 7.2 to determine the time/fixative combination that would retain the best morphology (n=2, 39, 40 weeks, CS). The fetal membranes were pinned onto cardboard backing to prevent curling during fixation. Subsequently tissues were fixed in 4% paraformaldehyde in 0.1M phosphate buffer pH7.2 for 4-6h. Fixed tissues were rinsed twice in 0.1M phosphate buffer pH7.2 and immersed in 70% EtOH. Samples were dehydrated through an EtOH gradient, cleared in xylene and impregnated with paraffin (Paraplast, Medos) in an automated tissue processor (Sakura, model RH12 EP4). The dehydration, clearing and impregnation profile was as follows: 1h in 50% EtOH, 1h in 70% EtOH, 2h in 90% EtOH, 2h in absolute EtOH bath 1 followed by 1h in EtOH baths 2 and 3, 1h each in 3 xylene baths and impregnated with wax for 1h each in 2 molten paraffin baths at 60°C. Samples were embedded in molten paraffin at 60°C on a tissue embedding console (Tissue Tek II). Sections between 4-6μm thick were cut on a Leitz 1202 microtome, placed on 3-aminopropyltriethoxysilane (Sigma) coated slides, incubated overnight at 37°C and stored over silica at -20°C.

2.6.2 Preparation of hybridization probes

Sense and antisense oligonucleotides 36-39mer in length were utilized as negative and positive hybridization probes respectively (Table 2.2). The probes were labelled to a high

specific activity by mixing 5pmoles of primer (≡50ng of a 36mer) with 10pmoles of [γ-^{32}P]ATP (3000Ci/mmol 10mCi/ml, Bresatec), 10-15U PNK in 1x forward reaction buffer (Progen) and incubated for 45min at 37°C. The reaction was terminated by heating to 65°C for 10min. Unincorporated radio-isotope was removed using spin columns (Qiaquick, Qiagen) and the labelled oligomer was diluted to 0.5ng/µl in fresh prehybridization buffer for oligonucleotides (composition is given in section 2.5.3; salmon sperm DNA was replaced with 1mg/ml DNA (degraded free acid, Sigma).

2.6.3 In situ hybridization

Frozen paraffin sections were allowed to reach room temperature in a dessicator then dewaxed in 3 xylene baths (5min in bath 1, 10 dips in baths 2,3), followed by 10 dips each in 3 absolute EtOH baths, immersed in sterile H_2O and rinsed twice in 4x SSC. Excess buffer was blotted off and sections prehybridized at 42°C for 2h in prehybridization buffer for oligonucleotides. Tissue sections were blotted free of the prehybridisation solution and lowered onto coverslips containing 25µl of diluted probe (≡12.5ng of labelled probe). The solution was spread evenly to remove air bubbles and cover the section. Slides were placed in a humidified container and hybridized overnight at 42-44°C for 14-16h.

Serial sections treated as follows were used as negative controls:

- treated with DNase free RNaseA (20µg/ml, Gibco) in 2x SSC, 2mM $MgCl_2$ at 37°C for 40min. Sections were washed twice in 2x SSC, prehybridized and hybridized with 12.5nl labelled anti-sense oligomer per slide.
- sections hybridized with 12.5ng sense oligomer per slide
- sections hybridized with cold anti-sense oligomer

After hybridization positive and negative control, slides were immersed in 250ml, 2x SSC then dipped in 500ml 1x SSC at room temperature. Stringency washes were carried out in 1x SSC at 42°C for 1h with gentle agitation. Sections were dehydrated in 3 absolute EtOH baths and air dried.

Initially the hybridization was analysed by autoradiography on X-ray film. Slides were taped onto a cassette, X-ray film (Hyperfilm, Amersham) was placed over the slides and exposed for 24h at room temperature. The X-ray film was developed by conventional methods. The strength of the signal was used as an indicator for the exposure time for liquid emulsion autoradiography.

2.6.4 Liquid emulsion autoradiography

The negative controls, slides with signal but minimal background and slides without visible signals after autoradiography were dipped in liquid photographic emulsion (K5, Ilford) diluted 1:1.5 in sterile water at 42°C. Slides were allowed to dry and stored under light safe conditions over silica for 3-7days. Following exposure the emulsion was developed for 4min in developer (D19, Kodak diluted 1:1with water), dipped in water for 10sec, fixed for 5min (emulsion fixative, Kodak), washed in water for 5min and allowed to slowly dry out. Sections were dehydrated in EtOH, stained in heamotoxylin/eosin and mounted. Slides were observed under bright and dark field on an Zeiss Axioplan 2 microscope and photographed using a Zeiss MC80 SLR camera with Kodak 400ASA colour film.

2.7 Analysis of TGFα & AR protein expression by Western hybridization

2.7.1 Sample preparation

Gestational tissues from 9 patients (CS=5, SD=4) and CT cells cultured for 12 or 96h (n=5, placenta delivered by CS) were used in this study. CT cells were seeded at a density of $0.5x10^5$ cells/ml in EMEM with 10% FCS and cultured as described in sections 2.2.4 & 2.2.5.

Approximately 0.1g of tissue from the villous cotyledons, amnion, chorio-decidua (previously rinsed in 0.8% NaCl solution, frozen in liquid nitrogen and stored at -80°C), was removed and cultured CT or ST cells grown in quadruplicate wells (surface

area=2cm^2/well) were washed free of culture media in 1x PBS. Cells, tissues were dispersed in 2ml 50mM Tris.Cl buffer pH8 using sterile sand and a pestle and mortar. Sand was removed by centrifuging at 15,000rpm for 1min, supernatant collected and stored at -80°C.

Cell culture media was also collected after 12 or 96h from quadruplicate wells as described in section 2.2.6.2. Cells and debris in the media was removed by centrifuging at 15,000rpm for 5min. The supernatant was passed through a sterile 0.45μm filter and stored at -80°C.

2.7.2 Estimation of total the protein concentration

The total protein concentration in the tissue, cell lysates and cell culture media was measured by a simplified version of the Lowry assay (Lowry *et al.*, 1951 & Peterson 1977). The samples were diluted 50x to 0.5ml with water, mixed with 0.5ml solution A (0.1ml 5% w/v $CuSO_4$, 0.9ml 1% w/v potassium tartarate, 10ml 10% w/v Na_2CO_3 in 0.5M NaOH) and heated for 10min at 37°C. Folin-Ciocalteu reagent (BDH) was diluted 1:10 with water to make solution B, 1.5ml was added to the mixture and vortexed. The solution was incubated at 52°C for 20min and absorbance at 680nm was measured. BSA solutions ranging from 0-50μg served as standards. Both standards and unknowns were assayed in duplicate.

2.7.3 Treatment with N-glycosidase F

Solubilised proteins from gestational tissues (n=3) were hydrolysed with N-glycosidase F (Boehringer Mannheim) to examine the extent of N-linked glycosylation present in TGFα and AR proteins. Cellular proteins were denatured by boiling for 5min with 1% SDS, (10μg/10μl and 50μg/50μl for TGFα, AR respectively). Added 4x vol of N-glycosidase buffer (20mM sodium phosphate, 10mM sodium azide, 10mM EDTA, 0.5% v/v Nonidet

P-40) and boiled for 2min. Cooled and added N-glycosidase F (0.4U and 2U for TGFα, AR respectively) and incubated at 37°C for 18h. Untreated samples served as controls. Treated and untreated samples of the glycoprotein alkaline phosphatase (Sigma) served as positive and negative controls of hydolysis respectively. After hydrolysis samples were lyophilised and reconstituted with water. The proteins were resolved by SDS-PAGE, immunoblotted and probed with antibodies directed against TGFα and AR as outlined in sections 2.7.4.1, 2.7.5 & 2.7.6 respectively.

2.7.4 Separation of proteins on polyacrylamide gels

Solubilised proteins present in the tissue, cell lysates and cell culture media were fractionated by size under denaturing conditions by SDS-PAGE and Tris-tricine PAGE.

2.7.4.1 SDS-PAGE

The discontinuous buffer system of Laemli, 1970 and Hames, 1990 was utilised for electrophoretic separation of proteins from gestational tissue, cell lysates and cell culture media. The stacking and resolving regions of the gel were made up in buffers of different composition and pH. The stacking gel consisted of 0.125M Tris.HCl pH6.8, 0.2% w/v SDS, acrylamide (T=4%, C=3%) polymerised by adding 0.02% v/v TEMED and 0.06% w/v ammonium persulphate (APS) added from a 10% stock solution.

The resolving gel consisted of 0.375M Tris.HCL pH8.8, 0.1875% w/v SDS, varying acrylamide percentages (T=10-15%, C=3%), 0.125% v/v TEMED and 0.0625% w/v APS. Mini and midi sized gels were cast; the thickness of the stacking and resolving gels was 0.75mm.

Samples (7.5-10μg protein/lane for mini gels, 40-50μg protein/lane for midi gels, concentrations estimated by the Lowry assay) and a protein molecular weight marker (Biorad) were denatured by boiling for 5min with an equal volume of 2x sample buffer.

The 2x sample buffer contained 0.0625M Tris.HCl pH6.8, 2% w/v SDS, 5% 2-βmercaptoethanol, 11.6% v/v glycerol and 0.01% bromophenol blue. Samples were loaded onto the stacking gel and run in 1x Tris-glycine electrode buffer (0.025M Tris pH8.3, 0.192M glycine, 0.1% w/v SDS) at 100-120V for mini gels and 20mA/gel for midi gels.

Samples were loaded onto two identical gels. After electrophoresis one gel was stained and the other used for Western transfer (section 2.7.5). Prior to staining gels were fixed in 4:1:5 absolute EtOH:HAC:water and stained in Coomassie blue R-250 (0.5% w/v dissolved in 10% HAC, 35% EtOH), for 45min with gentle shaking. Gels were destained in 10% HAC, 35% EtOH solution, washed free of HAC in water and dried in a gel drier (Gel Air, Biorad).

2.7.4.2 Tris tricine-PAGE

Proteins of low molecular weight present in the tissue, cell lysates and cell culture media were resolved by Tris-tricine PAGE using a modified procedure of Schagger & von Jagow, 1987. The Tris-tricine gels were made up of 3 layers; ie. stacking, spacing and resolving; their compositions are given in Table 2.3.

Between 40-50μg of protein and a protein molecular weight marker (Biorad) were diluted with 1vol of 2x tricine sample buffer (0.1M Tris pH6.8 with 0.08% w/v SDS, 33.3% w/v glycerol, 8% w/v SDS, 0.2M DTT, 0.02% w/v Coomassie blue-250) and denatured at 100°C for 5min. Samples were cooled, loaded and electrophoresed in anode and cathode buffers. Anode buffer contained 0.2M Tris.Cl pH8.9, cathode buffer 0.1M Tris, 0.1M tricine and 0.1% w/v SDS. Gels were run at 10mA/gel, stained and preserved as described in section 2.7.4.1.

2.7.5 Western transfer

Proteins resolved by SDS-PAGE or Tris-tricine PAGE were transferred electrophoretically at 15V, 0.1A overnight onto nitrocellulose membranes (0.2μM pore size, Biorad) in water cooled transfer cell (Trans-Blot, Biorad) set at 15°C. The transfer buffer consisted of 39mM glycine, 48mM Tris base pH9.2, 0.037% w/v SDS and 20% methanol. Membranes were recovered and proteins fixed onto the membranes in 0.2% v/v glutaraldehyde (BDH) in TBS buffer (0.05M Tris pH7.5, 0.15M NaCl) for 45min at room temperature. Membranes were not stored but were probed directly with antibodies. Transfer efficiencies were checked by staining transferred gels with Commassie blue as described earlier.

TABLE 2.3 Composition of stacking, spacing and resolving gels

Components	Gel type		
	Stacking	Spacing	Resolving
acrylamide (%T)*	4.0	10.0	16.5
M Tris.Cl pH8.45	0.74	1.0	1.0
and % w/v SDS**	0.074	0.1	0.1
% w/v glycerol	—	—	13.3
% v/v TEMED	0.04	0.15	0.06
% w/v APS***	0.02	0.06	0.02

* made from a 30% stock solution; C=3%

** made from a 3M Tris.Cl stock solution with 0.3% SDS

*** diluted from a 10% stock solution

2.7.6 Western hybridization and washing conditions

Immunoblots were immersed 1% w/v gelatine in TBST (TBS with 0.02% v/v Tween 20) solution for 45min to block the non-specific binding. The blocking solution was washed off in twice in TBST for 10min each. Similar washing conditions were used throughout

unless stated otherwise. Blot were incubated with the diluted Iry antibodies (Table 2.4) for 1h. Primary antibodies were washed off, diluted horse radish peroxidase (HRP) conjugated IIry antibody (Table 2.4) was applied and incubated for 1h. Blots were washed in TBS solution. The antigen antibody complex was detected with 4-Chloro-1-Napthol (4CIN, Sigma) and H_2O_2 (BDH) substrate solution prepared by dissolving 30mg 4CIN in 10ml absolute MeOH then mixing with 50ml TBS and 35µl H_2O_2. Blots were immersed in substrate solution, kept in darkness for 5-20min, washed in water, dried and stored away from light.

TABLE 2.4 Description and concentrations of the antibodies used for Western hybridization

Description and concentration	Target antigen TGFα	AR
Iry antibody type and host animal	monoclonal, anti-human raised in mouse	polyclonal, anti human raised in goat
concentration of Iry antibody	5µg/ml in TBST	2µg/ml in TBST
supplier of Iry antibody	Oncogene/Calbiochem	R&D Systems
description of IIry antibody	goat anti mouse IgG(H+L) HRP conjugated	rabbit anti goat IgG(H+L) HRP conjugated
dilution of IIry antibody	1:3000 in TBST	1:2000 in TBST
supplier of IIry antibody	Biorad	Dako

2.7.7 Laser densitometry

The relative amounts of TGFα, AR immunoreactive protein present was quantitated by laser densitometry. Immunoblots that had been probed with the antibodies were scanned on a laser densitometer (Ultrascan Xl, Pharmacia) and quantitated with the software program Gelscan XL version 2.1. The data was expressed as the mean and ±SEM (n=9 for tissues, n=5 for cultured cells and media).

2..8 Immunohistochemistry and immunocytochemistry

2.8.1 Preparation of tissue sections

Tissue embedded in paraffin wax for in situ hybridization analysis (section 2.4.1) were also utilized for immunohistochemistry studies (n=7, delivered by CS). Serial sections between 4-6μM in thickness were cut and placed on poly-L-lysine coated slides (Sigma). Sections were dewaxed as described earlier (section 2.6.3) and washed twice, 5min each in 1x PBS solution (10mM phosphate pH7.4, 0.8% NaCl).

2.8.2 Fixation of cells

CT cells were seeded and cultured as described in section 2.2.6.1 (n=6). Cells were washed free of media twice for 3min each in 1x PBS followed by a rinse in absolute MeOH. Cultures were fixed in pre-chilled methanol for 15min at -20°C and stored at -20°C over absolute EtOH.

2.8.3 Immunostaining prodedure

AR and TGFα proteins were detected using the Labelled Avidin Biotin-Strepavidin (LAB-SA) method of Elias *et al.*, 1989. The primary antibodies used for Western hybridization were also used for immunostaining; other reagents were purchased from Zymed Laboratories (Histostain Sp kit).

The endogenous peroxidase activity in tissue was quenched by immersing sections for 10min in a 1:1 solution of 30% H_2O_2 : absolute MeOH. The quenching solution was washed off thrice in 1x PBS for 2min each. Cell smears stored at -20°C were thawed out to room temperature and washed thrice in 1x PBS for 5min each prior to immunostaining.

Non specific binding was blocked with 10% non-immune serum in 1x PBS (goat serum for TGFα and rabbit serum for AR) for 40min. The non-immume serum was blotted off and samples incubated with diluted Iry antibody for 1h. The anti-TGFα antibody was diluted to a concentration of 3μg/ml and 1μg/ml for the anti-AR antibody. The primary antibodies were washed off thrice in 1x PBS. Similar rinses were carried out after every incubation step unless stated otherwise.

The biotinylated IIry antibody was applied, incubated for 15min and rinsed off. Samples were covered with the strepavidin-peroxidase enzyme complex solution, incubated for 10min and washed. The H_2O_2 substrate, diaminobenzidine chromogen mixture was applied, incubated for 10min and rinsed in water. Sections and cell smears were stained in heamotoxylin, rinsed in water, dehydrated in EtOH, cleared in xylene and mounted in Histomount.

Sections of breast, colon cancer tissue fixed in formalin and embedded in paraffin served as positive controls. The wax blocks were supplied by the Royal Melbourne Hospital, Melbourne. Sections of gestational tissue, cancers and cell smears incubated with 10% non immune serum instead of the primary antibody served as negative controls. Additional serial sections stained with heamotoxylin and eosin were used to assess the morphology of the tisssue sections. Sections and smears were observed on an Olympus BX50 microscope and photographed with an Olympus OM4Ti SLR camera using Kodak ASA 400 colour film.

2.9 Effects of TGFα on total cell number & aggregation of cytotrophoblast cells in vitro

CT cells isolated from eight placenta delivered by CS were treated with TGFα and its effects on proliferation was measured by total cell counts. The effects of TGFα on aggregation was examined by studying the distribution on nuclei among mono-nucleated and aggregated cells.

2.9.1 Estimation of total cell numbers

Cells were seeded @ 10^5 viable cells/ml/2cm^2 well in EMEM with 10% FCS in collagen coated 24 well plates and cultured as described in section 2.2.4.. Cells undergoing treatment were dosed with 10ng TGFα (Sigma) per well and cultured for 3, 6, 12 or 18h. For each incubation period cells in quadruplicate wells were treated per replicate while another 4 wells served as untreated controls for each replicate. The experiment was repeated with 20 and 50ng TGFα/well. After each incubation period treated and control cells were lifted with 200µl of trypsin solution added to each well and left standing for 3min at 37°C. The trypsin solution consisted of 1.5g trypsin, 6.8g NaCl, 0.4g KCl, 1.0g glucose, 2.2g NaHCO3 and 0.2g EDTA per litre. Trypsinisation was stopped by adding 300µl of FCS. Cells from sets of quadruplicate wells were pooled and evenly dispersed by repeated pipetting. A 10µl aliquot was stained with trypan blue, counted on a haemocytometer as described in section 2.2.2 and total number of cells/ml was determined.

For each concentration/time combination the data was expressed as the mean (n=8), ±SEM for treatments and controls. The differences between the mean values of pairs of treated and control cultures were calculated using the Student's *t* test; values were considered significant when $p \leq 0.05$.

2.9.2 Distribution of nuclei between mono-nucleated and aggregated cells

The procedure described by Kliman *et al.*, 1986 was used to study the distribution of nuclei in the cultured cells. CT cells were diluted to a density of 10^5 cells/ml in EMEM with 10% FCS. One ml of the cell suspension was placed on collagen coated sterile coverslips placed inside the wells. After 12h media was replaced with EMEM with 10% FCS with 10ng TGFα/ml. Cells were cultured for 6, 12 or 18h post-treatment. For each time interval quadruplicate wells were treated per replicate while another four wells served as untreated controls. After each incubation period the media was carefully removed and cells stained with haemotoxylin (Zymed) for 5min. The coverslips were

lifted from the well and cells observed under an Olympus BX50 microscope at 100x magnification. For each set of quadruplicate wells, 12 microscopic fields were observed and the number of nuclei in mononucleated cells and aggreates were tallied. The experiment was also repeated as described above with 20 and 50ng TGFα/ml of media.

The data was expressed as the mean, ±SEM (n=5) for each incubation period for treatments and controls. The differences in the mean values between sets of treatments and controls were analysed by the Student's *t* test; values of $p \leq 0.05$ were considered to be significant.

2.10 Determination of progesterone concentrations & of 3βHSD mRNA expression in cytotrophoblast cells in vitro

2.10.1 Treatment with TGFα

Freshly isolated cytotrophoblast cells were diluted to 0.75×10^6 cells/ml in EMEM with 10% FCS. Three million cells (ie. 4ml) were placed in sterile scintillation vials. A total of 12 vials were seeded per replicate (n=6). Cells in six vials were dosed with 15ng TGFα/0.75×10^6 cells/ml (ie. 60ng/vial). Cells in the balance six vials served as untreated negative control cultures. In addition to treatment and controls, cells isolated from two randomly selected patients were also treated with anti-human TGFα antibodies (Oncogene/Calbiochem). Six additional vials each containing 3×10^6 cells were treated with 60ng TGFα plus 6μg anti-human TGFα antibody. Vials containing treated and control cells were were loosely capped and partially immersed in a shaking water bath (50rpm) set at 37°C. Pairs of treated and control cultures were removed from the water bath after 0min, 15min, 30min, 1h, 2h and 3h. Cells were dispersed in their media using a chilled glass teflon homogeniser, centrifuged at 15,000rpm for 5min to remove debris, supernatants collected and stored at -20°C. The concentration of progesterone in the media was analysed by RIA.

For the second part of the experiment, additional cells from the same patients were seeded, dosed and cultured as described above. Cells from pairs of treated and control cultures were harvested after 0min, 15min, 30min, 1h, 2h and 3h, snap frozen in liquid nitrogen and stored at -80°C. Frozen cells were used for preparing RNA for analysis of 3βHSD mRNA expression (section 2.10.3).

2.10.2 Progesterone RIA

The concentration of progesterone in the culture media was determined using a RIA kit (Active progesterone DSL-3900, Zanofi Diagnostics). The RIA was performed according to the protocol provided by the manufacturer. The concentrations of progesterone standards were 0, 0.3, 1, 3.5, 16 and 60ng/ml. Standards and unknowns were assayed in duplicate.

For the assay 0.5ml ^{125}I-progesterone (<5Ci, 185kBq) and 25µl of cell culture media from treated or control cultures were pipetted into tubes coated with anti-human progesterone antibodies. The tubes were incubated for 1h at 37°C, solutions decanted, any remaining solution drained away and the radioactivity measured for 60sec on a gamma counter (Wizard 1470, Wallac). Samples containing fixed amounts of progesterone (1.5, 18.5ng/ml) were included in each assay for dtermining the inter and intra assay coefficients of variation.

Duplicate readings were averaged and progesterone concentrations in the media determined against the standard curve. The mean, ±SEM (n=6) for treatments and controls at each time interval was calculated. The Student's *t* test was used to compare the mean values between a pair of treatments and controls; *p* values ≤0.05 were considered to be significant.

2.10.3 Semi-quantitative RT-PCR analysis of 3βHSD mRNA expression

Total RNA was prepared from treated and control cells stored at -80°C using spin columns as described in section 2.3.2.1. The integrity of the RNA was assessed by formaldehyde agarose gel electrophoresis and RT-PCR for GAPDH mRNA (sections 2.3.3 & 2.4.2 respectively). To test the specificity of the 3βHSD primers, total RNA from two control cultures (1 and 1.5μg, T=0h) and human placenta (0.5 and 1μg) were reverse transcribed and amplified by PCR. The nucleotide sequences of the 3βHSD primers were:

RT, reverse PCR primer 5' AGA GTG GCT TAT ACG CCA GAT CTC GCT GA 3'

Forward PCR primer 5' CTT GCT GAG AAG GCT GTA CTG GCG GCT 3'

RNA was reverse transcribed into first strand cDNA at 42°C for 45min. Reagents from the RNA PCR core kit (Perkin-Elmer) were used for reverse transcription and PCR. The reverse transcription mix contained 0.75μM RT primer, 5mM $MgCl_2$, 1mM of each dNTP, 1U RNAse inhibitor and 2.5U Murine Leukemia Virus reverse transcriptase in 1x PCR buffer, total volume of 20μl. First strand cDNA was amplified by PCR after inactivating the reverse transcriptase at 95°C for 5min. First strand cDNA was diluted to 100μl and contained The PCR reaction mix contained 0.15μM each of the forward and reverse PCR primers, 2mM $MgCl_2$, 0.2mM of each dNTP and 2.5U Taq polymerase in 1x PCR buffer.

The PCR cycling parameters were as follows: one cycle at 94°C for 2min, followed by denaturation at 94°C for 30sec, annealing and extension at 63°C for 1min. Five μl samples were withdrawn after 20, 25 and 30 cycles and resolved on agarose gels. The cDNA was purified and the nucleotide sequence was determined as described in section 2.4.5 in order to determine the identity of the RT-PCR products.

Next, total RNA isolated from treated and control cells cultured for various periods of time was subjected to semi-quantitative RT-PCR. Reactions were set up using 1.5μg total RNA reverse transcribed and cycled 25 times by PCR. The composition as the RT, PCR mixes and cyling parameters were the same as described above. In each case GAPDH mRNA was also amplifed by RT-PCR using 1.5μg total RNA and GAPDH specific primers (Table 2.1). The compositions of the RT-PCR mixes and cycling parameters for GAPDH mRNA were identical to that used for RT-PCR of 3βHSD mRNA. The 3βHSD, GAPDH RT-PCR mixes were resolved on agarose gels, photographed and cDNA bands quantitated by laser densitometry. The data was expressed as the mean, ±SEM (n=5) relative to GAPDH for control or treated cultures at each time interval. The Students *t* test was used to compare the mean values between a pair of treated and control cultures; *p* values ≤0.05 were considered to be significant.

2.11 Representational Difference Analysis

2.11.1 Treatment of cytotrophoblast cells with TGFα

CT cells were isolated from a placenta delivered by Ceaserean section after a 38 weeks gestation (n=3). Cells were diluted to $0.75x10^6$ cells/ml in EMEM with 10% FCS. Four ml of the cell suspension (ie. $3x10^6$ cells) was pipetted into a sterile scintillation vial; a total of 12 vials were seeded. Six of the vials were treated with of TGFα (60ng/vial) while the remaining six vials served as untreated negative controls. Vials were loosely capped and cells cultured for 2h on a shaking water bath (50rpm) set at 37°C. Treated and control cultures were pooled separately into sterile, pre-chilled 50ml tubes. Cells were pelleted at 4200rpm for 5min at 4°C, media removed, cells snap frozen in liquid nitrogen and stored at -80°C.

Total RNA was prepared separately from treated and untreated cytotrophoblast cells by the spin column procedure outlined in section 2.3.2.1. RNA from treated cells were labelled 'tester' and RNA from control cells as 'driver'. The purity of driver and tester

RNA was assessed by measuring the ratio of A_{260}:A_{280} ratio. The integrity was assessed by observing the ratio and intensity of 28S, 18S ribosomal RNA bands on formaldehyde agarose gels (section 2.3.3) and amplifying GAPDH mRNA by RT-PCR as described in section 2.4.2.

2.11.2 Subtractive hybridization

mRNA being upregulated or transcribed in CT cells treated with TGFα was identified by subtracting the cDNA populations of treated and untreated control cells using a modified version of the RDA protocol of Hubank & Schatz, 1994. The modified protocol incorporated the technique of supression PCR developed by Siebert *et al.*, 1995.

The primers, reagents and enzymes used were purchased from Clontech Laboratories (CapFinder cDNA synthesis kit and PCR Select cDNA Subtraction kit) unless stated otherwise. The nucleotide sequences of the primers and adaptors used are given in Table 2.5. The protocol supplied by Clontech was followed throughout; however key steps and or steps where optimisation, concentrations were altered are outlined in the following sections.

2.11.2.1 General procedures

All incubations and thermal cycling were carried out in a Peltier PTC 200 thermal cycler. The cDNA and PCR generated fragments were resolved on 1.4% agarose gels containing 0.3μg/ml EtBr cast and run in 1x TBE buffer (10.8g Tris, 5.5g boric acid, 0.93g EDTA dissolved in one l). The size distribution of cDNA was estimated against a 1kb DNA ladder (Gibco) and size of PCR generated fragments against 100bp DNA ladders (Promega or Gibco). The cDNA concentrations were estimated against a 100bp DNA ladder of known concentration (Promega) electrophoresed alongside samples on agarose gels. Samples from intermediate steps were stored at -20°C.

Table 2.5 Nucleotide sequences of primers and adaptors used in RDA

Primer / adaptor	Sequence 5' → 3'
CapSwitch II primer	AAGCAGTGGTATCAACGCAGAGTACGCGGG
cDNA synthesis primer	AAGCAGTGGTATCAACGCAGAGTACT$_{(30)}$N$_{-1}$N (N=A,C,G orT; N-1=A.G or C))
PCR primer	AAGCAGTGGTATCAACGCAGAGT
Adaptor 1	CTAATACGACTCACTATAGGGCTCGAGCGCCGCCCGGGCAGGT CCCGTCCA
Adaptor 2	TGTAGCGTGAAGACGACAGAAAGGGCGTGGTGCGGAGGGCGGT GCCTCCCGCCA
PCR primer 1	CTAATACGACTCACTATAGGGC
PCR primer 2	TGTAGCGGTGAAGACGACAGAA
Nested PCR primer 1	TCGAGCGGCCGCCCGGGCAGGT
Nested PCR primer 2	AGGGCGTGGTGCGGAGGGCGGT

2.11.2.2 Synthesis of driver & tester cDNA

First strand cDNA was synthesised separately from driver and tester total RNA. Two reactions mixes one containing 1μg of tester the other 1μg of driver RNA, 1μM of each primer (cDNA synthesis primer, CapSwitch II oligomer), 1mM of each dNTP, 2mM DTT and 200U MMLV reverse transcriptase in 1x first strand buffer were set up in a total volume of 10μL. Reverse transcription was carried out by heating the mixes at 42°C for 1h. Thereafter the mixes were diluted with 40μL TE buffer (10mM Tris pH7.6, 1mM EDTA).

Before second strand cDNA was synthesised it was necessary to determine the optimum volume of diluted 1st cDNA and number of PCR cycles required. Reactions were set up with cDNA (1, 2μL of driver and 1, 3μL of tester diluted in TE buffer) into separate PCR mixes containing 0.2μM PCR primer, 0.2mM of each dNTP, 1μL KlenTaq polymerase mix in 1x KlenTaq PCR buffer in a total volume of 50μL. The mixes were cycled as follows: hot start 95°C for 15sec, annealing and extension at 68°C for 5min. After 15, 18, 21 and 24 cycles 5μL of the reaction mix was removed and electrophoresed on agarose gels. Optimum volume of diluted 1st strand cDNA and the maximum number of PCR cycles prior to saturation was determined by observing the EtBr stained cDNA in the

5μL samples on agarose gels. These values were used to synthesise second strand tester and driver cDNA that were subsequently used for substraction analysis.

Tester and driver cDNA were purified separately using the Chroma Spin-1000 columns provided. The cDNA concentrations in the eluted fractions were determined by electrophoresing 10μL from each fraction on agarose gels.

2.11.2.3 *Rsa1* digestion

Spin column purified tester and driver cDNA were digested with the restriction enzyme *Rsa* 1 to produce blunt ended fragments. Tester, driver fractions '320' and '75' (as described in the Clontech protocol) were digested separately with 10U of *Rsa* 1 (NEB) in 1x NEB buffer for 3h at 37°C. Digestion was monitored by spiking 10μL of tester and driver fractions with 0.5μg of PUC 18 plasmid DNA.

The *Rsa*1 digested tester and driver cDNA from column fractions 320 and 75 were purified separately using DNA binding columns (Qiaex II, Qiagen). Five μl aliquots were electrophoresed and yields of digested cDNA were estimated.

2.11.2.4 Ligation of adaptors

Adaptors 1 and 2 (Table 2.5) were ligated onto *Rsa*1 digested, purified tester cDNA. Three ligations were set up each with 120ng of cDNA mixed with adaptor 1, adaptor 2 and adaptors 1 and 2. The concentration of each adaptor in the three tubes was 1μM. Ligations were carried out overnight at 16°C with 1U T4 DNA ligase (Progen) in 1x ligase buffer (Progen) in a volume of 10μL. The success of the ligations were monitored before commencing the first and second subtractive hybridizations. This was achieved by

carrying out a primary PCR as outlined in section 2.11.2.7 and analysing the products on an agarose gel.

2.11.2.5 First subtractive hybridization

Excess *Rsa*1 digested, driver cDNA and a small amount of tester cDNA was heat denatured and allowed to anneal. Two reactions were set up; one with driver cDNA and tester cDNA liagted to adaptor 1, the second with driver cDNA and tester ligated to adaptor 2. The ratio of driver:tester in both tubes was 450ng:18ng in a total volume of 4μL. The cDNA was heated to 98°C for 90sec, hybridized at 68°C for 8h. This was followed immediately by the second subtractive hybridization.

2.11.2.6 Second subtractive hybridization

The two solutions from the first subtractive hybridization were pooled and held at 68°C. Fresh *Rsa*1 digested driver cDNA (300ng) was denatured at 96°C for 2min, cooled to 68°C and added into the pooled mixture. The cDNA was hybridized overnight at 68°C, diluted and stored as recomended.

The subtraction of common cDNA sequences were monitored by amplifying GAPDH cDNA in the subtracted and non-subtracted cDNA libraries using PCR. For the non-subtracted library 1μL of tester cDNA ligated with adaptors 1 and 2 (ie. tube 3 outlined in section 2.11.4) diluted 1:1000 in water was used to set up the PCR. One μL of cDNA removed after the second subractive hybridization diluted 1:200 in dilution buffer provided and used as template from the subtracted cDNA library. The GAPDH primers and PCR cycling parameters are described in Table 2.1 and section 2.4.2 respectively.

2.11.2.7 Primary & nested PCR

The primary and nested PCR reactions were set up and cycled as recommended in the Clontech protocol. Briefly the primary PCR was set up using 1μL of the 2nd subtractive hybridization mix diluted 1:200 in dilution buffer, PCR primers 1, 2 and cycled 30x. The nested PCR was carried out by combining 1μl of the primary PCR mix diluted 1:9 with water and nested primers 1, 2 and cycling a further 11-13x. Negative control reactions for both primary and nested PCR were set up using 1μl unsubtracted tester DNA ligated with adaptors 1 and 2 diluted 1:1000 in water.

2.11.2.8 Identification of subtracted cDNA

One tenth volumes from the nested PCR mixes were resolved on agarose gels. Unique DNA bands appearing in the subtracted tester library were identified by comparing against the cDNA bands from the non-subtracted tester. Note the protocol that follows was not given in the Clontech manual. One μl was removed from each of the unique cDNA bands in the subtracted tester on agarose gels with and autopipettor then re-amplified by PCR. The re-amplification mixes contained 2mM $MgCl_2$, 0.2mM of each dNTP, 0.1μM each of nested primers 1, 2, 2.5U Taq polymerase (Perkin Elmer) in 1x PCR buffer (Perkin Elmer) in a total volume of 50μl. The PCR profile was 95°C for 1min followed by 20 cycles at 94°C for 30sec, 68°C for 30sec and 72°C for 2.5min. One tenth volumes of the PCR mixes were resolved on agarose gels and concentrations of the cDNA fragments estimated. Without further purification between 5-9ng of the amplified cDNA was ligated into the TA plasmid vector (TA cloning kit, Invitrogen). Plasmids were transformed into *E. coli* strain Top10' cells by the cold shock method and plated on Luria broth agar plates containing 100μg/ml ampicillin. Transformants were selected from agar plates and cultured overnight at 37°C in 100μL of Luria broth containing 100μg/ml ampicillin in 96 well ELISA plates.

Next the insert cDNA was amplified by PCR. One μL of the overnight culture was mixed with 24μL PCR mix and cycled 20x. The composition of the PCR mix and cyling

parameters were as described above. The amplified cDNA was purified and sequenced as described in 2.4.6 using nested primers 1 or 2. The identity of the cDNA was determined by aligning the sequence against known nucleotide sequences using the ANGISS database.

2.11.2.9 Detection of upregulated mRNA

In order to determine whether the upregulated mRNA was present in tester samples from other patients, tester cDNA libraries from two other patients were probed by Southern hybridization. Tester and driver cDNA (which served as the negative control) were prepared from CT cells isolated from another two patients (CS, 38 weeks) as outlined in section 2.11.1. Equivalent amounts of tester and driver cDNA were resolved on 1.2% agarose gels cast and run in 1xTBE. Southern blots were set up and the cDNA transferred overnight in 10x SSC buffer by capillary electrophoresis at room temperature onto nylon membranes (Hybond N^+, Amersham).

Insert cDNA cloned into the TA vector amplified by PCR (section 2.11.2.8) were used as hybridization probes. The amplified DNA was purifed by spin columns (Wizard, Promega) and labelled with α-^{32}PdCTP by random priming as described earlier in 2.4.6 and 2.5.3 respectively. Southern blots were prehybridized in 5x Denhards solution, 6x SSC, 0.5% w/v SDS and 50µg/ml denatured salmon sperm DNA at 65°C for 6h. Labelled probe was incorporated into the prehybridization solution and hybridized overnight at 65°C. Following stringency washed autoradiograms were set up. X-ray films were exposed for 2-3 days at -80°C and developed by conventional procedures. The sizes of hybrid molecules were determined by comparion against a DNA size markers.

CHAPTER 3

Expression of EGF / EGFR Related Growth Factor and Receptor mRNA in gestational tissue

This study examines whether CR, BTC, HRG, HB-EGF, AR, TGFα growth factor and c-erb-B2, c-erb-B3, c-erb-B4 receptor mRNAs are present in gestational tissue obtained from women undergoing caesarean section or spontaneous vaginal deliveries close to term. It also examines the expression of the same EGF/EGFR related mRNA in cytotrophoblast and syncytiotrophoblast cells in vitro.

3.1 Introduction

The placenta, amnion and chorion are composed of heterologous cells that perform functions which are vital for normal fetal growth and development. Up to about the middle of the second trimester the placenta increases exponentially in size due to the rapid, synchronised proliferation of fetal trophoblasts, vascular elements, fibroblasts and maternal decidual cells (Boyd & Hamilton, 1970). This period is also characterised by the differentiation of trophoblast cells into the villous ST and the EVT cells that invades the endometrium (Pijnenborg, 1990; Benirschke & Kaufmann 1995).

Stem cells in the amnion and chorion also proliferate rapidly during the first two trimesters, increasing the size of the amniotic cavity where the fetus is suspended, obliterating the uterine and chorionic cavities (Moore 1989). During this period the amnion and chorionic membranes fuses with each other and the decidua parietalis (Moore 1989). Thereafter the growth rate and invasive capacity of the fetal membranes and placenta gradually diminishes towards term (Moore 1989; Benirschke & Kaufmann 1995).

The temporal and spatial growth of the placenta and fetal membranes is regulated in a very precise and coordinated manner during the parallel morphogenesis of the gestational tissue. Growth factors and their cognate membrane bound receptors that are produced endogenously are thought to be the principal molecules that regulates the temporal and spatial growth of the gestational tissue (Ohlsson 1989; Graham & Lala 1992; Graham *et al.*, 1993). Studies *in vitro* have shown that growth factors also regulate vital functions such as placental hormone synthesis and eicosanoid production in the fetal membranes (Ohlsson 1989; Blay & Hollenberg 1989; Lundin-Schiller & Mitchell 1991; Lala & Hamilton 1996)

Previous studies have shown that the EGFR mRNA and / or protein is expressed throughout gestation in the human placenta, amnion, chorion and decidua (Chegini & Rao 1985, Hoffmann *et al.*, 1992). The EGF, TGFα, AR, BTC and HB-EGF are structurally related peptides that have been shown to interact with the EGFR (Fisher & Lakshmanan 1990; Higshiyama *et al.*, 1993; Watanabe *et al.*, 1994). EGF, TGFα mRNA and / or protein has been detected in the human placenta throughout gestation (Bissonnette *et al.*,

1991), while AR protein has been detected in the placenta up to 18 weeks gestation (Lysiak *et al.*, 1995). It is uncertain whether AR mRNA and protein is expressed in the fetal membranes or if BTC and HB-EGF expression occurs in the gestational tissue. *In vitro* BTC and HB-EGF have been found to act as potent mitogens on fibroblast and vascular smooth muscle cells (Shing *et al.*, 1993; Watanabe *et al.*, 1994; Blotnick *et al.*, 1994).

The mRNA and or protein expression of two EGFR related proteins namely c-erb-B2, c-erb-B3 is also reported to occur in human term placenta (Kraus *et al.*, 1989; Mulhauser *et al.*, 1993; Jokhi *et al.*, 1994). However it is uncertain whether the human placenta expresses c-erb-B4 mRNA or protein or if the three EGFR related receptors are expressed in the fetal membranes. HRG protein which is also structural homologue of the EGF has been shown to interact with the EGFR related receptors (Holmes *et al.*, 1992; Plowman *et al.*, 1993a; Beerli *et al.*, 1995). Whether HRG is expressed endogenously in the gestational tissue at any stage during pregnancy however remains uncertain. In cancerous cells the EGFR related receptors and HRG are believed to play important roles in regulating cell division, differentiation, transformation and hormone synthesis (Prigent and Lemoine 1992; Kung *et al.*, 1994; Dougall *et al.*, 1994; Karunagaran *et al.*, 1996).

The endogenous expression and the effects exerted by growth factors in the placenta have been widely studied using dispersed trophoblast cell cultures. Studies carried out by Chen (1988) and Ameniya (1994), have demonstrated that placental CT and ST cells express EGFR mRNA and protein. The protein c-erb-B2 has also been found in CT cells isolated from the third trimester (Aboagye-Mathiesen *et al.*, 1997). However it is uncertain whether the CT and ST cells express c-erb-B3, c-erb-B4 mRNA and protein.

There have been conflicting reports of the endogenous expression of EGF by CT and ST cells. Ameniya and co-workers (1994), reported that EGF mRNA is expressed in CT, ST cells while Bass and colleagues (1994), reported that they were unable to detect EGF or TGFα mRNA in CT cells isolated from the third trimester. It remains uncertain whether CT, ST cells express AR, BTC, CR, HRG or HB-EGF mRNA *in vitro*.

In order to gain a better understanding of the expression of the EGF / EGFR related growth factor and receptor mRNA in the gestational tissue where the ligands and receptors may be exerting regulatory control this study was undertaken with the aims of:

- determining if BTC, HB-EGF, AR, TGFα, HRG, CR, c-erb-B2, c-erb-B3, c-erb-B4 mRNA are expressed in the villous cotyledons, amnion and chorion-adherent decidua close to term

- determining if the EGF / EGFR related growth factor and receptor mRNA are expressed in CT and ST cells *in vitro* and

- to measure the levels of some of the growth factor and c-erb-B2, c-erb-B3, c-erb-B4 mRNA expressed in the gestational tissue and cultured cells relative to GAPDH mRNA.

3.2 Materials and methods

Villous CT cells were isolated from placenta delivered by Ceasarean section and vaginally (n=5). Cells were characterised by immunoctyochemical staining against cytokeratins 8, 18 and the amount of total hCG synthesised. Cells were seeded at a density of $0.75x10^6$ cells per ml in EMEM with 10% FCS and cultured for 12 and 96h. The isolation, characterisation and culture conditions are given in Chapter 2, section 2.2.

Total RNA was prepared from amnion, chorion-adherent decidua and villous cotyledons (n=9; CS=5, SD=4) and CT cells cultured for 12 and 96h. Total RNA was quantitated and its quality assessed by formaldehyde agarose gel electrophoresis. Details of the RNA isolation, quantitation and quality assessment are given in Chapter 2, section 2.3. Total RNA from gestational tissue and cultured cells was used for RT-PCR and semi-quantitative RT-PCR analysis.

Between 0.5-1.0μg total RNA isolated from villous cotyledons (VC), amnion, chorion-adherent decidua, CT cells cultured for 12 and 96h was subjected to RT-PCR using

primers designed specifically for each EGF/EGFR related growth factor and receptors. The primers used for reverse transcription, PCR, the RT-PCR procedure and agarose gel electrophoresis of RT-PCR generated amplicons are given in Chapter 2, Table 2.1 and sections 2.4.1-2.4.2, 2.4.4 respectively.

Three randomly selected samples of total RNA from each of the gestational tissue and cultured cells was also subjected to semi-quantitative RT-PCR using primers specific for AR, BTC, HB-EGF and c-erb-B2, c-erb-B3 and c-erb-B4. Semi quantitative analysis was carried out with one μg of RNA. GAPDH mRNA was used as an internal standard for semi-quantitative RT-PCR. The amount of receptor and growth factor mRNA present in the cultured cells and gestational tissue was measured relative to GAPDH mRNA by laser densitometric analysis of amplicons generated by semi-quantitative RT-PCR. Details of the semi-quantitative RT-PCR analysis are given in Chapter 2, section 2.4.3-2.4.5.

3.3 Results

3.3.1 Characterisation of cells isolated from villous cotyledons

The isolated cells were rounded, mono-nucleated and between 10-18μM in diameter. After 12h in culture, the cells remained mono-nucleated and had nearly reached confluence. After 96h in culture the cells were no longer round in appearance but were aggregated into large, dense, irregularly shaped clusters that contained many nuclei (Fig. 3.1 A, B, C). Freshly isolated cells and cells maintained in culture for 12h or 96h showed intense immunostaining against cytokeratins 8 and 18 (Fig.3.1 D, E, F).

After an initial seeding density of $0.75x10^6$cells/ml, the mean number of cells increased to $0.86x10^6$, SEM $\pm 0.14x10^6$ cells per ml after 12h in culture (n=5). Total hCG was not detected in media conditioned by CT cells for 12h using a fluorescent immunoassay. The lower level of sensitivity of the immunoassay was 2mIU/ml. After a total of 96h in culture the mean concentration of hCG was 9mIU/ml, SEM ±2mIU/ml (n=5).

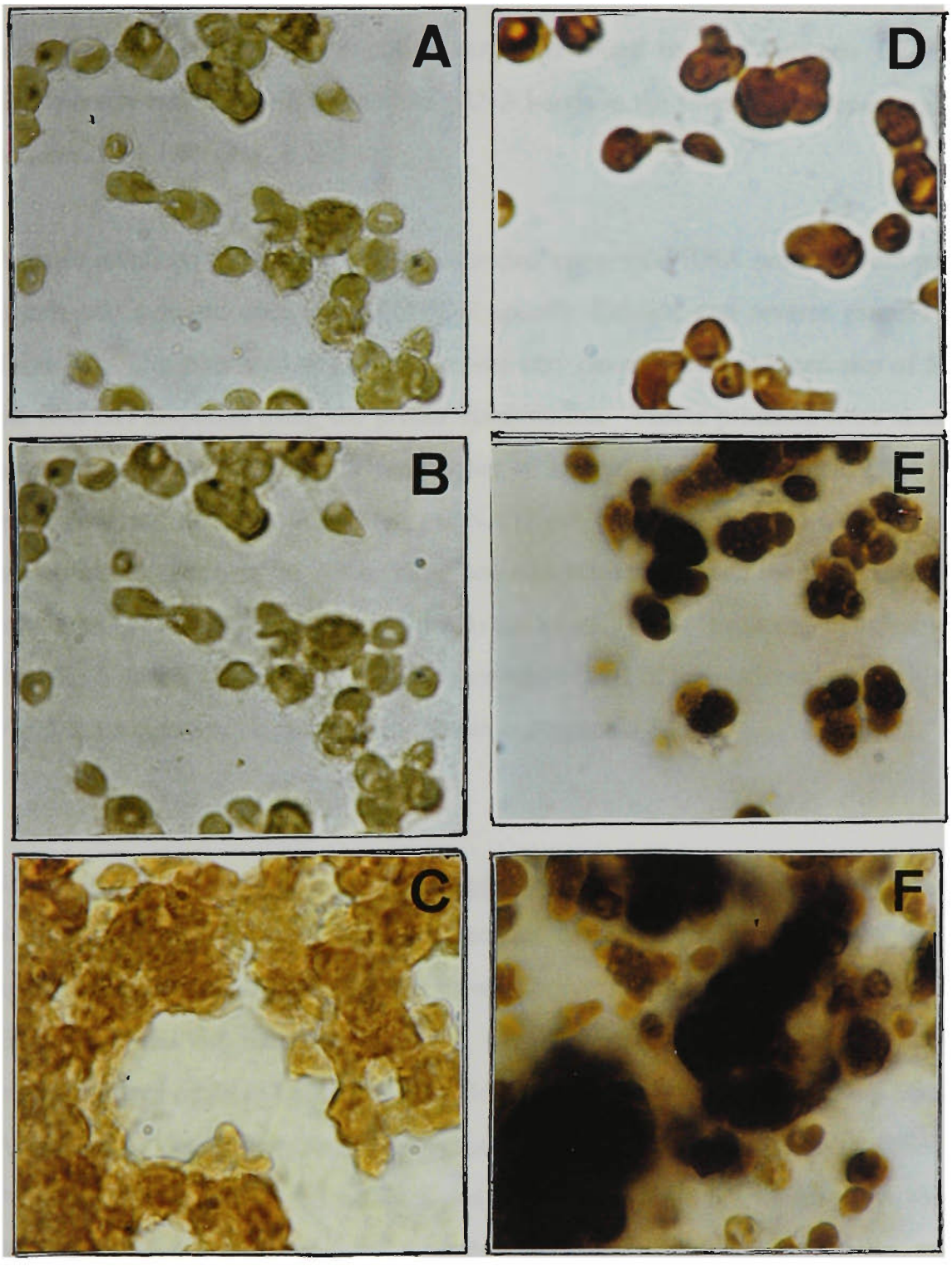


FIG. 3.1 Morphology and immunocytochemical staining of cells isolated from villous cotyledons after 39 weeks gestation. Freshly isolated CT cells (A), CT cells cultured for 12h (B) and after 96h in culture (C). Positive immunocytochemical staining for cytokeratin 18 by freshly isolated cells (D), cells after 12h in culture (E) and after 96h in culture (F). Magnification=150x in A-C and 350x in D-F.

3.3.2 Quality of total RNA preparations used for RT-PCR analysis

The A_{260}:A_{280} ratios of the total RNA preparations from gestational tissues and cultured cells measured by UV spectrophotometry was found to vary between 1.7-2.0. The size:intensity ratio of the 28S and 18S rRNA bands in the total RNA preparations ranged between 1.54-1.85 (Fig. 3.2A).

A single amplicon 531bp in size was generated using total RNA samples from gestational tissues and cultured cells using GAPDH specific forward and reverse primers given in Table 3.1. The estimated size of 531bp was very close to the predicted size of 529bp for the GAPDH amplicon using the primer combination. Visible products were absent in the negative control reactions which consisted of human placental genomic DNA, RT mix minus reverse transcriptase and yeast tRNA (Fig.3.2B). Following DNA sequencing, the nucleotide sequence of the 531bp amplicon was found to match the nucleotide sequence of human GAPDH cDNA reported by Arcari *et al.*, 1984. Base mis-matches were less than 0.6% over a stretch of 481 identifiable bases (part of the nucleotide trace is shown in Fig. 3.3; complete nucleotide trace is given in Appendix 12a).

3.3.3 Specificity of the primers and optimisation of RT-PCR parameters

Starting with 1μg total RNA from the placenta, amplified products were visible following agarose gel electrophoresis using the forward, reverse primer pairs specific for AR, HB-EGF, BTC and the receptors c-erb-B2, c-erb-B3 and c-erb-B4 after 35 cycles. The estimated sizes of these fragments were 365, 341, 355, 470, 331 and 473bp respectively. Amplicons of similar size were obtained starting with 0.5μg total placental RNA after 40 PCR cycles with each of the receptor and HB-EGF specific primer pairs. In the case of AR and BTC however visible cDNA bands were absent and only a background smear was observed following agarose gel electrophoresis of the RT-PCR mix using 0.5μg RNA.

RT-PCR analysis of 1μg of total RNA and the CR, HRG and TGFα reverse and forward primer pairs produced very faint bands of expected size plus a number of non-specific bands in a background smear. Only a smear was detected when reactions were set up with 0.5μg of total placental RNA and CR, HRG and TGFα specific reverse and forward

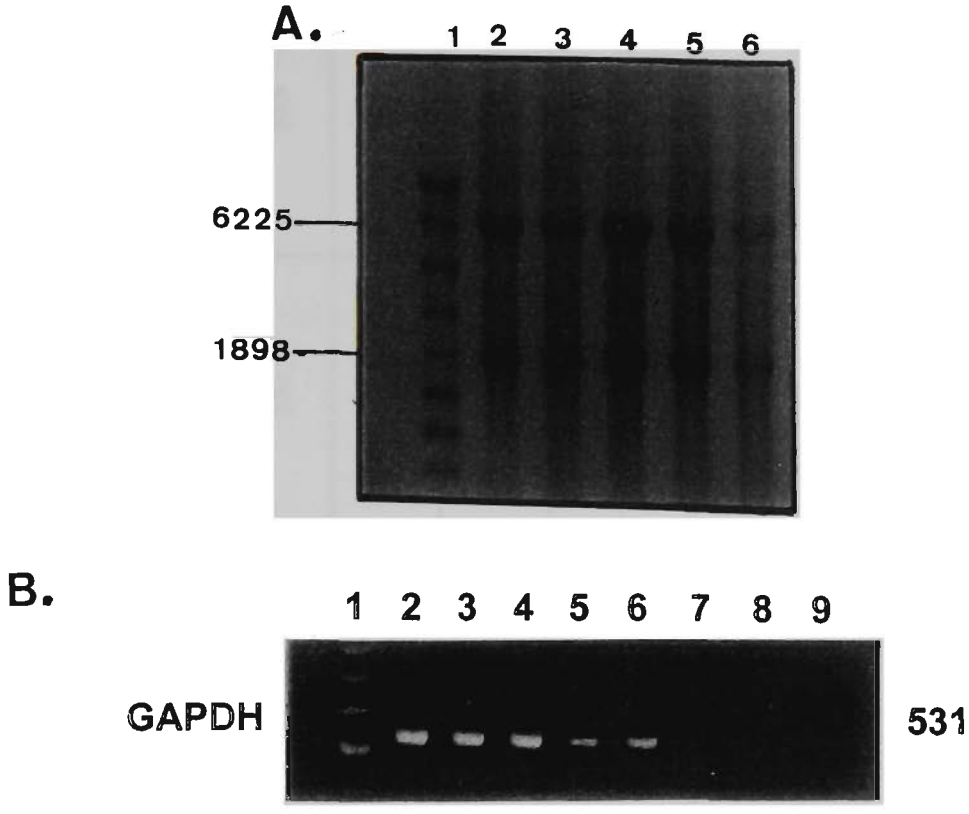


FIG. 3.2 **A)** total RNA separated on 1% formaldehyde agarose gel. Lanes 2-6 contain samples from amnion, chorio-decidua, villous cotyledons, CT and ST cells respectively. Lane 1- RNA marker. **B)** RT-PCR analysis of total RNA using GAPDH specific primers. 1/10th volume of the reaction mixes from amnion, chorio-decidua, villous cotyledons, CT, ST cells resolved on a 1.3% EtBr stained agarose gel (lanes 2-6 respectively). Lanes 7- genomic DNA, 8- yeast tRNA and 9- total RNA from placenta minus reverse transcriptase. Lane 1- DNA marker, spiked band=500bp.

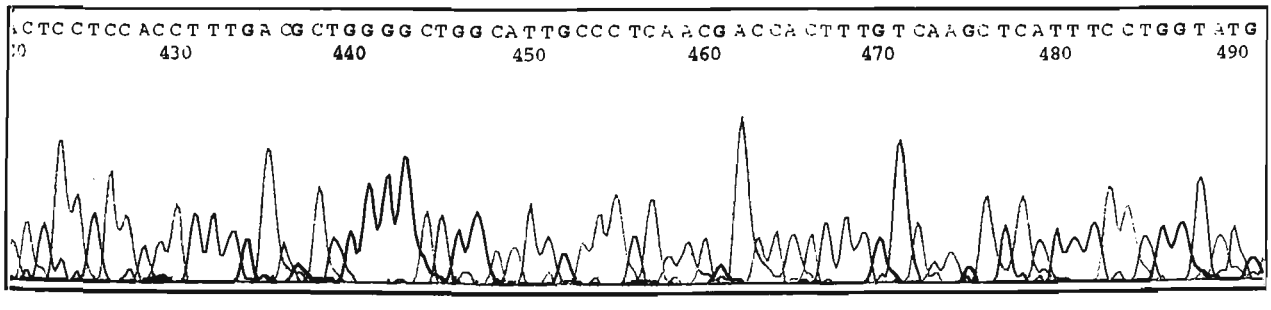


FIG.3.3 Nucleotide sequence trace of a part of the 531bp amplicon generated with the GAPDH specific primers and a total RNA preparation from amnion. Complete trace is given in Appendix 12a.

Table 3.1 Primers used in reverse transcription and PCR

Specific for	Primer sequence 5'→3'; RT (reverse transcription), R (reverse), F (forward)	Position on cDNA sequence	Reference	R & F primers spans intron / exons	Predicted size of fragment with R and F primers (bp)	Size of fragment estimated on gels (bp)
GAPDH	R-AGGTCCACCACCCTGTTGCTGTA F-TCCTGCACCACCAACTGCTTAGC	1040-1018 511-533	Daneau *et al.*, 1996	yes	529	531
BTC	R-GATAGGAGTTATATCTTTACCCAG F-GAGGAAAACTGTGCAGCTACCACC	799-776 442-465	Sasada *et al.*, 1993	?	357	355
CR	RT-GCTTTGTATAGAAAGGCAGATGCC R-GGTAGAAATGCCTGAGGAAAGCAG F-CAGAGATGACAGCATTTGGCCCCA	805-782 687-664 385-408	Ciccodicola *et al.*, 1989	yes	302	302
HRG	RT-GGCTGACACATACCTTTCACTATG R-GGCATGCCTGAGGAAGCTGTTACA F-CCAGAAGAGAGTGCTGACCATAAC	1732-1709 1356-1333 849-872	Holmes *et al.*, 1992	?	507	511
HB-EGF	R-GATGACCAGCAGACAGACAGATGA F-CAAGAGGCAGATCTGGACCTTTTG	795-772 454-477	Fen *et al.*, 1993	yes	341	341
AR	R-CTGACATTTGCATGTTACTGCTTC F-GTTACCTCAAGAAGTGAGATGTCT	729-706 360-383	Plowman *et al.*, 1990a	yes	369	365
TGFα	RT-AGTGGCAGCAAGCGGTTCTTCCCT R-TGTATCAGCACACATGTG F-TGCCCAGATTCCCACACT	500-477 399-382 173-190	Derynck *et al.*, 1984	yes	226	228
c-erb-B2	R-TTCCATCCTCTGCTGTCACCTCTT F-GACATCTTCCACAAGAACAACCAG	1132-1109 661-684	Tal *et al.*, 1987	yes	471	470
c-erb-B3	R-TGCACCATACCATGTTCCTCAAGG F-ACTGTGCACAAAGGAGTGTGGATC	2693-2670 2362-2385	Plowman *et al.*, 1990b	yes	331	331
c-erb-B4	R-GTCCCCATGAATACCAGTGACTAG F-AGATGCTACGGACCTTACGTCAGT	1161-1138 691-714	Plowman *et al.*, 1993b	?	470	473

primer pairs were used. However when total placental RNA was reverse transcribed with the 'RT' primer, followed by 35 PCR cycles with primer pairs specific for HRG, CR and TGFα, single cDNA bands were detected. The estimated sizes of the cDNA were 511, 302 and 228bp for HRG, CR and TGFα respectively.

The estimated size of the amplicons generated using the growth factor or receptor primer combinations were found to be identical to their calculated sizes or differed by ±2-5bp (Table 3.1).

Visible products were absent in reaction mixes with total RNA minus reverse transcriptase and genomic DNA implying that the amplified products were produced by reverse transcription of RNA and not by amplification of genomic DNA. Amplified products were also absent in the yeast tRNA negative control reactions.

Using the results from the optimisation trials, 1μg of total RNA samples from the amnion, chorion-adherent decidua, villous cotyledons and cultured cells were subjected to RT-PCR analysis.

3.3.4 RT-PCR analysis of total RNA

3.3.4.1 Gestational tissues

Amplified products were detected using primers specific for the growth factors AR, TGFα, HB-EGF in all total RNA preparations from all villous cotyledons, amnion and chorio-decidua tested (Caesarean section, n=5). The estimated sizes of the AR, TGFα, HB-EGF cDNA fragments were 365, 228 and 341bp respectively. Amplified products were also detected following agarose gel electrophoresis from some of the villous cotyledon, amnion and chorion samples analysed with the HRG and CR specific primers (Table 3.2). The estimated sizes of the CR and HRG amplicons were 302 and 511bp respectively. In the case of BTC 4/5 of the total RNA samples from the villous cotyledons tested generated cDNA fragments 355bp in size. Amplified products were not detected from any of the total RNA samples from the amnion and chorio-decidua amplified with the BTC specific primers. However faint non specific bands of 200, 245

and 493bp were visible in some amnion and chorio-adherent decidual preparations using the BTC specific primer combination following agarose gel electrophoresis.

Amplified products were detected from all total RNA preparations from gestational tissues delivered by Caesarean section and tested with the primers specific for the receptors c-erb-B2, c-erb-B3 and c-erb-B4.

RT-PCR analysis of total RNA preparations obtained from tissues delivered spontaneously after 38-40 weeks gestation (n=3) showed no significant differences in the growth factor or receptor mRNA present compared with tissue obtained following Caesarean section. (Table 3.2).

The sizes of the RT-PCR generated products remained constant irrespective of the tissue type or patient and were also equivalent in size to products generated during the optimisation trials (section 3.3.3, Fig. 3.4, Table 3.1).

Very faint non-specific bands of variable size were observed with AR, TGFα and HRG specific primers in a few of the villous cotyledonary RNA. Faint non specific bands of variable size were also present in some RNA from amnion and chorion using primers for CR and c-erb-B3.

Visible bands were not detected in the negative control reaction mixes that consisted of yeast tRNA, genomic DNA and reaction mixes without reverse transcriptase after agarose gel electrophoresis.

3.3.4.2 Cultured cytotrophoblast and syncytiotrophoblast cells

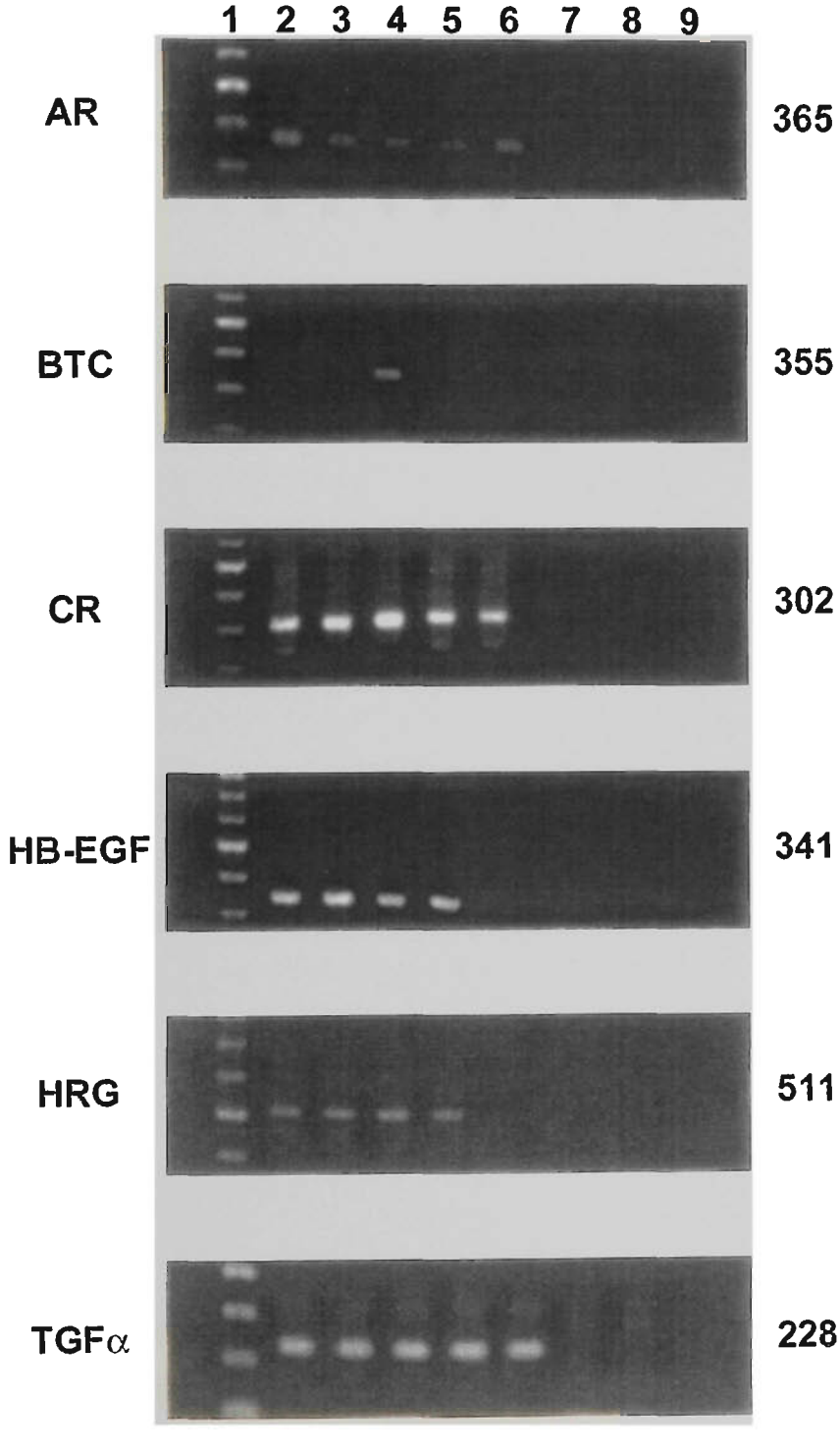
Amplicons of expected size were detected in the RNA preparations from cytotrophoblast cells cultured for 12h (n=5) using primers specific for AR, TGFα, CR, HRG, HB-EGF and c-erb-B2. Very faint bands were present two CT RNA samples after amplification with the c-erb-B4 primers. Amplicons of predicted size were not detected with the BTC

and c-erb-B3 primers in the RNA samples from CT cells under the cylcing parameter used.

After the cells had differentiated into syncytia and had been in culture for a total period of 96h, cDNA fragments of expected size were detected using the AR, CR and TGFα primer combinations only. HRG, c-erb-B2 and c-erb-B4 did not appear to be expressed although in two samples very faint bands of predicted size was detected using the c-erb-B2 primers. Some non-specific banding was observed with the c-erbB-2 and c-erbB-3 primers in both CT and ST cell preparations that were absent in the tissue preparations. Visible products were absent in the negative control reactions (Fig. 3.4).

Table 3.2 Distribution of EGF related growth factor mRNA in gestational tissue

Growth factor	Number of samples showing positive expression					
	Caesarean section (N=5)			Spontaneously delivered (N=3)		
	amnion cotyledons	chorion	villous	amnion cotyledons	chorion	villous
BTC	0	0	4	0	0	3
CR	4	3	4	3	2	3
HRG	3	3	5	3	3	3
AR	5	5	5	3	3	3
HB-EGF	5	5	5	3	3	3
TGFα	5	5	5	3	3	3


1 2 3 4 5 6 7 8 9
AR
365
BTC
355
CR
302
HB-EGF
341
HRG
511
TGFα
228

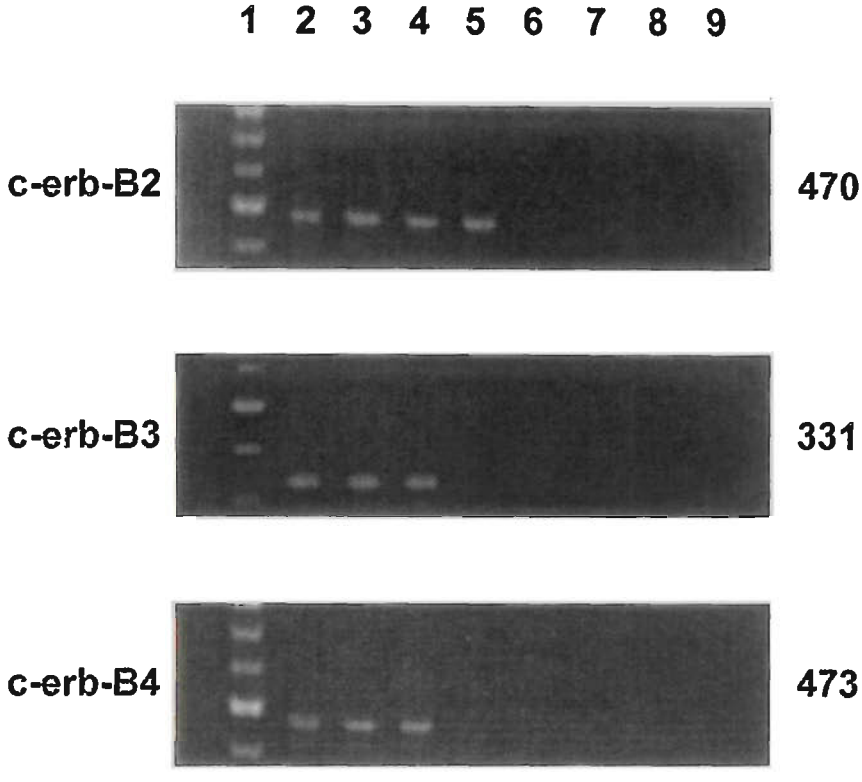


Fig. 3.4 RT-PCR analysis of total RNA. 1/10th volume of RT-PCR mixes resolved on EtBr stained 1.3% agarose gels. Lanes 2-6 contain samples from amnion, chorion-adherent decidua, villous cotyledons, CT cells 12 and 96h in culture respectively. Lanes 7-9 are negative control reactions consisting of yeast tRNA, genomic DNA and reaction mixes without reverse transcriptase respectively. Lane 1- 100bp DNA marker, spiked band=500bp. The sizes of the amplicons are given in bp.

3.3.5. DNA sequencing of RT-PCR generated cDNA fragments

Neglecting the 5' and 3' ends of the amplicons where the sequence was sometimes unclear, the nucleotide sequences of amplicons from gestational tissue and cultured cells were found to match published data. The percentage of base mis-matches between the published and experimental data and was very minor and ranged between 0.4-1.2% (Fig. 3.5). The published cDNA sequences and the complete nucleotide sequence traces are given in Appendices 4-11 and 4a-11a respectively.

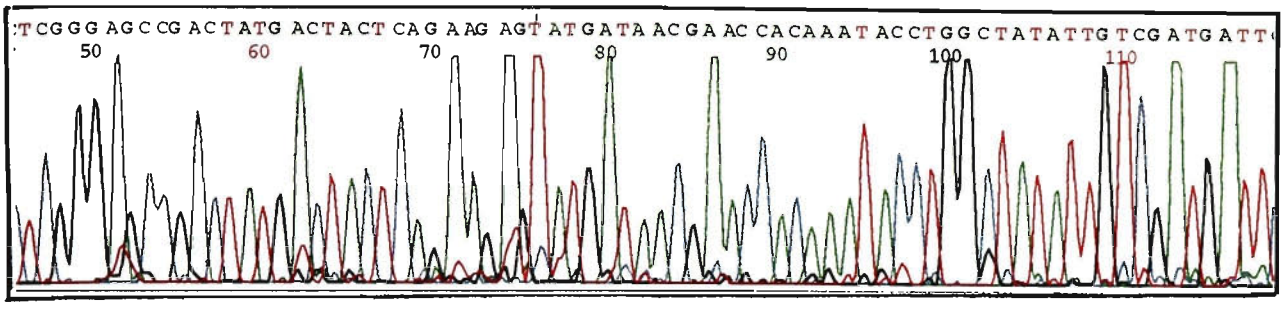
3.3.6 Relative levels of growth factor and receptor mRNA expression in tissues and cells

Semi quantitative RT-PCR analysis revealed that per μg of total RNA, the amount of AR mRNA was higher in the villous cotyledonary specimens compared with the fetal membranes. The amount of HB-EGF mRNA was also higher in the villous cotyledons compared with the fetal membranes. BTC mRNA was detected only in the villous cotyledons. In the case of the cultured CT cells similar amounts of AR mRNA was detected in CT cells cultured for 12 and 96h. HB-EGF mRNA was only present in the CT cells cultured for 12h.

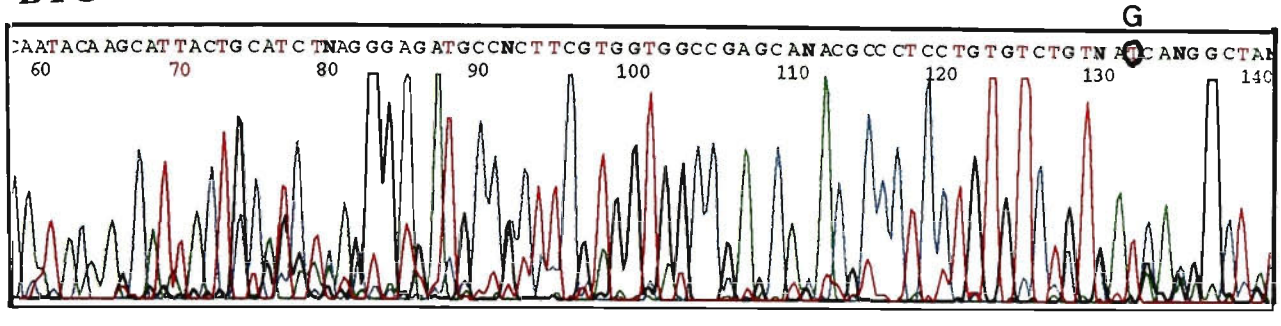
In the case of the EGFR related receptors, the villous cotyledonary samples were found to contain higher amounts of c-erb-B2, c-erb-B3 and c-erb-B4 mRNA compared with the fetal membranes. The relative amounts of c-erb-B3 and c-erb-B4 in the chorion-adherent decidua and amnion were found to be similar. In the chorion however the relative amount of c-erb-B2 mRNA was found to be 0.6x the amount present in the amnion.

In the CT cells cultured for 12h the relative levels of c-erb-B4 mRNA was approximately 2.2 times greater compared with cells grown for 96h (Fig. 3.6).

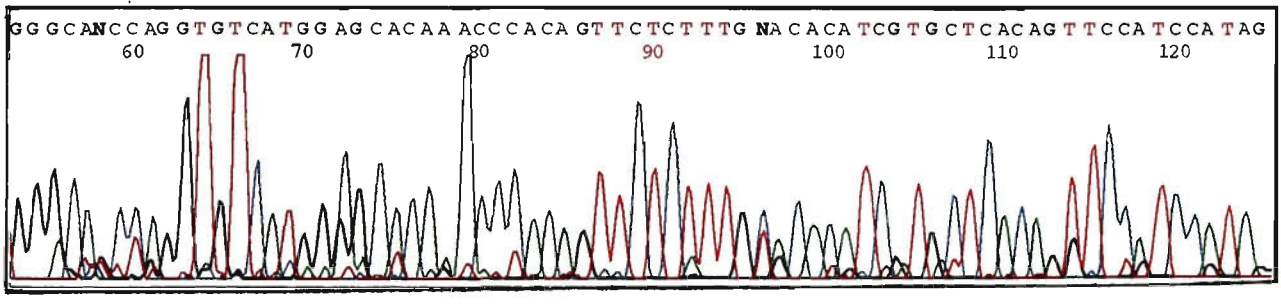
AR



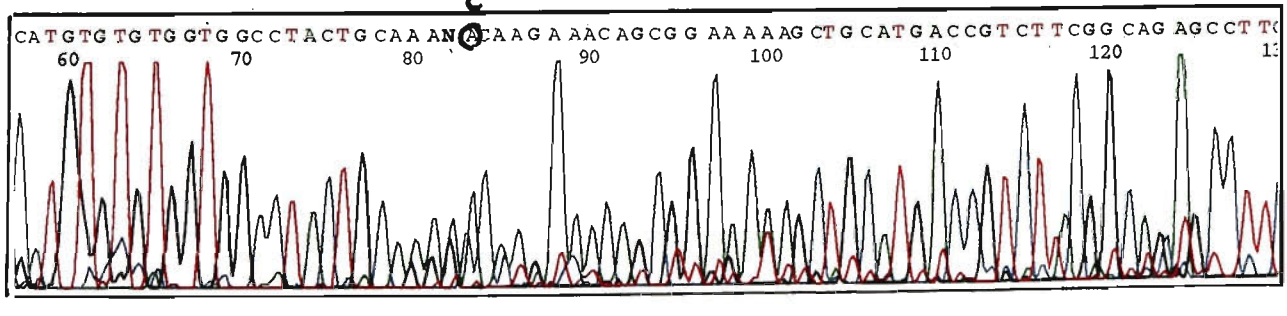
BTC



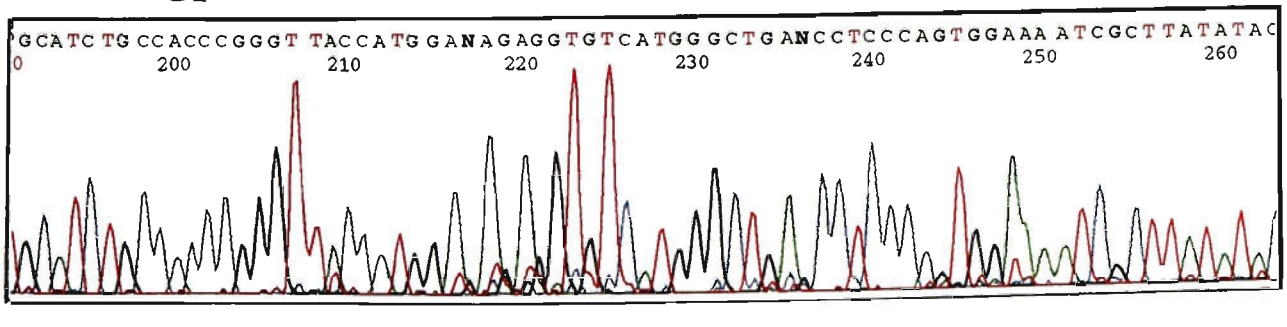
CR



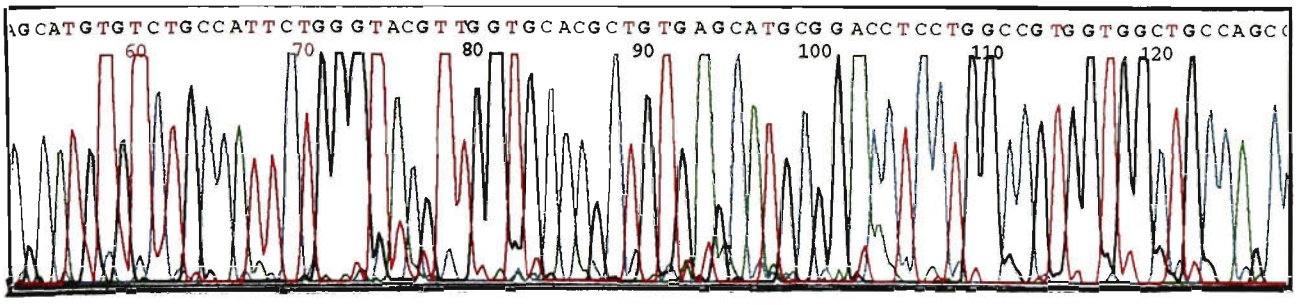
HRG



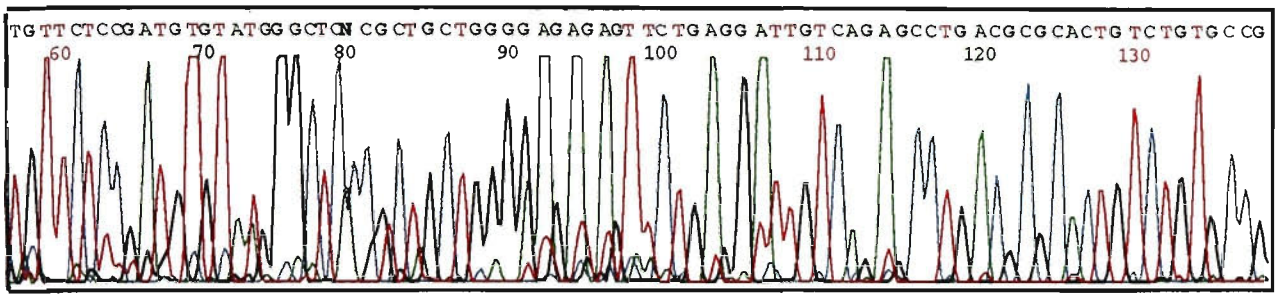
HB-EGF



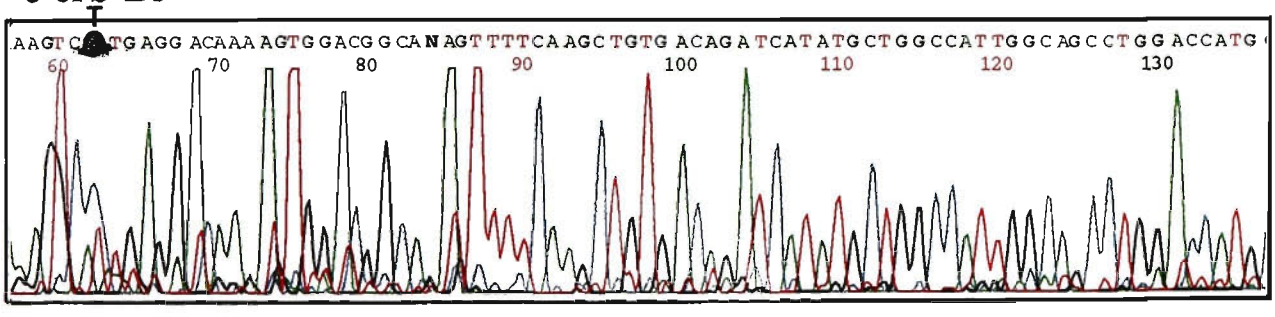
TGFα



c-erb-B2



c-erb-B3



c-erb-B4

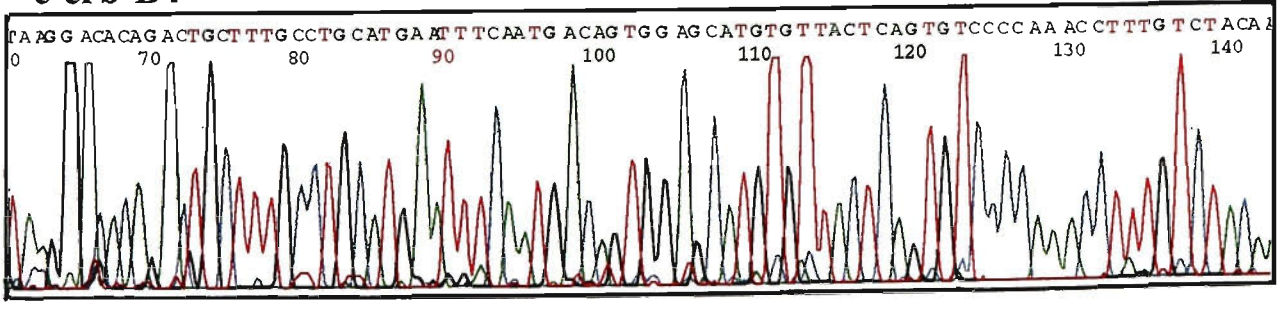
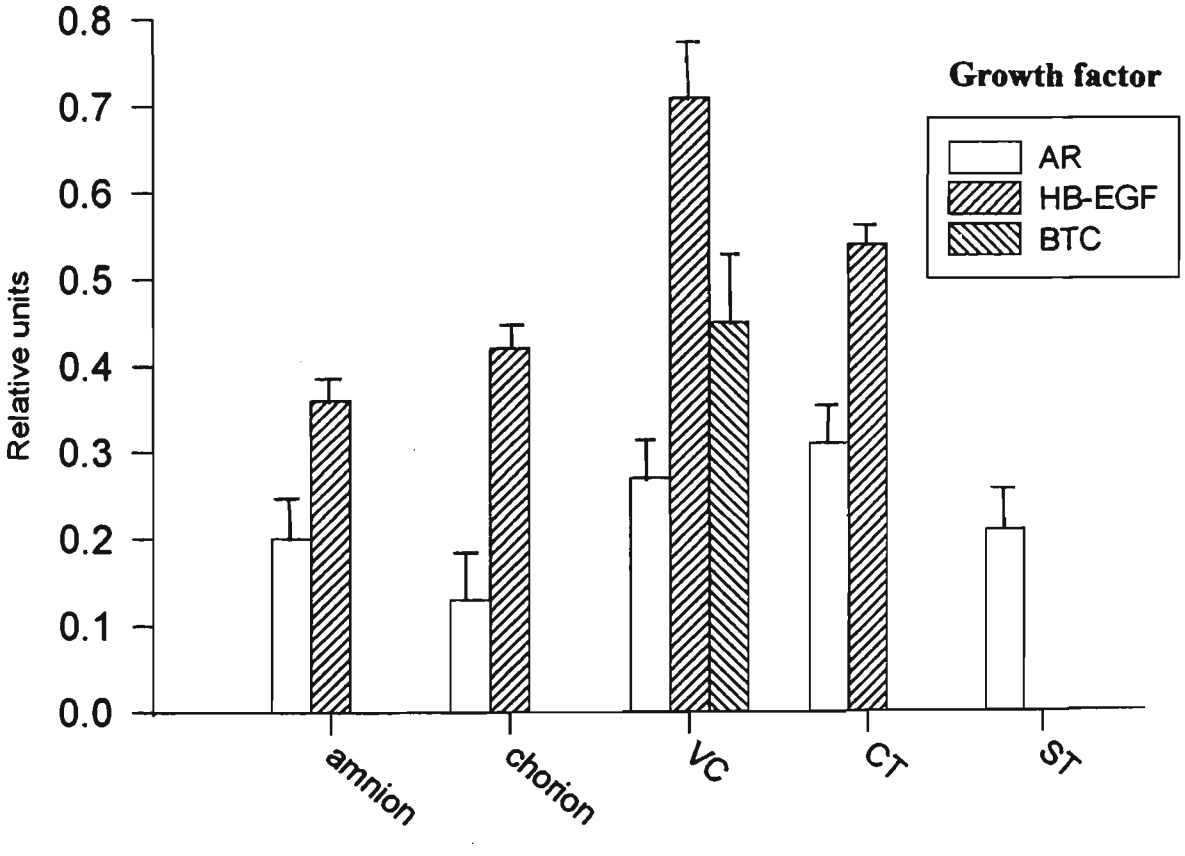


Fig. 3.5 Nucleotide sequence traces of RT-PCR generated cDNA. Base mis-matches between published sequence data (references given in Table 3.1) and nucleotides on the trace are circled and the difference shown above the circled base. Only parts of the traces are depicted; the complete traces are given in Appendices 4a-11a.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Relative units
Growth factor
AR
HB-EGF
BTC
amnion
chorion
VC
CT
ST

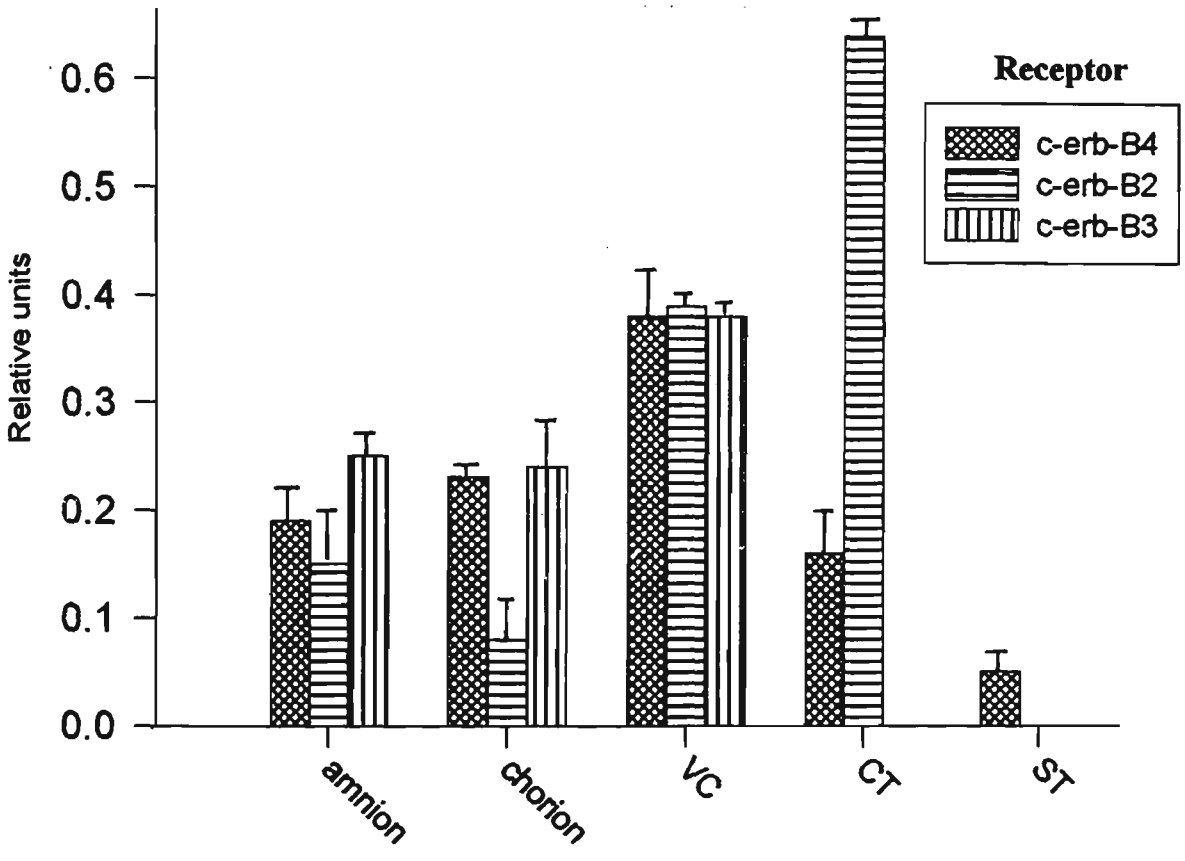


FIG. 3.6 Relative levels of growth factor and receptor mRNA in gestational tissue and cultured cells. Growth factor and receptor amplicons generated by RT-PCR were quantitated relative to RT-PCR generated GAPDH cDNA by laser densitometry. Values shown are the mean, ±SEM of the laser densitometric measurements of amplicons (n=3).

3.4 Discussion

The findings of this study have demonstrated for the first time that close to term CR, HB-EGF, HRG mRNA is expressed in human villous cotyledons, amnion, chorion-adherent decidua while BTC mRNA is expressed in the villous cotyledons but not in the fetal membranes.

In a previous study Watanabe and colleagues (1997), immunolocalised CR protein to the ST cells of human term chorionic villi. We detected CR mRNA in the villous cotyledons by RT-PCR analysis and our data supports the findings of Watanabe (1997). In their study Watanabe (1997), also showed that CR protein was present in the ST cells of chorionic villi during the first two trimesters. Studies in mice indicate that CR may play a crucial role in the early stages of gestation. CR mRNA has been localised by in situ hybridization to the trophectoderm layer of the blastocyst which gives rise to the placenta and to the chorion during gastrulation (Johnson *et al.*, 1994). The expression of CR mRNA in the murine gestational tissue appears to be a transient feature in early stages of embryonic development. In latter stages of pregnancy, CR mRNA has been found only in the myocardium and truncus arteriosus suggesting a role in fetal heart organogenesis in mice (Dono *et al.*, 1993; Johnson *et al.*, 1994). Although we cannot speculate that CR mRNA is expressed throughout gestation in the human placenta and fetal membranes the pattern of CR mRNA expression in the human gestational tissue appears to be different to that of the mouse.

Previous studies have shown that fibroblasts, macrophages and smooth muscle cells of the vascular elements produce abundant amounts of HB-EGF mRNA and the growth factor has been found to be one of the most potent mitogens of smooth muscle cells and fibroblasts (Higashiyama *et al.*, 1991; Temizer *et al.*, 1992; Nakano *et al.*, 1993). The high levels of HB-EGF mRNA that we detected in the placenta may be from these cellular sources. HB-EGF is also thought to play an important role in the early stages of pregnancy in mice. Murine uterine decidual cells have also been found to express HB-EGF mRNA where the growth factor is thought to play a role in decidualisation in readiness for implantation (Zhang *et al.*, 1994b). However whether HB-EGF is expressed in the human decidua or fetal membranes at any stage of gestation remains uncertain.

In contrast to our findings, Holmes and co-workers (1992), did not detect HRG mRNA in human term placenta by Northern hybridization. In our study, HRG mRNA was detected in the placenta by RT-PCR analysis. The enhanced sensitivity of RT-PCR, compared with Northern hybridization may account for the different findings. Four different types of HRG mRNA's namely α, β1, β2, β3 that are characterised by divergent regions close to the amino and carboxy acid termini have been described (Holmes *et al.*, 1992). The four HRG mRNA's are thought to be expressed in a tissue specific manner, as Northern hybridisation studies have shown that HRG mRNA transcripts of several different sizes are present in various tissues (Holmes *et al.*, 1992). The forward and reverse PCR primers that were used in this study were generated from the homologous regions of the four HRG mRNA species. Therefore, the specific HRG mRNA sub-type/s that may be expressed in the gestational tissues was not be identified in this study.

There have been no previous reports of BTC mRNA or protein expression in the placenta. Previous studies have shown that BTC mRNA transcripts, that are approximately 3kb in size are expressed in the murine liver, kidney, intestine and heart (Sasada *et al.*, 1993).

We also found that AR and TGFα mRNA were present in the villous cotyledons, amnion and chorion-adherent decidua. Plowman and colleagues (1990a), found that AR mRNA transcripts, 1.8kb in size, were highly expressed in the human placenta. Although our findings are consistent with this report, we found that the amount of AR mRNA in the placenta, relative to the constitutively expressed GAPDH mRNA, was fairly low. Whether immunoreactive AR protein is expressed in term placenta however remains questionable. Lysiak and colleagues (1995), reported that AR protein was absent in the human placenta after 18 weeks gestation and in the decidua throughout gestation. There have been no previous reports of AR mRNA or protein expression in the fetal membranes.

Bissonnette and co-workers (1991), detected TGFα transcripts, 4.8kb in size in human placenta at 38 and 40 weeks gestation and our findings are consistent with this report. In the case of the fetal membranes, dispersed epithelial cells from term amnion have been found to express TGFα mRNA *in vitro* (Tahara *et al.*, 1994). The amniotic epithelial cells may be the source of TGFα mRNA that we detected in the amnion tissue. In

another study, low copy numbers of TGFα mRNA were detected human term decidua by RT-PCR analysis (Haining *et al.*, 1991). In our study TGFα mRNA was easily detected in the chorion-adherent decidua and it is possible that the higher levels of TGFα mRNA that we detected were from cells located in the chorion.

Our findings also show that c-erb-B2, c-erb-B3 and c-erb-B4 receptor mRNA are present in the villous cotyledons, amnion and chorion-adherent decidua at term and that these mRNA were present in moderately high levels relative to GAPDH mRNA. Our findings are consistent with that of Kraus and co-workers (1989), who reported that c-erb-B3 mRNA, 6.2kb in size were present in human the placenta. Our data also supports the findings of Mulhauser and colleagues (1993), who localised c-erb-B2 mRNA to human villous and EVT cells throughout pregnancy. Mulhauser (1993), also showed that c-erb-B2 protein was present only in the apical membranes of the differentiated ST cells of human term placenta and were absent in the undifferentiated trophoblast populations.

Although our studies do not reveal the cellular sites of mRNA synthesis or if functional proteins are synthesised, on the basis of the mRNA expression it is very likely that the EGF/EGFR related growth factor and receptor proteins are being co-expressed in the gestational tissue close to term. The presence of both ligand and cognate receptor suggests autocrine, paracrine and juxtacrine regulation by endogenously produced EGF related growth factors in the gestational tissue. Previous studies have shown that TGFα and EGF interacts with the EGFR protein in the gestational tissue (Massague 1983, Tavare *et al.*, 1987). Our findings suggests that other ligands such as AR, BTC and HB-EGF may be competing with EGF, TGFα for EGFR receptor binding sites. AR has been shown to interact with EGFR in MDA, MCF-10A breast cancer cells and SK-OV3 ovarian cancer cells (Johnson *et al.*, 1993a). BTC and HB-EGF have been found to interact with the EGFR in A431 epidermoid carcinoma and MCF-7 breast cancer cells respectively (Watanabe *et al.*, 1994; Riese *et al.*, 1996b). The presence of other EGFR binding ligands may at least partly explain why the number of EGFR receptors and binding sites increase with gestational age in the human placenta (Chen *et al.*, 1988), even though it has been reported that immunoreactive EGF levels decline progressively towards term (Bissonnette *et al.*, 1991).

HRG on the other hand has been shown to interact with the EGFR related receptors. This growth factor has been shown to bind to c-erb-B2 and c-erb-B3 receptor proteins on rat hepatocytes (Carver *et al.*, 1996) and c-erb-B4 receptor on NIH 3T3 cells (Cohen *et al.*, 1996). Co-expression of c-erb-B2 and c-erb-3 receptors in breast carcinoma cells are thought to act as high affinity receptors for HRG and this interaction has been demonstrated to inhibit the interaction of EGF with the EGFR (Sliwkowski *et al.*, 1994). HRG is also thought to induce the formation of heterodimers between the receptors c-erb-B2 and c-erb-B3 in NIH 3T3 cells (Wallasch *et al.*, 1995). The presence of the receptors c-erb-B2, c-erb-B3, c-erb-B4 and the ligand HRG in the placenta, amnion and chorion suggests that regulatory effects may be exerted through the interaction of these receptor/s and HRG.

Although CR protein possesses structural homology to EGF and is considered to be a member of the EGF family, it does not appear to interact with the EGFR or related receptors. For example in Chinese hamster ovary cells, it has been demonstrated that CR protein does not bind to the EGF receptor protein (Brandt *et al.*, 1994). In a recent study however CR was found to interact with the intracellular, mitogen activated protein kinase (MAPK) protein and initiate the Shc signal trandsduction pathway (Kannan *et al.*, 1997). It is possible that similar interactions between CR and MAPK proteins may be occurring in the gestational tissues.

This study demonstrates that a greater number of EGF/EGFR related growth factor and receptor mRNA are present in the placenta, chorion-adherent decidua and amnion than was previously thought. Further studies particularly in situ hybridization and immunohistochemical analyses are required to determine the cellular sites of mRNA and protein synthesis respectively. As the growth factor, receptor mRNA were present in tissue collected close to term, whether these growth factors may play a role in parturition can also be questioned. Previous studies have shown that TGFα and EGF increase the prostaglandin E2 synthesis in term amnion cells *in vitro* (Tahara *et al.*, 1994). Prostaglandin E2 is believed to influence myometrial contractions during labour (Bennett *et al.*, 1990) and in the rat uterus EGF has been shown to stimulate contractions of the uterine muscles pre-treated with estradiol (Gardner et al 1987).

At the present time we can only speculate on the autocrine, paracrine and juxtacrine regulatory loops that may be exerted by the EGF/EGFR related growth factors and receptors in the gestational tissue close to term. The effects exerted maybe of a redundant, antagonistic, synergistic or of an exclusive nature. Therefore further investigations are required to unravel the complex interactions and regulatory effects of the EGF/EGFR related growth factors and receptors in human gestational tissue.

In the dispersed trophoblast cultures, CT cells were found to express CR, AR, TGFα, HB-EGF, HRG growth factor and c-erb-B2, c-erb-B4 receptor mRNA. Following differentiation into ST cells however, HB-EGF, HRG, c-erb-B4 mRNA were absent, but the cells continued to express CR, AR, TGFα and low levels of c-erb-B2 mRNA. Although the ST cultures contained a few scattered CT cells, our findings showed that there were differences in the EGF/EGFR related growth factor and receptor mRNA expressed in the CT and ST cells. We found CR mRNA in the undifferentiated CT and the differentiated ST cells. Ciccodicola and co-workers (1989), suggested that CR may be expressed only in undifferentiated cells as CR mRNA transcripts, 2.2kb in size were found only in undifferentiated embryonal teratocarcinoma NTERA clone NT2D1 and were absent in differentiated cells. Our findings suggest that CR may play a role in differentiation of CT in to ST cells and secondly that it may exert regulatory effects in the differentiated ST cells.

Although we detected BTC and c-erb-B3 mRNA in the villous cotyledons, these mRNA's were absent in the cultured CT and ST cells. It is possible that these mRNA's may be expressed by cells other than CT or ST cells in the placental villi *in vivo*.

In this study we found TGFα message in CT, ST cells; however our findings differ from that of Bass and co-workers (1994), who reported that TGFα mRNA was absent in CT isolated from the first and third trimester placenta and ST cells derived from these cultures. These differences may be due to several reasons including the protocol used to isolate the CT cells, culture conditions employed, period of culture and that primary isolates of CT cells which had not been previously passaged were used in our study. Furthermore, we used an internal downstream primer in a secondary PCR as the primary

RT-PCR failed to generate visible cDNA bands. Similar approaches using a nested, internal primers have been successful in detecting EGF, AR and TGFα mRNA expression in human and porcine gestational tissues (Haining *et al.*, 1991, Kennedy *et al.*, 1994).

The expression of AR mRNA in cultured CT, ST cells and HB-EGF mRNA in CT *in vitro* has not been previously reported.

Our findings also revealed that c-erb B2 and c-erb B4 mRNA expression occurs in CT cells whereas after differentiation into ST, the levels of c-erb-B2 declined sharply and that c-erb-B4 mRNA was absent. Although the expression of c-erb-B2 has not been reported in cultured term villous CT cells, very low amounts of c-erb-B2 protein was detected in non invasive CT cells isolated from first trimester after 18-20h in culture by quantitative ELISA (Aboagye-Mathiesen *et al.*, 1997). We also found HRG mRNA expressed in the CT cells. Based on the mRNA expression, it suggests that HRG and the receptors c-erb-B2 and c-erb-B4 proteins may be involved in the differentiation of CT cells in to ST cells.

Immunocytochemical studies would clarify whether endogenous production of growth factors such as AR, TGFα, CR, HB-EGF, HRG and the receptors c-erb-B2 and c-erb-B4 occurs in cultured CT cells and would provide a basis for investigating the autocrine and paracrine regulatory loops that may be exerted in the CT and ST cells.

CHAPTER 4

Localization of Cripto, Heregulin, Heparin Binding Epidermal Growth Factor and Amphiregulin mRNA in Gestational Tissue

In the previous study we found that CR, HRG, HB-EGF and AR mRNA were present in villous cotyledons, amnion and chorio-adherent decidua between 38-40 weeks gestation. The cellular sites of CR, HRG, HB-EGF and AR mRNA synthesis in the gestational tissues were investigated in this study.

4.1 Introduction

Cripto, HRG, HB-EGF and AR are potent growth regulatory peptides that exhibit amino acid and structural homology and belong to the EGF family (Ciccodicola *et al.*, 1989; McKay & Leigh 1993). Previous studies have shown that AR, CR and HRG mRNA are often co-expressed in human tissues. For example AR and CR mRNA are co-expressed in human colorectal tumours (Ciardiello *et al.*, 1991) while AR, HRG and CR mRNA have been isolated from human breast carcinoma cells (Normanno *et al.*, 1995). HB-EGF mRNA is reported to be expressed in macrophages (Higashiyama *et al.*, 1991), vascular smooth muscle cells (Dluz *et al.*, 1993), and the lung, heart, kidney and testis (Vaughan *et al.*, 1992).

The HRG, HB-EGF and AR mRNA's are believed to encode precursor proteins that contain amino acid residues which are associated with intra-cellular trafficking, glycosylation and hydrophobic residues that enables the precursors to form integral membrane bound proteins in addition to the residues that comprise the mature, secreted growth factors (Plowman *et al.*, 1990a; Wen *et al.*, 1992; Fen *et al.*, 1993). In the case of CR mRNA however, nucleotides encoding amino acids associated with intracellular trafficking and membrane spanning hydrophobic regions have not been identified although potential glycosylation sites have been identified (Brandt *et al.*, 1994). AR and HB-EGF mRNA's possess a common feature in that the mRNA's encode very basic amino acid residues that are capable of binding heparin (Plowman *et al.*, 1990a; Fen *et al.*, 1993).

Studies *in vitro* using A431 epidermoid carcinoma cells and vascular smooth muscle cells have shown that HB-EGF interacts with the EGFR found on cell membranes (Higashiyama *et al.*, 1991). AR protein has also been shown to interact with the EGFR present on mammary epithelial cells (Johnson *et al.*, 1993a). HRG has been reported to interact with the three EGFR related receptors c-erb-B2, c-erb-B3 and c-erb-B4 in transfromed cell lines (Holmes *et al.*, 1992; Tzahar *et al.*, 1994). While it has been shown that CR protein does not interact with the EGFR, c-erb-B2, c-erb-B3 or c-erb-B4, the receptor protein that CR may interact with has not been identified (Brandt *et al.*, 1994; Kannan *et al.*, 1997). However Kannan and colleagues (1997), reported that CR

phosphorylates Shc and MAPK proteins in murine mammary epithelial cells which leads to signal transduction (Kannan *et al.*, 1997).

Investigations into the paracrine and autocrine effects of these EGF related growth factors have shown that HB-EGF increases the proliferation of vascular smooth muscle cells and keratinocytes (Higashiyama *et al.*, 1991; Hashimoto *et al.*, 1994). CR was found to enhance proliferation of murine mammary epithelial cells (Ciardiello *et al.*, 1991), while AR and HRG are reported to exert mitogenic effects on human breast cancer cells (Johnson *et al.*, 1993a; Kung *et al.*, 1994). HRG is also reported to decrease the expression of estrogen receptor mRNA and protein in MCF-7 breast cancer cells (Mueller *et al.*, 1995) and intracellular adhesion molecule 1 mRNA in human adenocarcinoma cells (Bacus *et al.*, 1993).

There have been very few reports on the mRNA or protein expression these growth factors in the placenta and fetal membranes. In a previous study however we detected AR, HRG, CR and HB-EGF mRNA transcripts in the villous cotyledons and fetal membranes obtained towards the end of pregnancy using RT-PCR analysis (Chapter 3). Plowman and co-workers (1990a), also reported that relatively high levels of the 1.4kb AR transcripts are present in the human placenta while HRG mRNA has been reported to be absent in the human placenta (Holmes *et al.*, 1992). The cellular sites of AR, HRG, HB-EGF and CR mRNA expression in the placenta and fetal membranes however remains uncertain.

The expression of AR and CR protein in the human placenta has been examined previously. One study showed that AR protein is present in the ST up to the second trimester and that AR enhanced the proliferation of EVT cells isolated from first trimester human placenta (Lysiak *et al.*, 1995). In another study CR protein was immunolocalised to the ST cells of the human placenta collected from each trimester (Watanabe *et al.*, 1997). The effects exerted by CR on ST cells however remains uncertain.

In this study, AR, HB-EGF, CR and HRG mRNA expression in situ was examined in to identify the production sites of these growth factors in the gestational tissues.

The specific aims of the study were to:

- to determine the specificity of the AR, CR, HRG and HB-EGF sense and anti-sense oligonucleotide probes by Northern hybridization analysis and

- to identify the cellular sites of CR, AR, HB-EGF and HRG mRNA synthesis within the placental villi and fetal membranes by in situ hybridization.

4.2 Materials & Methods

Initially tissue samples from the villous cotyledons and fetal membranes were fixed in 4% paraformaldehyde, neutral buffered formalin and Bouins solution for 4, 6, 12 and 24h to determine the most suitable time/fixative combination that provide adequate fixation and retain tissue morphology. Details are given in Chapter 2, section 2.6.1 (n=2, CS, 39, 40 weeks gestation). Thereafter specimens from the villous cotyledons that were fixed for 4-6h and fetal membranes fixed for 4h in 4% paraformaldehyde solution then embedded in paraffin wax were used for in situ hybridization analysis (n=6, CS at 38-40 weeks gestation). Details of the fixation and paraffin embedding are given in Chapter 2, section 2.6.2.

Oligonucleotides, 33-39mer in length, specific for CR, AR, HRG and HB-EGF were used as sense and anti-sense probes for in situ hybridization. The synthesis, purification, quantitation of the sense and anti-sense oligonucleotide probes are given in Chapter 2, section 2.4.1 and their nucleotide sequences in Chapter 2, Table 2.2. The specificity of the oligonucleotide probes was tested prior to in situ hybridization by labelling the sense and anti-sense oligonucleotides with [γ-^{32}P]ATP and carrying out Northern hybridizations with total RNA samples prepared from villous cotyledons, amnion and chorion-adherent decidua (n=5). The preparation of total RNA and Northern blotting are described in Chapter 2, sections 2.3.1, 2.5.1 respectively. The labelling of probes with [γ-^{32}P]ATP and Northern hybridization are outlined in Chapter 2, sections 2.5.2 and 2.5.3 respectively.

The cellular sites of CR, AR, HB-EGF and HRG mRNA expression in the gestational tissue were localized by in situ hybridization. Tissue sections, 4-6μm in thickness were cut from paraffin embedded tissues and probed with [γ-^{32}P]ATP labelled sense and anti-sense probes. All probes were labelled to high specific activities as described in Chapter 2, section 2.6.2. The in situ hybridization procedure is described in detail in Chapter 2, sections 2.6.3-2.6.4.

4.3 Results

4.3.1 Tissue fixation studies

Paraffin embedded tissue sections fixed in Bouins solution for 4 and 6h stained with heamotoxylin and eosin exhibited good tissue morphology but were found to be brittle and difficult to section. The cell walls of the tissues fixed for 12 and 24h Bouins solution appeared to be thickened and tissues appeared to be over fixed.

Sections of gestational tissues fixed in neutral buffered formalin for 4 and 6h contained ruptured cells in the chorionic trophoblast and ST cell layers with the tissues appearing to be under-fixed in some regions. The morphology of fetal membranes and placental villi fixed for 12 and 24h in neutral buffered formalin was well preserved and different cells within the tissues were easily identified.

Heamotoxylin eosin stained tissue sections of fetal membranes and placental villi fixed in 4% paraformaldehyde solution for 4 and 6h were found to have retained morphological features characteristic of fetal membranes and chorionic villi from the third trimester. In tissues specimens fixed for longer periods in 4% paraformaldehyde, the different cell types were clearly visible in the fetal membranes and villi although some parts appeared to be over fixed.

As preliminary studies showed that fetal membranes fixed for 4h and chorionic villi fixed for 4 or 6h in 4% paraformaldehyde exhibited the best morphology, these conditions were used for fixing specimens used for in situ hybridization studies (Fig. 4.1).

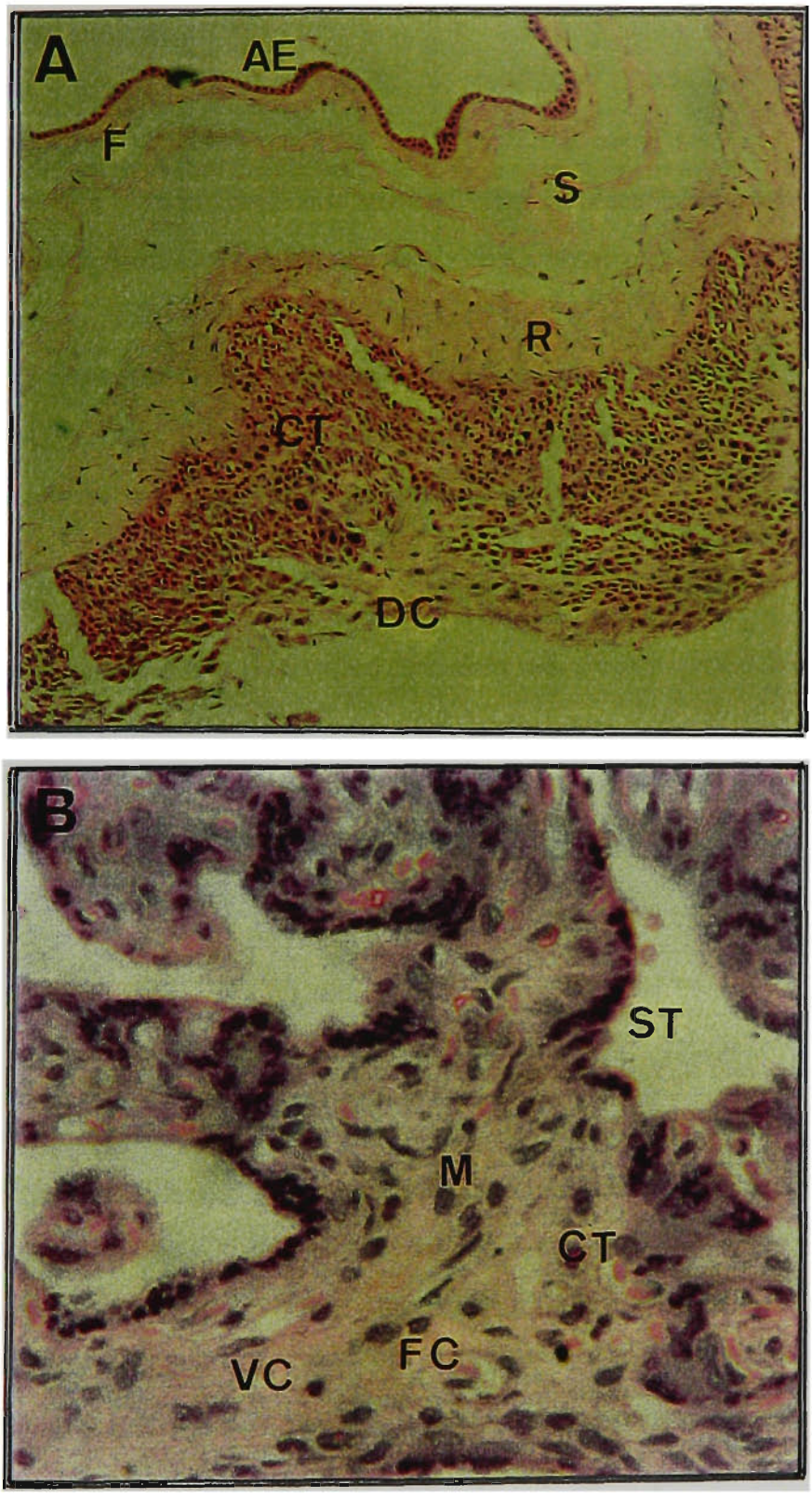


FIG. 4.1 Gestational tissue fixed in 4% paraformaldehyde solution (CS, 39weeks). A) fetal membranes fixed for 4h. B) chorionic villi fixed for 6h. Both sections have been stained with haemotoxylin and eosin. AE=amniotic epithelial cells, CT=chorionic trophoblasts, DC=adherent decidual cells, F=fibroblast layer, S=spongy layer, R=reticular layer, CT=cytotrophoblast cells, ST=syncytiotrophoblast cells, M=macrophages, VC=villous stroma, FC=fetal capillaries. Magnification A=100x and B=250x.

4.2 Northern blot analysis

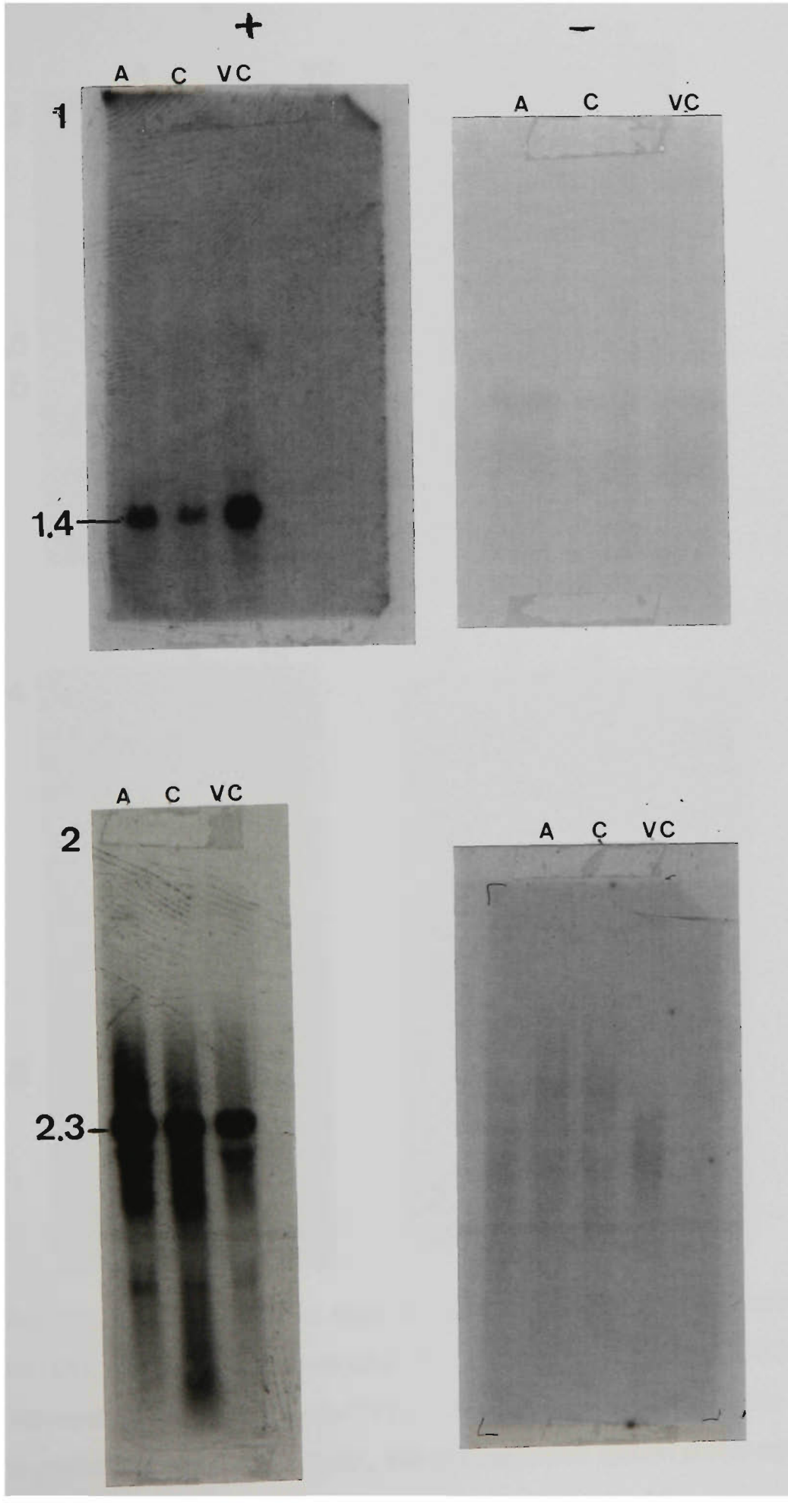
The CR anti-sense probe was found to hybridize to transcripts 2.3kb in size present in the total RNA preparations from the villous cotyledons, amnion and chorion-adherent decidua. Northern blots probed with the labelled HRG anti-sense probe were found to hybridize to transcripts 1.6kb in size in the villous cotyledons, chorion and amnion RNA preparations. The anti-sense AR probe hybridized to transcripts 1.4kb in size in the total RNA preparations from the villous cotyledons, amnion and chorion-adherent decidua. Two mRNA species 2.5 and 1.4kb present in the amnion and villous cotyledons and 2.5kb transcripts present in the chorion total RNA preparations were found to hybridize with the anti-sense HB-EGF probe (Fig. 4.2).

Hybridization products were not detected on autoradiographs when Northern blots containing total RNA preparations from each of the gestational tissues were hybridized with labelled CR, AR, HB-EGF and HRG sense probes (Fig. 4.2). If less stringent washing conditions were used, the sense HRG and CR probes were found to hybridize weakly with the 28S rRNA bands.

4.3 3 In situ hybridization analysis

The [γ-^{32}P]ATP labelled AR, HB-EGF, CR and HRG anti-sense probes were found to hybridize to the amniotic epithelial cells. In all cases the surface of the epithelial cells facing the amniotic cavity showed a more intense hybridization compared with the surface facing the basement membrane. Non-specific hybridizations in the fibroblast and spongy layers of the amnion were observed and intensity found to vary between specimens when a constant amount of CR, HRG, HB-EGF and AR anti-sense probes (ie. 12.5ng/slide) were used in the hybridization.

In the chorion intense reactions were observed in the decidual cells adhering onto the chorion and the chorionic trophoblast cells with the CR and HRG anti-sense probes. In addition strong hybridization was also detected in the reticular layer of the chorion.


+
−
A C VC
1
1.4
A C VC
A C VC
2
2.3
A C VC

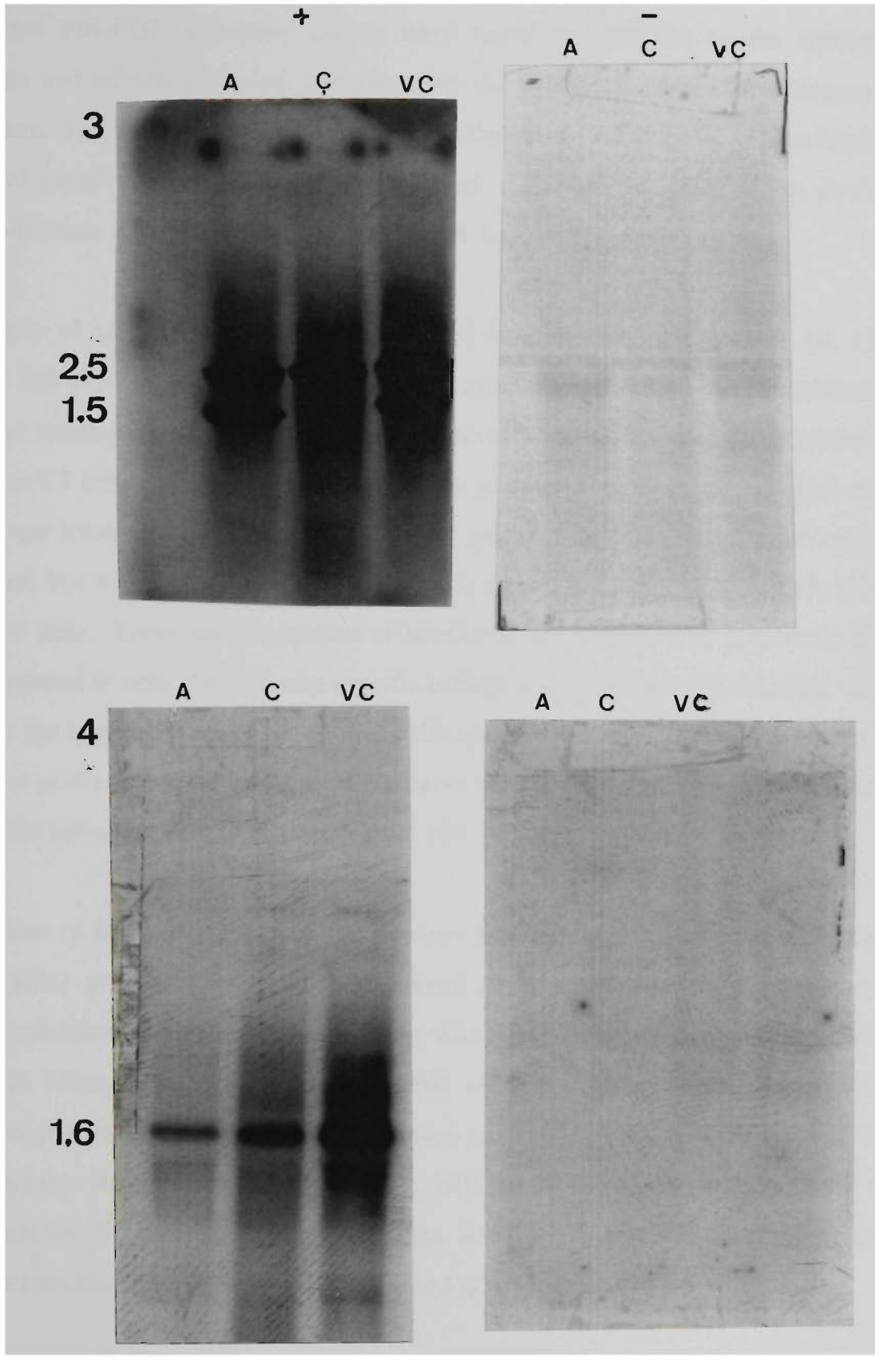
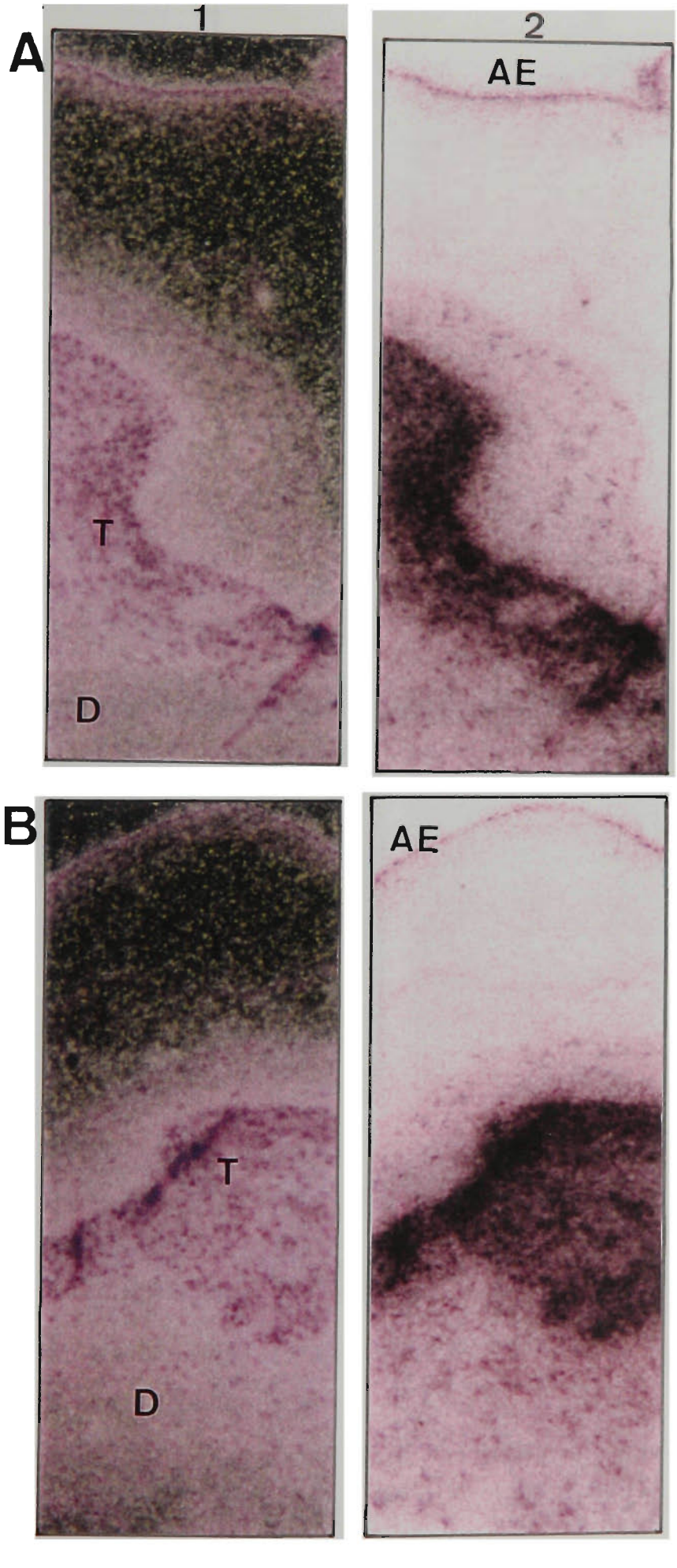


FIG. 4.2 Northern blot analysis. Northern blots containing 20μg total RNA per lane from the amnion (A), chorion-adherent decidua (C) and villous cotyledons (VC) hybridised with anti-sense (+) and sense (-) [γ-^{32}P]ATP labelled oligonucleotide probes. The hybridization products detected with AR, CR, HB-EGF and HRG specific probes are shown in panels 1-4 respectively. Sizes of the hybrid molecules are given in kb.

The AR and HB-EGF anti-sense probes were found to hybridize to the chorionic trophoblasts and adherent decidual cells, however the hybridization was more intense in the chorionic trophoblasts compared with the adherent decidual cells. Hybridization reactions of variable intensities were also observed in the reticular layer of the chorion with the anti-sense AR and HB-EGF probes (Fig. 4.3).

The ST layer of cells of the placental villi reacted intensely with the labelled AR, CR, HRG and HB-EGF anti-sense probes. In all cases however there was considerable background staining in the villous stroma that prevented localisation to other scattered cells such as CT cells and macrophages. When the amount of labelled probe applied onto sections were lowered from 12.5ng to 10ng per section, the background hybridization was lessened but was present in sufficient amounts to prevent localisation to cells other than the ST cells. Lowering the amount of labelled probe applied onto sections to 5ng per slide resulted in only uniform, non-specific background staining being detected. Fig. 4.4 depicts the hybridizations observed using CR specific anti-sense probe with 12.5 and 5ng labelled probe per slide. Similar results were obtained with the AR, HB-EGF and HRG specific anti-sense probes in the placental villi.

Serial sections of the fetal membranes and villous cotyledons probed with cold anti-sense AR, CR, HRG or HB-EGF probes that served as negative controls did not show detectable hybridization signals under bright or dark field. Sections of gestational tissue treated with RNase1 then probed with labelled anti-sense probes showed some non specific background reactions in the villous stroma and compact and spongy layers of the fetal membranes. When labelled sense CR, AR , HRG and HB-EGF probes that served as negative controls were used in the hybridization, sections of the villous cotyledons and fetal membranes showed minimal and uniform background staining.


A
1
T
D
2
AE
B
T
D
AE

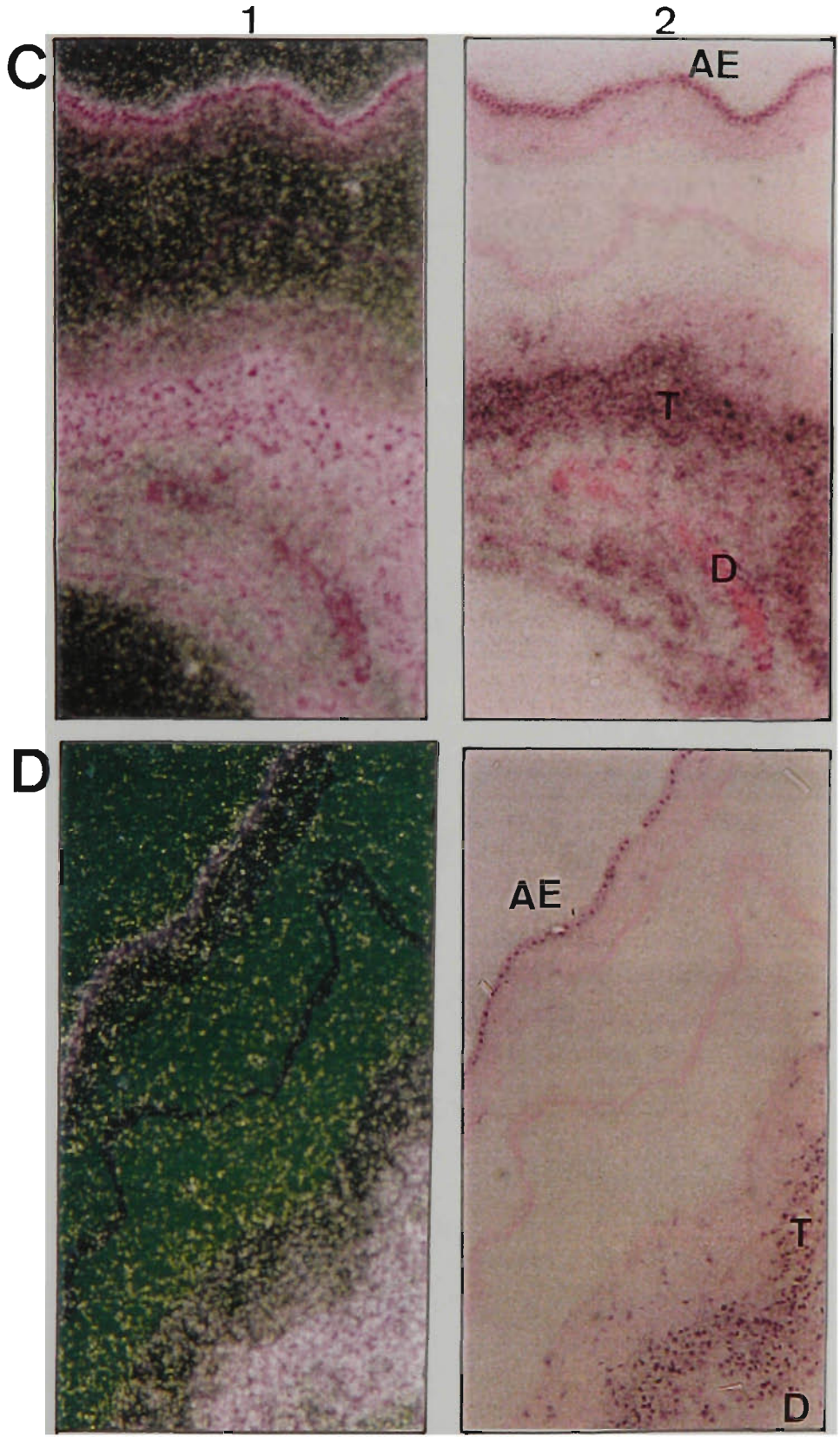


FIG. 4.3 In situ hybridization of paraffin embedded sections of the fetal membranes delivered after 40 weeks gestation probed with labelled anti-sense oligonucleotide probes specific for A) CR B) HRG C) AR and D) HB-EGF. Panel 1 depicts sections photographed under dark field microscopy and panel 2 under bright field. AE=amniotic epithelium, T=chorionic trophoblast and D=adherent decidua. Magnification=100x.

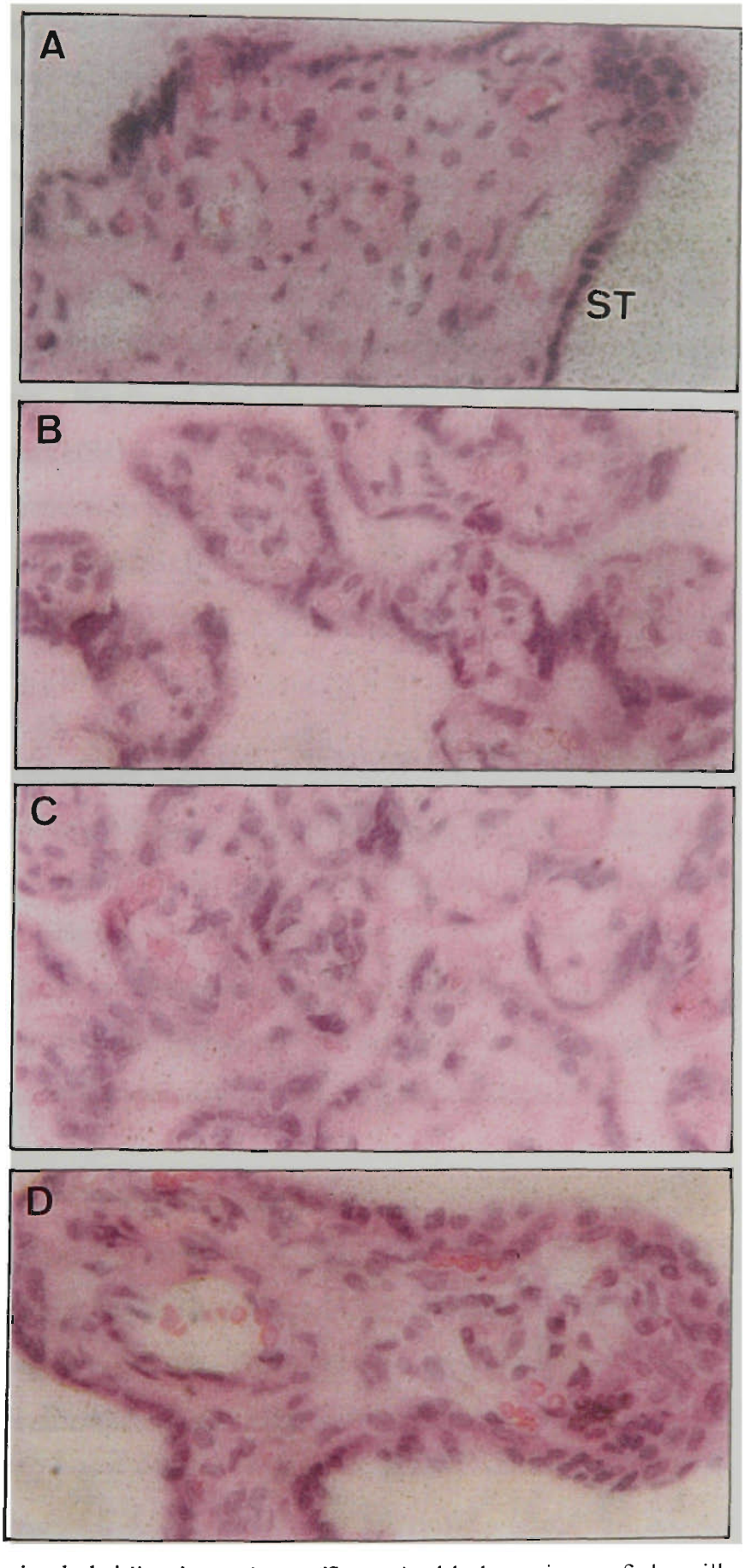


FIG. 4.4 In situ hybridization of paraffin embedded sections of the villous cotyledons (CS, 39 weeks) probed with labelled anti-sense oligonucleotide probe specific for CR. The amount of labelled probe used in A, B and C were 12.5, 10 and 5ng/slide respectively. D) section probed with sense probe specific for CR. A-D photographed under bright field. ST=syncytiotrophoblast, magnification=250x.

4.4 Discussion

In the present study we investigated the cellular sites CR, AR, HB-EGF and HRG mRNA synthesis in placental villi and fetal membranes delivered close to term by in situ hybridization. We used anti-sense oligonucleotides that were complimentary to these growth factor mRNA and sense oligonucleotides as negative control probes for in situ hybridization. The positive and negative control probes have not been used previously for in situ analysis therefore their specificity was determined by Northern hybridization prior to in situ analysis. We found that the anti-sense AR probe hybridized to 1.4kb sized transcripts, CR and HRG anti-sense probes to transcripts 2.3kb and 1.6kb respectively in total RNA preparations from the villous cotyledons and fetal membranes. The anti-sense HB-EGF probe was found to hybridize with 2.5 and 1.5kb sized transcripts in total RNA samples from amnion and villous cotyledons and only to 2.5kb sized transcripts in the chorion samples.

Our findings are consistent with that of Plowman and co-workers (1990a), who reported that high levels of the 1.4kb AR mRNA transcripts are present in the human placenta. Their report however did not indicate the gestational age of the placental samples nor was it extended to include the fetal membranes. AR mRNA transcripts of similar size have also been found in other tissues such as ovary, testis, breast and colon (Plowman *et al.*, 1990a), indicating that AR transcripts are spliced in a similar manner in the different tissues and that a single gene copy is present in the human genome.

In a previous study Ciccodicola and co-workers (1989), reported that CR was not expressed in the placenta and this findings differs from our results. CR mRNA transcripts that are similar in size to the ones we detected are reported to be present in the human epidermoid carcinoma cell line NTERA cells (ie. 2.1kb, Brandt *et al.*, 1994). Johnson and colleagues (1994), found 2.1kb CR mRNA in whole murine embryos at 6-7 days gestation and transcripts of 2.1 and 3.5kb in embryos at mid and late gestation. Further they found that in the adult murine liver, heart, brain and kidneys expressed only the 3.5kb transcripts (Johnson *et al.*, 1994). These investigators suggested that the differences in temporal expression were due to differential splicing in embryonic, fetal and adult tissues or due to the activation of a closely related gene. In support of this view Southern blot analyses

have shown that a number of CR related sequences are present in human and mouse genomes but whether the genes are transcribed remains uncertain (Dono *et al.*, 1993).

In another study Holmes and colleagues (1992), reported that HRG mRNA was not expressed in the human placenta but the report did not indicate the gestational age of the placental samples. HRG mRNA transcripts 6.6, 2.5 and 1.8kb in size have been found in the human uterus, 2.2kb in the brain and a 1.5kb transcript that is similar in size to the one we detected in the testes (Holmes *et al.*, 1992). The size heterogeneity of HRG transcripts have been attributed to the different HRG isomers α, β1, β2, β3 and β4 that probably result from differential splicing rather than transcription of other closely related genes (Holmes *et al.*, 1992).

HB-EGF mRNA transcripts of 2.5 and 1.5kb have been found in the human lymphoma cell line U-937 (Higshiyama *et al.*, 1991) while only the 2.5kb mRNA transcript has been reported from murine uterine epithelial and stromal cells (Zhang *et al.*, 1994a). The two transcripts are probably a result of differential splicing since there appears to be only one HB-EGF gene in human genome (Fen *et al.*, 1993).

As our preliminary Northern hybridization experiments showed that the anti-sense oligonucleotides hybridized to transcripts that were similar in size to those described previously for growth factors AR, CR, HRG and HB-EGF they were used as probes for in situ analysis.

In situ hybridization studies showed that AR, CR, HRG and HB-EGF mRNA's are co-expressed in the amniotic epithelial, chorionic trophoblast and decidual cells although considerable sample to sample variation was noted in the intensity of the hybridization in these cells. There have been no previous reports of the mRNA expression in situ of any of these growth factors in the human fetal membranes. Studies in mice however have shown that CR mRNA is expressed in the extraembryonic ectoderm, mesoderm and ectoplacental cone at 7-8 days post conception during embryonic development (Johnson *et al.*, 1994). In their study Johnson and co-workers (1994), found that CR mRNA was transiently expressed and was not absent after day 8 in the fetal membranes. Studies have

shown that in mice CR may play a significant role in embryonic cardiac morphogenesis as the mRNA has been localized to the mesoderm tissue of the heart, truncus arteriosus, (Dono *et al.*, 1993), epi and myocardium (Johnson *et al.*, 1994).

HB-EGF and AR mRNA are known to be expressed during embryonic development in the uterine tissues. HB-EGF mRNA and protein have been localized to the glandular epithelium, stroma and decidual cells lining the gravid murine uterus (Das *et al.*, 1994). AR mRNA was found to accumulate rapidly in the luminal epithelial cells just prior to the attachment of the murine blastocyst on day four post conception (Das *et al.*, 1995). Hence it is believed that AR and HB-EGF may play roles in the hatching of the blastocyst and its embedding in the uterus. It has also been shown that AR and HB-EGF mRNA expression is stimulated by progesterone in the gravid uterine tissues (Das *et al.*, 1994; Das *et al.*, 1995).

In the placental villi our findings showed that AR, CR, HB-EGF and HRG mRNA appeared to be expressed in the ST layer. High background levels were present in the villous stroma in sections of the placental villi and this hindered localization of the mRNA to other cell types. As routine haemotoxylin-eosin staining showed that the placental villi were adequately fixed and had retained its morphology, inadequate fixation of tissues did not appear to be cause for high levels of background staining. Therefore the labelled probe concentrations were reduced and post hybridization washes lengthened in an effort to reduce the background and non-specific hybridization. However these attempts were not successful. Use of other radioactive isotopes such as ^{3}H and ^{35}S that are known to give better cellular resolution for labelling probes or use of non radioactive labels such as dioxygenin instead of [γ-^{32}P] may therefore be beneficial (Woods & Ellis 1994).

To our knowledge there have been no previous reports of the localization of these mRNA in the placenta at any gestational age. In a recent report by Watanabe and co-workers (1997), however immunoreactive CR protein was localized to the syncytium of placental villi from the third trimester and this data supports our finding CR mRNA in the ST cells of the placental villi. Further they reported that CR immunoreactivity increased with

gestational age and found immunostaining to be highest in term tissues. There have been no reports however of the effects exerted by CR in the placenta. The receptor protein that binds with the CR protein has not been identified with certainty, but in a recent report CR protein was shown to phosphorylate substrates in the ras and raf induced MAPK pathway in mouse mammary epithelial cells (Kannan *et al.*, 1997).

Although we localized HRG mRNA to the ST cells, there have been no previous reports of protein expression in the placenta. The presence of HRG protein in the gestational tissue may be quite significant as it is a known ligand of the c-erb-B2 and c-erb-B3 receptor proteins ((Holmes *et al.*, 1992; Tzahar *et al.*, 1994). C-erb-B2 protein is known to be expressed in the human placenta and has been localized to the ST cells in the third trimester (Mulhauser *et al.*, 1993). C-erb-B3 mRNA has been detected in human placenta (Kraus *et al.*, 1989), and is likely that the receptor protein is also present It is therefore possible that the c-erb-B2 and c-erb-B3 receptor proteins in the placenta may be involved in regulatory activities following the interaction of HRG. Although very few studies have been carried out on the regulatory activities of HRG it has been shown that in breast cancer cells HRG regulates the expression of the estrogen receptor mRNA and protein (Mueller *et al.*, 1995). It is possible that similar effects may be occurring in the placenta.

We found AR and HB-EGF mRNA being co-expressed in the ST cells and chorionic trophoblasts and amniotic epithelium. There have been no reports of HB-EGF and AR protein expression in term placenta or fetal membranes but their common receptor protein the EGFR is found on CT, ST decidual, chorionic trophoblasts and amnion epithelial cells (Ladines-Llave *et al.*, 1991; Rao *et al.*, 1984). If AR and HB-EGF protein expression does occur paracrine and autocrine interactions could occur within the gestational tissues. It is also interesting to note that interaction of AR and HB-EGF with the EGFR is probably modulated by their ability to interact with heparin sulphate proteoglycans that are found on cell surfaces and in this respect differ from other members of the EGF family (Johnson & Wong 1994; Higashiyama *et al.*, 1993).

In summary localization of AR, CR, HRG and HB-EGF mRNA to cells in the gestational tissues implies that these growth factors are involved in growth and functional regulation

in the gestational tissues. Further studies particularly those involving immuno-histochemical techniques are required to determine their cellular sites of synthesis and potential action.

CHAPTER 5

Transforming Growth Factor Alpha, Amphiregulin Protein Expression in Gestational Tissue & Cytotrophoblast Cells *In Vitro*

In a previous study we found that TGFα and AR mRNA were present in the villous cotyledons, amnion and chorion-adherent decidual tissues collected from placenta delivered close to term. CT cells isolated from the same placenta and cultured for 12 and 96h were also found to express the mRNA of these growth factors (Chapters 3 & 4). The presence of immunoreactive TGFα and AR proteins in each of these tissues and their cellular sites of synthesis are examined in this study. The expression and secretion of AR, TGFα by CT and ST cells ***in vitro*** *was also investigated.*

5.1 Introduction

Amphiregulin and TGFα are potent growth regulatory peptides that share derivatory, structural and functional homologies. Previous studies have shown that AR and TGFα are initially synthesised as large glycosylated transmembrane precursors, composed of 252 and 160 amino acid residues respectively and that the smaller, mature growth factors are released from the precursors as a result of proteolytic cleavage (Derynck *et al.*, 1984; Plowman *et al.*, 1990a). Mature AR peptides are monomers composed of 78 or 84 residues that vary between 17-22kDa in size (Shoyab *et al.*, 1988 & 1989), while mature TGFα is a 50 residue monomer of 6kDa (Marquardt *et al.*, 1984). Frequently however multiple TGFα species between 15-22kDa known as meso-TGFα are encountered and they are thought to arise as a result of incomplete precursor processing (Bringman *et al.*, 1987; Texido *et al.*, 1987). Mature AR and meso-TGFα are also heterogeneously glycosylated proteins (Texido & Massague 1988; Shoyab *et al.*, 1988). The mature AR, TGFα peptides share 32% amino acid sequence homology and all secretory isoforms possess the six cysteine motif $X_nCX_7CX_{4\text{-}5}CX_{10}CXCX_8CX_n$ that is characteristic of members of the EGF family (Fisher & Lakshmanan 1990). A major difference between the two mature peptides is that AR possesses a 43 residue amino terminal extension which has two putative nuclear localisation signals and is composed primarily of very basic residues that enables it to bind heparin (Cook *et al.*, 1991; Plowman *et al.*, 1990a).

Both AR and TGFα interact with the 170 kDa EGF receptor resulting in intrinsic tyrosine phosphorylation and receptor dimerisation that precede signal transduction (Reynolds *et al.*, 1981; Johnson *et al.*, 1993a). The two growth factors have been found to exert mitogenic effects on a variety of normal and transformed epithelial cells, fibroblasts and keratinocytes (Coffey *et al.*, 1987, Shoyab *et al.*, 1989, Plowman *et al.*, 1990a; Johnson *et al.*, 1991, Stromberg *et al.*, 1992).

TGFα is also believed to be an important mediator of feto-placental growth. Immunolocalisation studies have revealed that at 4-6 weeks post conception it is present in the CT, ST and IT cells, hence it has been suggested that it may play a role in implantation (Hoffmann *et al.*, 1993). There are conflicting reports however on its localisation in the placenta following development of the chorionic villi. One study

reported that TGFα was found in the CT and ST only during the first two trimesters (Horowitz *et al.*, 1993) whereas Lysiak and co-workers (1993), detected immunoreactive TGFα in the CT, EVT, ST cells from each of the trimesters. *In vitro* studies have shown that it acts as a mitogen on EVT cells from the first trimester (Lysiak *et al.*, 1993). It also acts as a mitogen on CT cells isolated from the first and second trimesters and is equipotent to EGF in inducing their proliferation (Filla *et al.*, 1993). Scattered amniocytes, chorionic plate trophoblasts and decidual cells from each trimester have also been reported to express TGFα protein (Lysiak *et al.*, 1993).

Lysiak and co-workers (1995), also investigated the expression of AR protein in the human placenta and found that it was present in the ST cells of the chorionic villi up to 18 weeks of gestation but did not observe immunostaining in placental tissue obtained between 23 weeks and term. They also found that the proliferation of EVT cells isolated from first trimester placenta increased in a dose dependant manner up to 100ng/ml of AR protein. Further they reported that AR was absent in the decidua between 11 weeks and term. Previous studies have shown however that AR mRNA is present in both human and sheep placentae (Plowman *et al.*, 1990a; Forsyth *et al.*, 1997) and our recent findings also showed that AR mRNA is present in the villous cotyledons close to term. Further, we found that AR mRNA is present in the chorion-adherent decidua and amnion tissues collected towards the end of gestation. Taken together these findings suggests that AR protein may be more widely distributed in the gestational tissues close to term than previously thought and warrants further investigation.

During gestation TGFα produced in the maternal endometrial and/or decidual cells may exert paracrine regulation on adjacent ST and CT cells of the placental villi (Horowitz *et al.*, 1993). It is probable that AR protein is also synthesised in endometrial cells as AR mRNA has been found in the gravid murine and porcine endometrium (Das *et al.*, 1994; Kennedy *et al.*, 1994) and if this were the case, AR may exert paracrine regulation on the adjacent ST and CT cells. On the other hand autocrine and juxtacrine regulation by membrane bound TGFα, AR precursors may be occurring within and between the ST and CT cells of the villi. Placental CT cells cultured *in vitro* in the absence of secretory factors from maternal sources provides a means of investigating the synthesis and

secretion of these ligands enabling the examination of the possible regulatory loops in these cells. Very few studies have examined the synthesis and secretory profile of TGFα and AR proteins in CT cells. Filla and co-workers (1993), found that CT cells isolated from first and, second trimesters synthesised and secreted TGFα, however their study was not extended to include the differentiated ST cells. There have been no previous reports of AR protein expression by CT or ST cells *in vitro*. Both of these cell types are however known to have functional EGF receptors *in vitro* (Lai & Guyda 1984).

Because of the limited data available presently on the synthesis, localisation of AR, TGFα proteins in human gestational tissue and their possible regulatory loops in CT and ST cells *in vitro* this study was undertaken with the specific aims of:

- determining whether immunoreactive TGFα and AR proteins are present in the villous cotyledons, amnion, chorion-adherent decidua tissues collected from placenta close to term;
- characterising the immunoreactive AR, TGFα proteins by examining their extent of N-linked glycosylation;
- determining the cellular sites of TGFα, AR expression in the villous cotyledons, chorion-adherent decidua and amnion; and
- determining whether TGFα, AR proteins are expressed and secreted by CT cells isolated from placenta delivered close to term and cultured for 12 and 96h.

5.2 Materials & methods

CT cells were isolated and characterised as described in Chapter 2, sections 2.2.1 & 2.2.3. CT cells were seeded at a density of 0.75×10^6 cells/ml in EMEM with 10% FCS and cultured as described in Chapter 2, section 2.2.4-2.2.5 for 12, 96h. Cultured cells were dispersed and used for Western hybridization studies. CT cells (50µl≡25,000 cells in EMEM with 10% FCS) were also placed on coverslips and cultured for 12, 96h. These cells were used for immunocytochemical analyses.

Immunoreactive AR and TGFα proteins present in lysates prepared from gestational tissue and cultured cells were detected by Western hybridization. Tissue proteins (n=9; CS=5, SD=4, 38-40 weeks), cell proteins in lysates prepared from CT cells cultured for 12, 96h and media conditioned by CT cells grown for 12, 96h (n=5, CS, 38-40 weeks) were resolved by SDS-PAGE. The proteins present in the conditioned media were also resolved by Tris-tricine PAGE. Proteins were transferred onto nitrocellulose membranes and probed with AR antiserum (2μg/ml) and TGFα antiserum (5μg/ml). Details of SDS-PAGE, Tris-tricine PAGE and Western hybridization are given in Chapter 2, section 2.7. The relative levels of AR and TGFα protein present per μg of total protein was analysed by laser densitometry performed on immunoblots probed with AR and TGFα antisera (Chapter 2, section 2.7.7).

Cellular sites of AR and TGFα protein expression were investigated by immunohistochemistry. Villous cotyledons and fetal membranes (n=7; CS, 38-40 weeks) were fixed in 4% paraformaldehyde. Sections between 4-6μm in thickness were probed with AR and TGFα antisera diluted to 1 and 3μg/ml respectively. Immunohistochemistry was performed by the labelled avidin biotin strepavidin amplification (LABSA) method and immunostaining detected with diamminobenzidine chromogen. Details of the immunohistochemistry procedures are given in Chapter 2, section 2.8.

Immunocytochemistry was performed on CT cells cultured on poly-L-Lysine coated coverslips for 12, 96h by the LABSA method (Chapter 2, sections 2.8.2-2.8.3). The concentration of AR and TGFα antisera used were the same as described for immunohistochemistry.

5.3 Results

53.1 TGFα protein expression in gestational tissue

Western blot analysis of proteins resolved by SDS-PAGE revealed the presence of a single protein in the amnion, chorion-adherent decidua and villous cotyledon tissue lysates

that reacted very strongly with the mouse monoclonal anti-human TGFα antibody. The relative molecular mass of the immunoreactive species in each of the tissue lysates was estimated to be 32kDa. The immunoblots contained resolved proteins that varied between 15 and 120kDa however the anti-TGFα antibody did not react even weakly with any protein other than the 32kDa species (Figs. 5.1-I & 5.2-I). The immunoreactive protein was present in all tissue samples analysed (n=9; CS=5, SD=4).

Immunoblot analysis of TGFα after hydrolysis with N-glycosidase F for 18h to remove N-linked carbohydrate moieties showed that the size of the immunoreactive species in each of the gestational tissues had decreased from 32kDA to 25kDa. In the untreated control samples the antibody bound only to the 32kDa protein. The relative molecular mass of the protein alkaline phosphatase that was used as a positive control to monitor hydrolysis by N-glycosidase F hydrolysis was found to be 83kDa in untreated samples and 58kDa in treated samples (Figs. 5.1-II- & 5.2-II).

5.3.2 AR protein expression in gestational tissue

Immunoblot analysis of protein lysates from the villous cotyledons, amnion and chorion-adherent decidua resolved by SDS-PAGE and probed with the polyclonal anti-AR antibody revealed the presence of a single immunoreactive species in some of the tissue samples that tested positive for TGFα. The relative molecular mass of the immunoreactive AR species was estimated to be 52kDa. The number of samples that were found to co-express this protein and the 32kDa TGFα species are given in Table 5.1. The AR antibody did not show any cross reactivity with the 6kDa synthetic TGFα peptide that served as a negative control (Fig. 5.3-I).

Following hydrolysis of tissue proteins with N-glycosidase F, immunoblot analysis revealed that the relative molecular mass of the reactive isoform had decreased from 52kDa to 40kDa whereas in the untreated control samples the antibody reacted with the 52kDa species (Fig. 5.3-II).

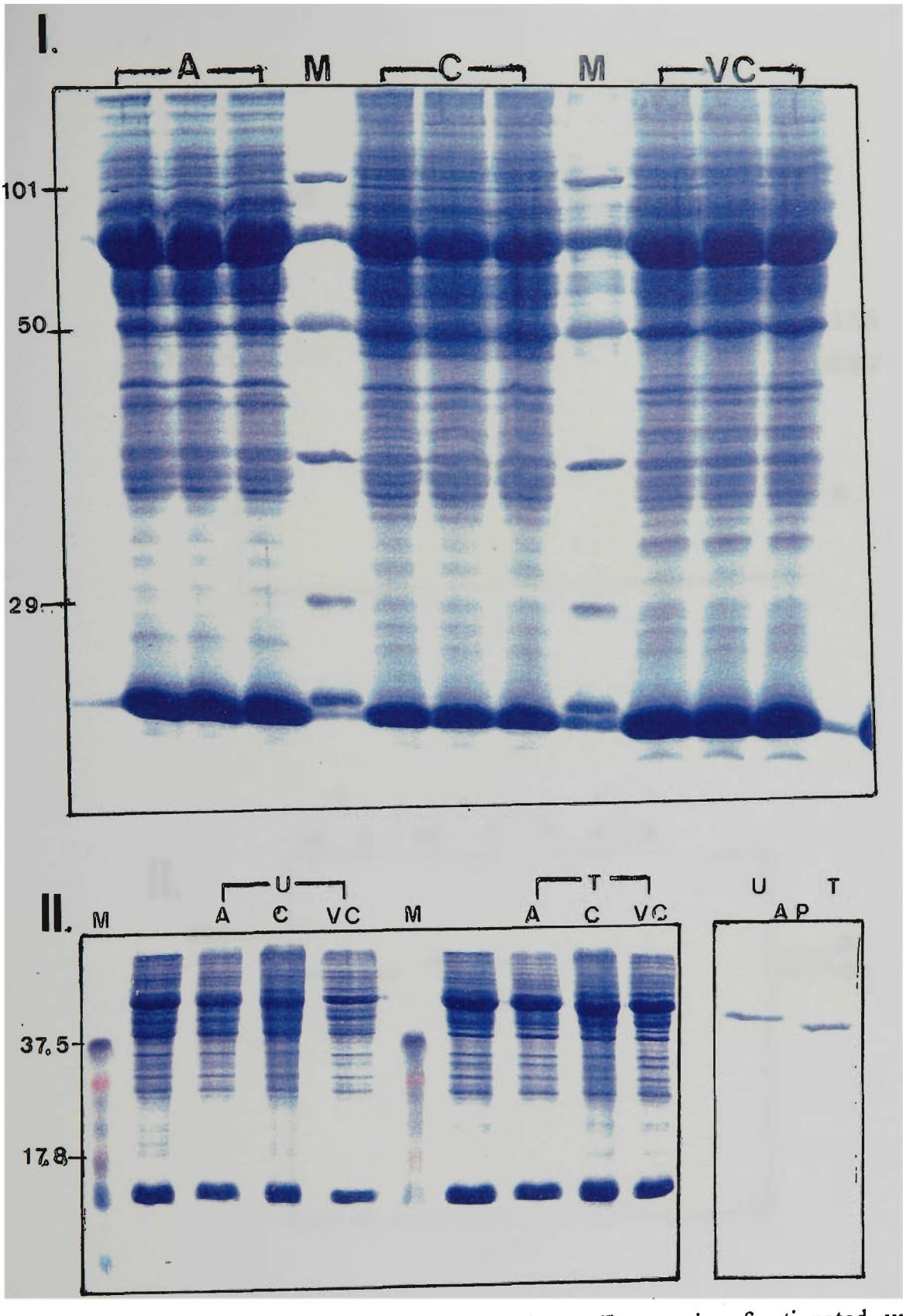


FIG. 5.1 Electrophoretic separation of proteins. I) proteins fractionated under denaturing conditions by SDS-PAGE on a gel containing 12% polyacrylamide. II) proteins hydrolysed with N-glycosidase F. T=treated samples, U=untreated controls and alkaline phosphatase (AP) that was used to monitor hydrolysis by N-glycosidase-F resolved on a 10% polyacrylamide gel by SDS-PAGE. Thirty, ten μg of total protein were loaded / lane in I and II respectively and stained with Coomassie blue after electrophoresis. A=amnion, C=chorion-adherent decidua, VC=villous cotyledons and M=protein molecular weight standard. Sizes are given in kDa. Similar gels were immunoblotted and probed with antibodies directed against TGFα or AR.

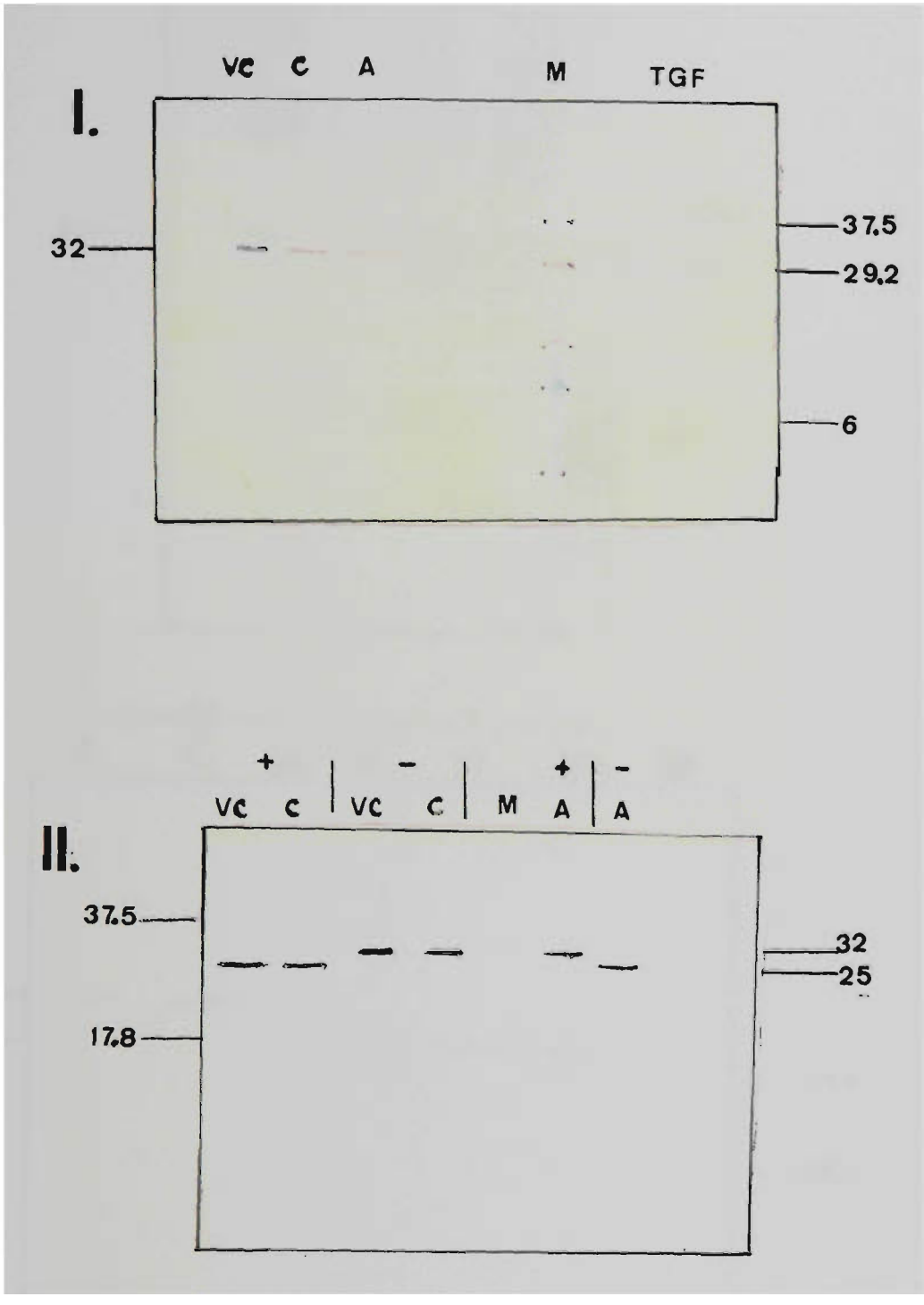


FIG. 5.2 Immunoblots probed with the anti-human TGFα monoclonal antibody. Immunoblots contain proteins fractionated by SDS-PAGE (I) and proteins hydrolysed with N-glycosidase F (II). Blots contain 10μg of sample protein per lane. A=amnion, C=chorion-adherent decidua, VC=villous cotyledons, TGF=recombinant TGFα peptide that served as the positive antigen control, T=treated with N-glycosidase F, U=untreated controls and M=protein molecular weight standard. Sizes of the immunoreactive proteins and standards are given in kDa.

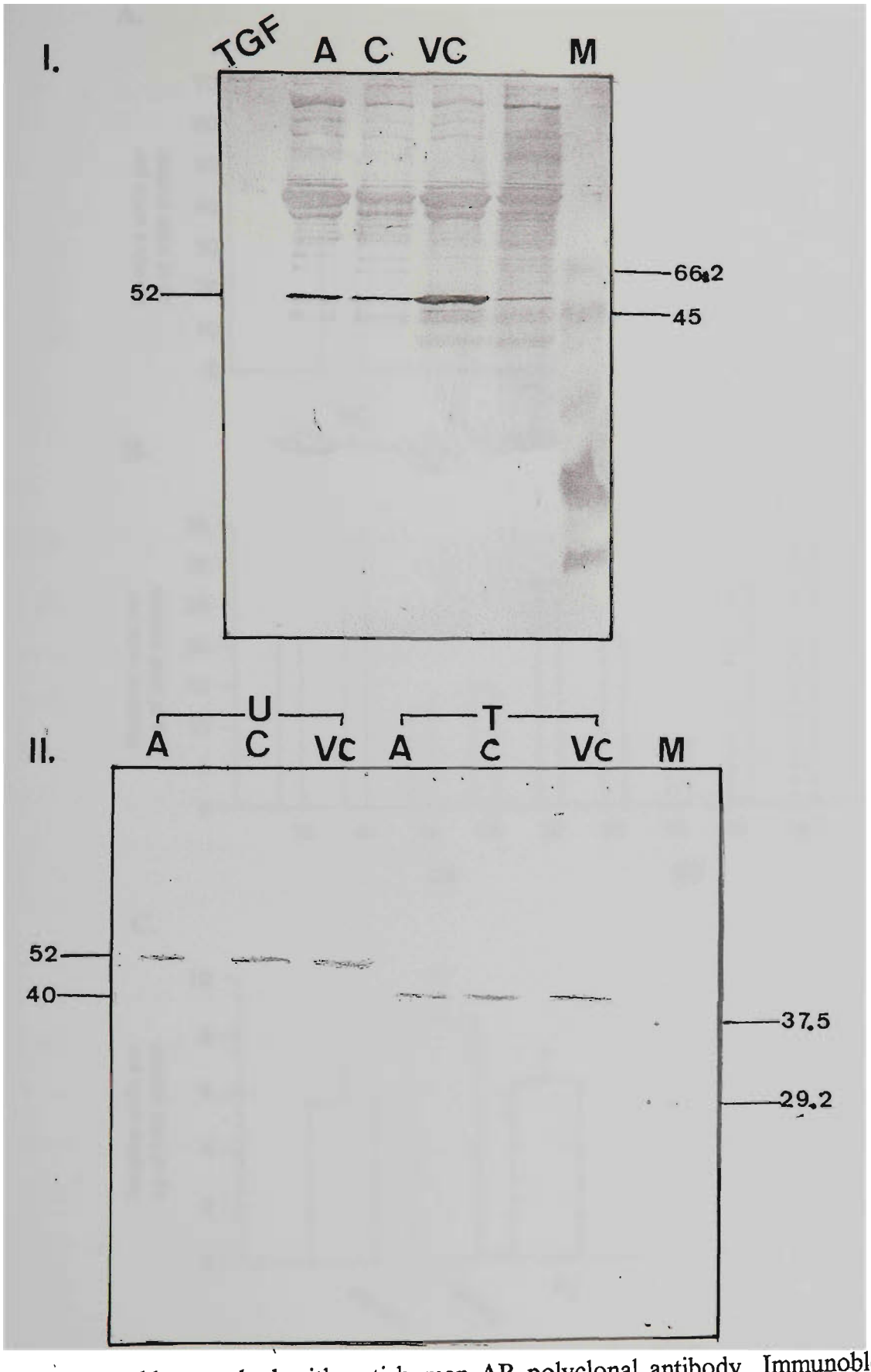


FIG. 5.3 Immunoblots probed with anti-human AR polyclonal antibody Immunoblots contain proteins fractionated by SDS-PAGE (I) and SDS-PAGE after hydrolysis with N-glycosidase F (II). Blots contain 50μg of sample protein per lane. A=amnion, C=chorion-adherent decidua, VC=villous cotyledons, T=treated with N-glycosidase F, U=untreated controls, TGF=6kDa TGFα synthetic peptide that served as a negative control and M=protein molecular weight standard. Sizes of the immunoreactive proteins and standards are given in kDa.

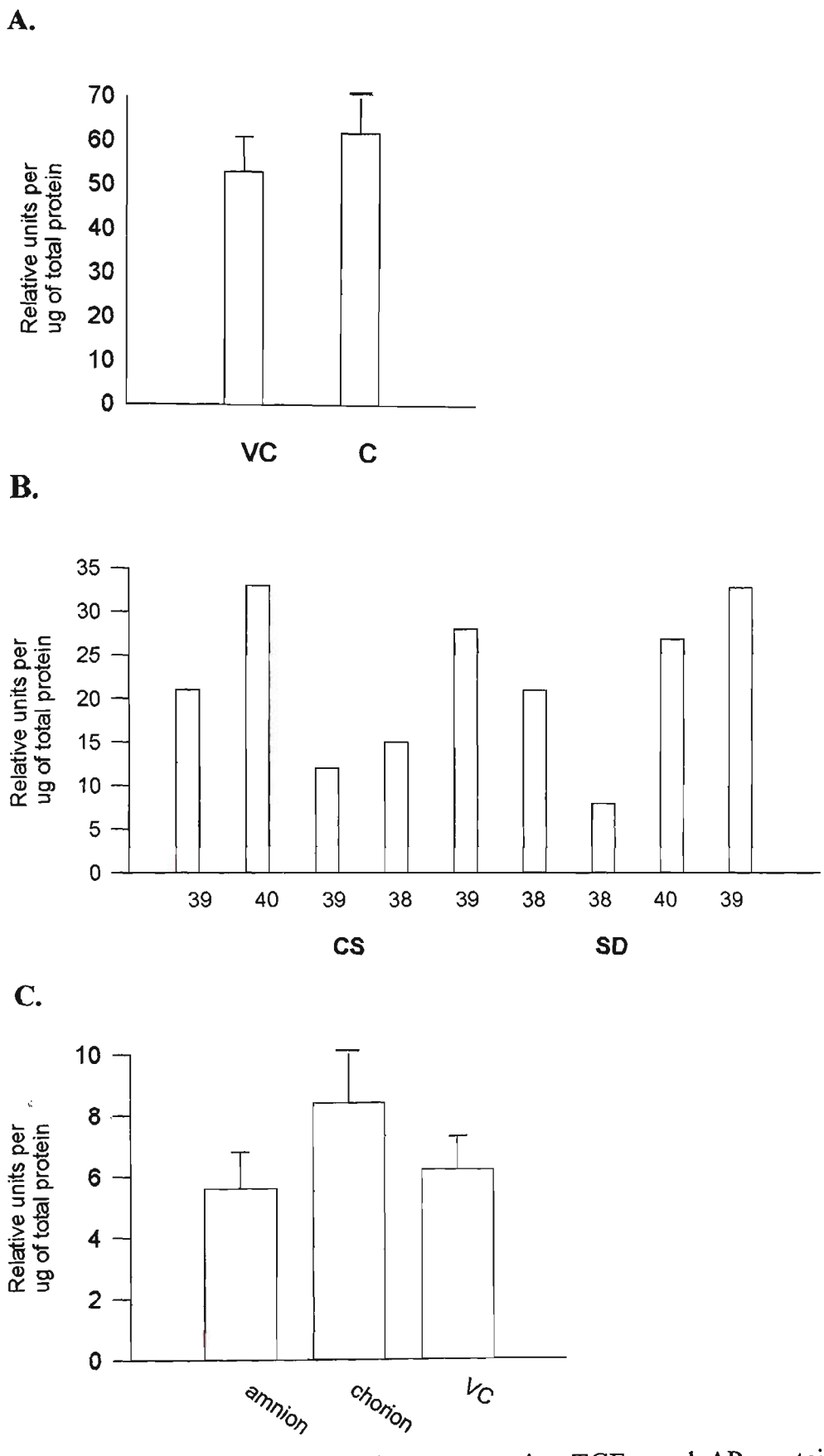


FIG. 5.4 Laser densitometric analysis of immunoreactive TGFα and AR proteins. A) mean, ±SEM (n=9; CS=5, SD=4) of 32kDa TGFα species in lysates prepared from chorion-adherent decidua (C) and villous cotyledons (VC). B) denstometric readings of the 32kDa TGFα isoform from 9 amnion samples delivered by CS or SD. The gestational ages are in weeks. C) mean, ±SEM of laser densitometry performed on the 52kDa protein that reacted with the anti-AR antibody, amnion n=6, chorion-adherent decidua n=7, villous cotyledons n=5.

TABLE 5.1 Number of samples showing positive immunoreactivity to anti-AR and anti-TGFα antibodies by immunoblot analysis of lysates resolved by SDS-PAGE

tissue	CS (n=5)	SD (n=4)
amnion	3	3
chorion-adherent decidua	3	4
villous cotyledons	3	2

5.3.3 Relative levels of TGFα and AR protein expression in gestational tissue

Quantitation of the 32kDa protein by laser showed that the amount of TGFα per μg of total protein in the chorion-adherent decidua and villous cotyledons were fairly constant with minor, inter subject variability (Fig.5.4A). The amount of TGFα protein in the amnion however was found to vary considerably with over a 6 fold difference between samples from the different subjects (Fig. 5.4B). Among the 3 tissues, the chorion-adherent decidual samples consistently had the highest amounts of immunoreactive TGFα per μg of total protein as shown by the desitometric readings.

Immunoreactive AR protein was present only is some samples that contained TGFα protein (Table 5.1) and laser densitometry performed on the immunoreactive AR proteins showed the amount of AR present in each of the gestational tissues were relatively constant except for minor inter subject variability. For immunoprecipitation of the 52kDa AR species however 4-5 times the amount of total protein used for TGFα immunoprecipitation was required (Fig. 5.4C).

5.3.4 Localisation of TGFα & AR proteins in gestational tissue

Immunohistochemistry performed on tissue sections from placental chorionic villi with the anti-human TGFα antibody exhibited strong staining in the apical membranes of scattered

ST cells. CT cells also showed variable staining where some cells were weakly stained while other CT cells lacked immunoreactivity. Non-specific staining was observed in the villous stroma but Hofbauer cells and fibroblasts embedded in the stroma usually lacked staining.

Serial sections of the chorionic villi probed with the anti-human AR antibody exhibited a pattern of staining that was very similar to sections reacted with the TGFα antibody. Staining was observed along the apical membranes of isolated stretches of ST cells, a few CT cells and the villous stroma. Serial sections of the villi incubated with non-immune sera that served as negative controls for TGFα and AR immunolocalisation studies showed negligible non-specific staining (Fig. 5.5A-D).

Cuboidal epithelial cells of the amnion showed positive TGFα immunostaining on their distal and apical surfaces. Scattered fibroblasts and/or macrophages in the connective tissue layer also reacted positively to the TGFα antibody. In contrast scattered epithelial cells were uniformly stained on their apical surfaces when serial sections of the amnion were stained with the anti-AR antibody with little or no staining in the connective tissue layer.

Decidual cells adhering onto the chorion exhibited intense staining against the anti-TGFα antibody. Isolated cells of the trophoblast layer extending from the reticular layer to the decidua also showed strong immunostaining. In serial sections incubated with the anti-AR antibody intense staining was also observed in the cells of the decidua however the chorionic trophoblast cells generally lacked immunostaining. Negative control sections of the amnion and chorion incubated with non-immune sera that served as negative controls exhibited some non-specific staining in the compact layer of the amnion and cellular, reticular layers of the chorion. The non-specific staining made it difficult to analyse the immunoreactivity in the fibroblasts and/or macrophages found in these layers (Fig. 5.6A-D).

The immunostaining described previously was observed in the majority of tissue sections collected from different patients and these findings are summarised in Table 5.2.

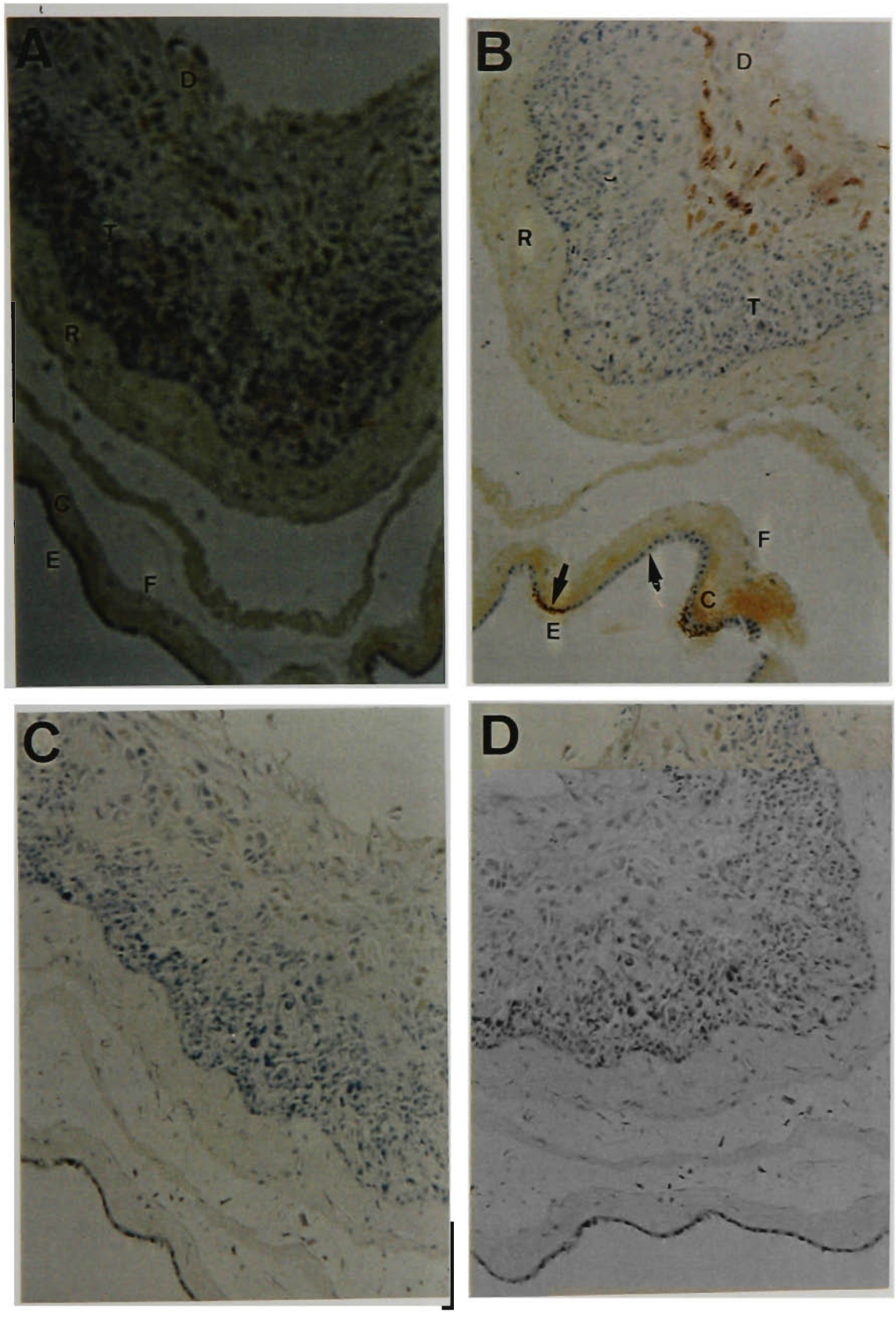


FIG. 5.5 Immunolocalisation of TGFα and AR in the fetal membranes (CS, 39 weeks). A) reacted with anti-human TGFα antiserum. B) serial section stained with anti-human AR antiserum. E=epithelial cells, C=compact layer, F=fibroblasts, R=reticular layer, T=trophoblast cells, D=decidua, ▼=cells with staining and ▲=cells without labelling. Micrographs C & D are serial sections incubated with non-immune serum in place of TGFα and AR antibodies respectively that served as negative controls. Magnification=100x.

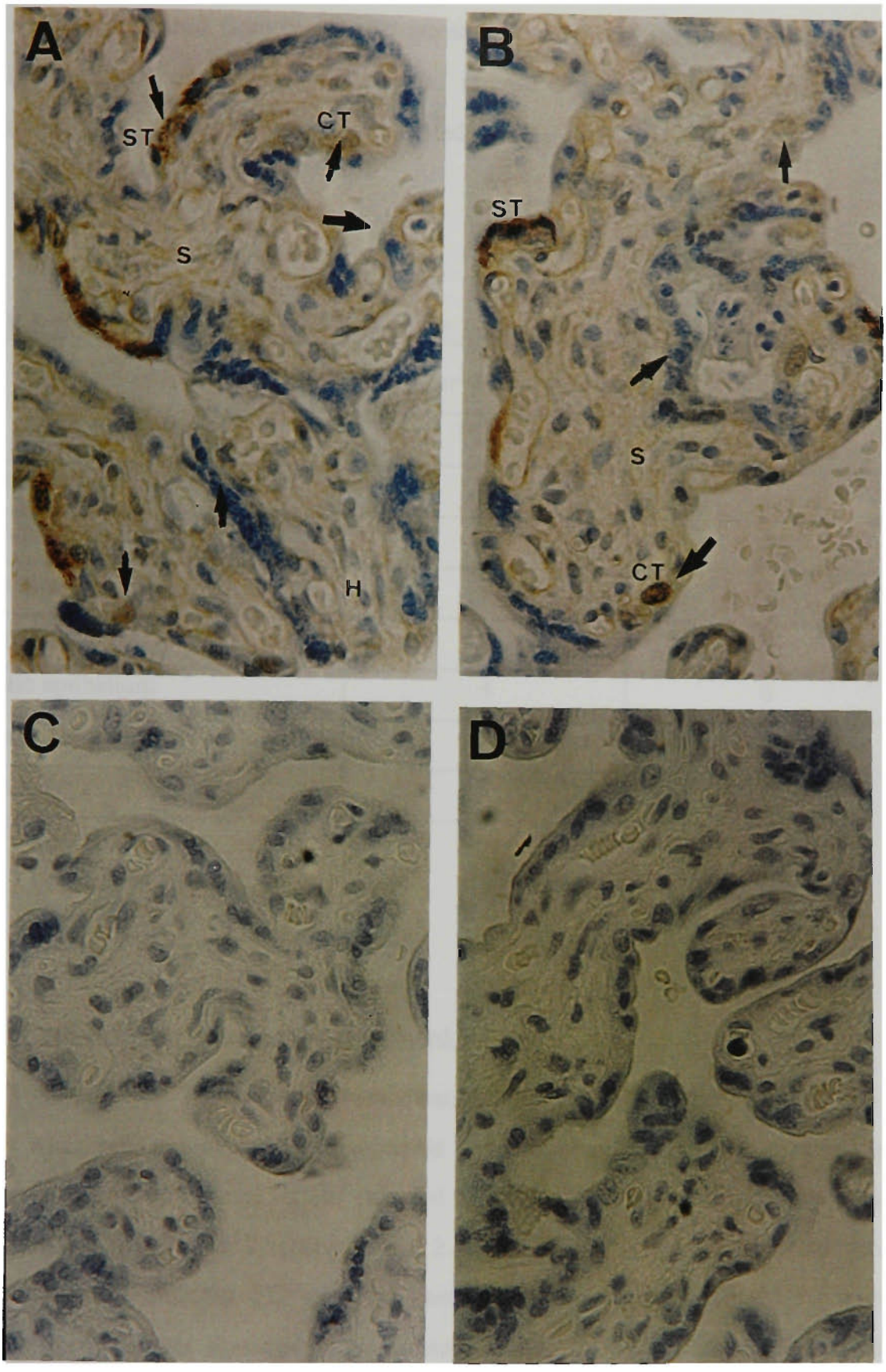


FIG. 5.6 Immunolocalisation of TGFα and AR in placental villi (CS, 40weeks). A) reacted with anti-human TGFα antiserum. B) section reacted with anti-human AR antiserum. CT=cytotrophoblast cell, ST=syncytiotrophoblast cells, H=Hofbauer cells, S=villous stroma, ▼=cells with staining and ▲=cells without labelling. C & D are sections incubated with non-immune serum in place of TGFα and AR antibodies respectively that served as negative controls. Magnification=250x.

Tissue sections prepared from breast and intestinal carcinomas used as positive controls for TGFα and AR immunostaining showed intense localised staining whereas serial sections incubated with non-immune sera lacked staining (Fig. 5.7).

TABLE 5.2 Summary of positive TGFα, AR immunoreactivity in gestational tissue (n=7 delivered by CS)

Cell or tissue type	TGFα	AR
syncytiotrophoblast cells	5	5
cytotrophoblast cells	6	4
villous stroma	7	7
Hofbauer cells	4	3
amniotic epithelium	4	6
chorionic trophoblasts	7	2
adherent decidua	7	7

5.3.5 Morphology & characteristics of cultured CT cells

CT cells cultured for 12h were mono-nucleated, rounded cells between 12-24 μm in diameter while cells cultured for 96h appeared to be multinucleated and compacted into dense irregular shaped clusters. Cells cultured for 12 and 96h stained positively against cytokeratins 8, 18 (also see Chapter 2, Fig. 2.1). hCG was not detected in the media conditioned by CT cells for 12h using an immunofluorescent assay specific for human hCG whereas hCG was detected in media conditioned by the irregularly shaped cell clusters; the mean, ±SEM (n=5) were 7.1mIU/ml, ±2.1 respectively.

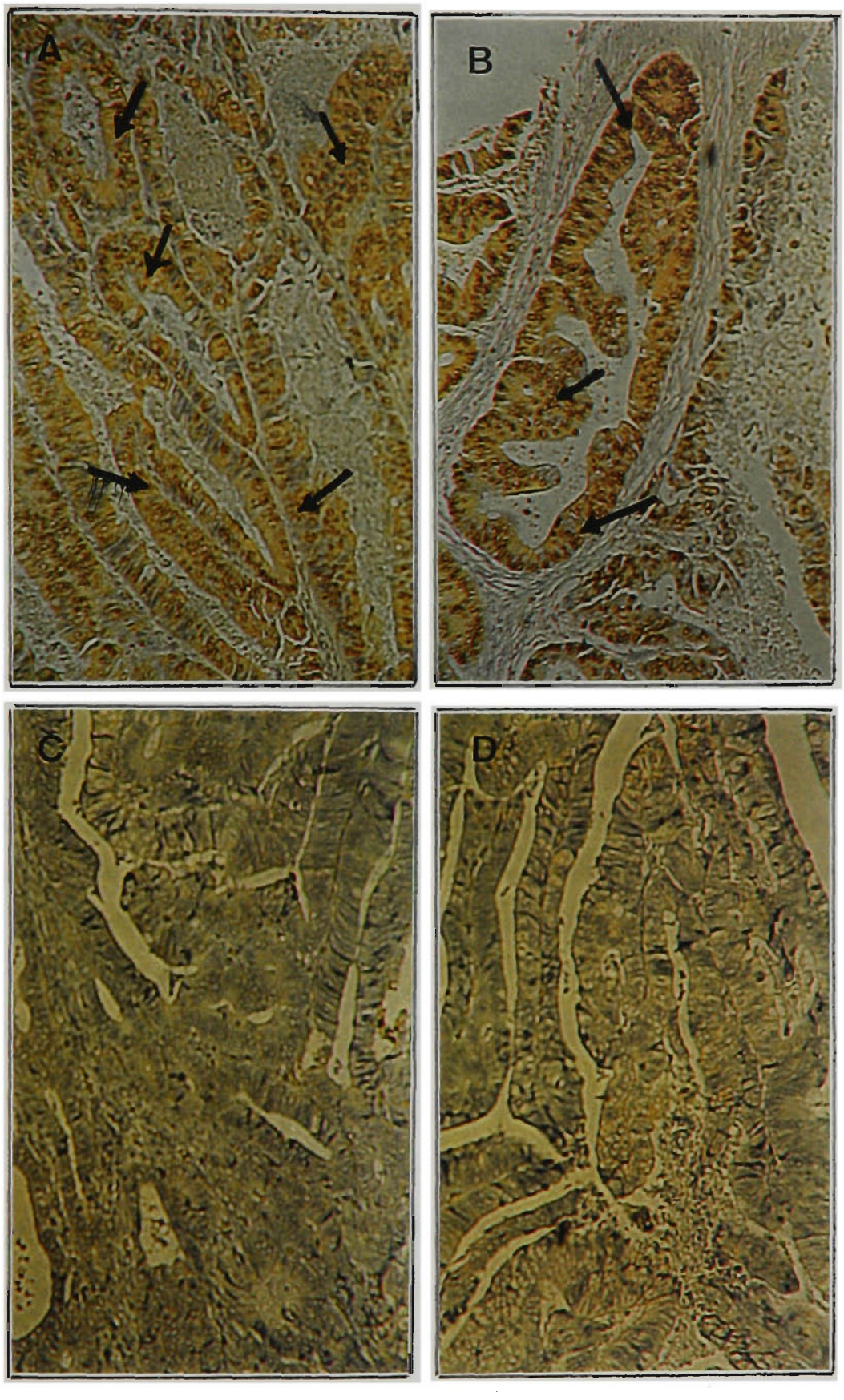


Fig. 5.7 Immunolocalisation of TGFα and AR in intestinal carcinoma cells. A) section stained with anti-human TGFα antiserum. B) section stained with anti-human AR antiserum. C & D are sections incubated with non-immune sera that served as negative controls. Arrows indicate positive immunostaining. Magnification 100x.

5.3.6 Expression, secretion of TGFα, AR proteins by cultured CT & ST cells

Immunoblot analysis of cell lysates from 12 and 96h cultures resolved by SDS-PAGE probed with the anti-TGFα and AR antibodies gave results that were very similar to that obtained from the gestational tissues. CT and ST cell lysates contained only two immunoreactive proteins with estimated sizes of 32 and 52kDa that hybridized to the anti TGFα and anti AR antibodies respectively (Fig.5.8 & Fig. 5.9).

Culture media conditioned by CT or ST cells for 12, 96h fractionated by SDS-PAGE, probed separately with AR and TGFα antibodies were found to contain only the 32kDa TGFα species. The amount of TGFα in the media was very small as observed by the very weak immunostaining on Western blots and it was only possible to detect the TGFα isoform when the blots contained between 40-50μg of transferred protein per lane. The amount of protein loaded per lane was not increased above 50μg as a 73kDa protein from the FCS that was present in the cell culture media caused distortion of the gel resulting in poor resolution.

Samples of media conditioned by CT, ST cells, fractionated by Tris-tricine PAGE were found to contain proteins of the size range 7-18kDa. However, the proteins in this size range did not react with the anti-TGFα or AR antibodies. Media that was not conditioned by CT or ST cells, resolved by SDS-PAGE or Tris-tricine PAGE which served as negative controls did not contain any proteins that reacted with the two antibodies.

5.3.7 Relative levels of immunoreactive TGFα & AR proteins in cultured CT & ST cells

Densitometric analysis of immunoblots containing proteins from CT and ST cells probed with the TGFα antibody showed that the amount of the 32kDa protein present in the two cell types was very similar. Laser densitometry performed on immunoblots probed with the AR antibody showed that per μg of total protein the ST cells had on average a 3.4 fold greater amount of AR compared with the CT cells. In CT and ST cells, the 32 kDa

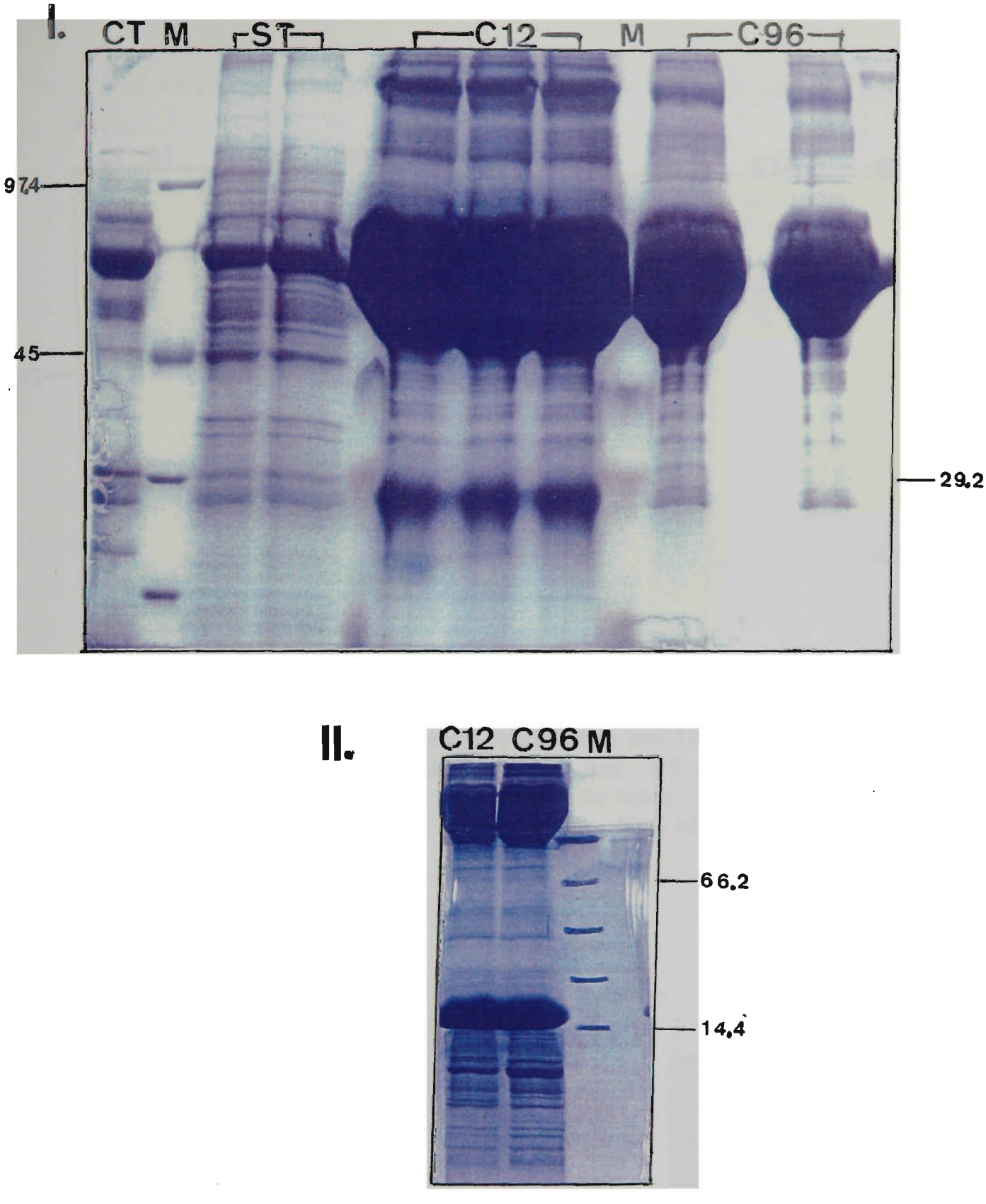


FIG. 5.8 Electrophoretic separation of proteins. I) proteins from cells and culture media fractionated by SDS-PAGE on a 10% polyacrylamide gel. II) proteins from conditioned media resolved by Tris-tricine-PAGE. Spacing, resolving gels with 10% and 16.5% polyacrylamide respectively. Forty μg of protein was loaded / lane Proteins are stained with Coomassie blue. CT=cytotrophoblast cells cultured for 12h, ST=cells cultured for 96h, C12=media conditioned by cells for 12h, C96=media conditioned by cells grown for 96h and M=protein molecular weight standard. Sizes are given in kDa. Similar gels were immunoblotted and probed with antibodies directed against TGFα or AR.

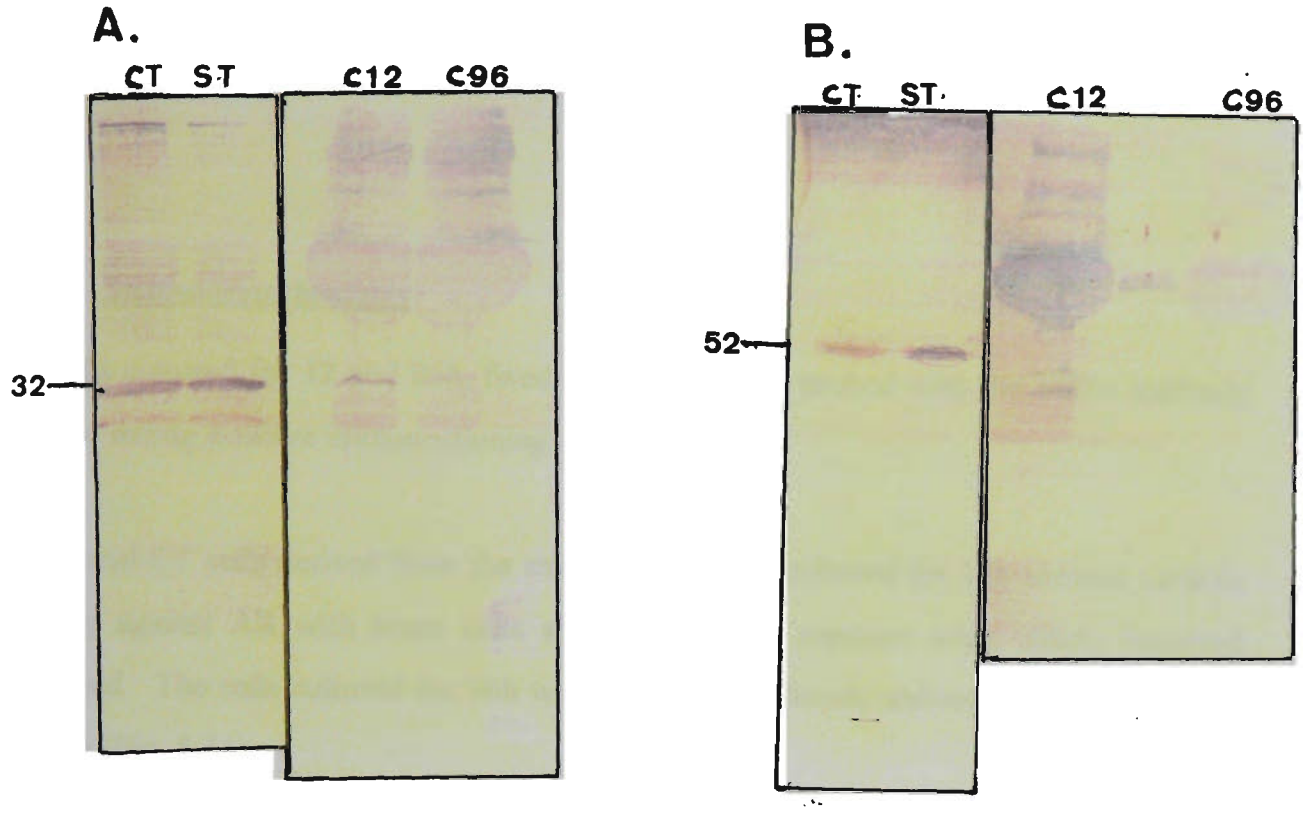


FIG. 5.9 Immunoblot analysis. A) Western hybridization anti-TGFα and B) anti-AR antibodies. Blots contain 40μg of sample protein per lane. CT=cytotrophoblast cells cultured for 12h, ST=syncytiotrophoblast cells cultured for 96h, C12=media conditioned for 12h, C96=media conditioned by cells grown for 96h. Sizes of the immunoreactive proteins are given in kDa.

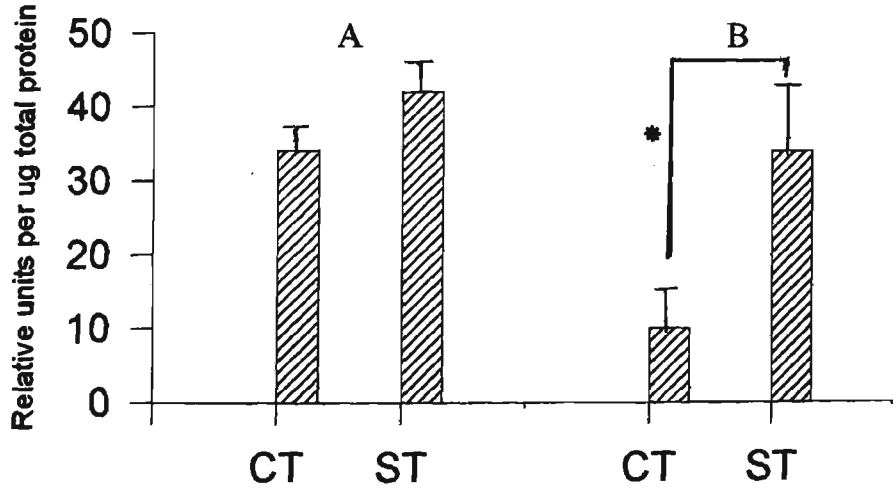


FIG. 5.10 Laser densitometric analysis of immunoreactive TGFα and AR proteins. **A & B** mean, ±SEM (n=5) of 32 and 52kDa TGFα, AR proteins respectively that were present in the CT and ST cell lysates. * indicates a significant difference in the amount of AR protein found in CT cells compared with ST cells calculated by the Students's *t* test ($p \leq 0.01$).

TGFα species was present at consistently higher levels than the 52kDa AR species per μg of total protein (Fig. 5.10).

5.3.8. Immunocytochemistry

CT cells cultured for 12 and 96h, fixed in methanol and probed with the TGFα antibody showed strong positive immunostaining.

Additional CT cells derived from the same subjects and cultured for 12h showed variable staining against AR with some cells showing positive reaction while others remained unstained. The cells cultured for 96h were far more uniformly stained than cells cultured for 12h (Fig. 5.11).

5.4 Discussion

The findings of the present study revealed that immunoreactive AR and TGFα proteins are present in chorionic villi, amnion and chorion-adherent decidua towards the end of gestation. Using antibodies directed against the mature peptides, a N-glycosylated 32kDa TGFα species was found to be consistently present in the placenta and fetal membranes while a N-glycosylated 52kDa AR species was found in the majority of samples expressing the TGFα protein. Immunoreactive AR and TGFα proteins of similar size to the ones we detected have been described from microsomal preparations of mammary glands removed from non-pregnant, pregnant ewes and a number of ovarian carcinomas (Forsyth *et al.*, 1997; Kohler *et al.*, 1992). The initial translation products, ie. proTGFα, proAR have been found to be proteins of 17-22kDa and 40kDa respectively (Martinez-Lacaci *et al.*, 1996, Teixido *et al.*, 1987, Wong *et al.*, 1989). However a number of previous reports have described AR and TGFα proteins of variable size that do not appear to correspond to the precursors or to the proteolytically processed mature growth factors in tissue, cell lysates and conditioned media. For example AR species of 20-35kDa

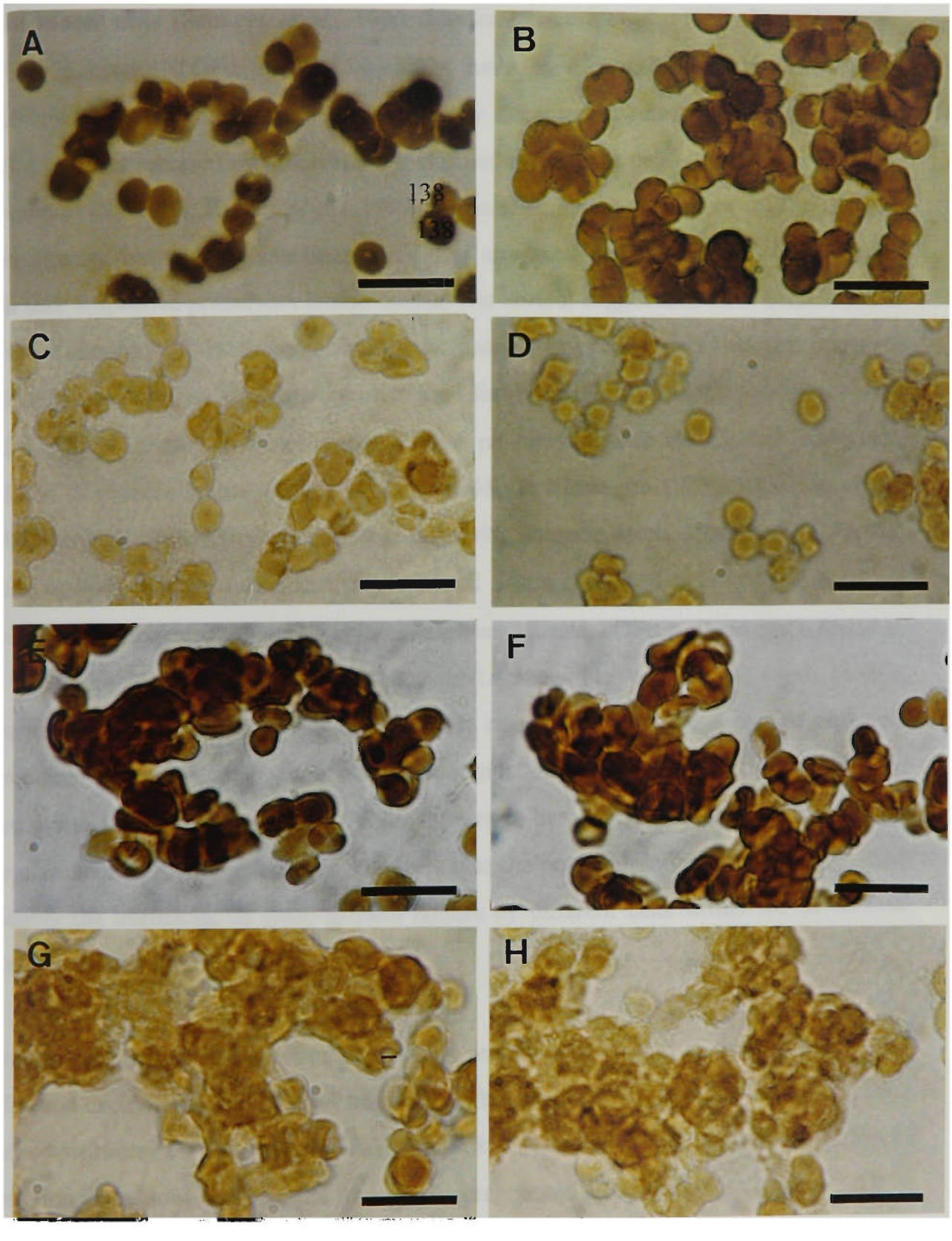


FIG. 5.11 CT and ST cells stained with anti-TGFα and anti-AR antibodies. A-B, CT cells cultured for 12h stained with the TGFα and AR antibodies respectively. E-F, ST cells stained with TGFα and AR antibodies respectively. C-D, G-H are CT and ST cells incubated with non-immune sera only. Scale bar = 30μm.

AR species have been found in mouse embryonic lung tissue, 27kDa in extracts, media conditioned media by ovine mammary explants and 55-60kDa species secreted by MCF-7 breast cancer cells (Schuger *et al.*, 1996; Martinez-Lacaci *et al.*, 1996; Forsyth *et al.*, 1997). Likewise TGFα species 20-48kDa have been described in human ovarian carcinomas, 30, 68kDa in media conditioned by mammary carcinoma cells and 24, 40, 42kDa proteins secreted by retrovirus transformed rat embryo cells (Linsley *et al.*, 1985; Dickson *et al.*, 1986; Kohler *et al.*, 1992). The significance and necessity for the size heterogeneity however remain uncertain. The structural relationships of these species with their respective precursors is also unclear but several suggestions have been made as to their identity. It is thought that these species may correspond to the complete precursors, partially or alternatively processed forms of the precursors, modified by the addition of N- and/or O-linked carbohydrates, palmitoylation in the case of TGFα and addition of glucose amine glycans in AR (Teixido & Massague 1988; Brachman *et al.*, 1989; Derynck 1988; Martinez-Lacaci *et al.*, 1996; Johnson *et al.*, 1993b). The AR and TGFα species that we found in the gestational tissue contained N-linked carbohydrate moieties. The extent of N-linked glycosylation of AR and TGFα proteins were found to be consistent with the findings of Martinez-Lacacaci (1996), and Luetteke (1988), respectively. Therefore these species probably correspond to either a part or complete precursor molecules that have undergone post translational modification by N-glycosylation Since both AR and TGFα appears to be glycosylated to similar extents in the placenta and fetal membranes it suggests that the post-translational modifications and possibly the trafficking of these growth factors by different cells within the gestational tissues appears to be similar.

Immunolocalisation studies revealed that AR protein was present in the villous CT, ST cells, and decidua during the third trimester. These findings differed from that of Lysiak and co-workers (1995), who reported that AR was absent in ST cells after 18 weeks gestation and villous CT, decidua throughout pregnancy. Moreover they observed AR in the nuclei of villous ST cells in samples collected between 11-18 weeks whereas nuclear AR staining was not observed in the ST or CT cells during our studies. Two nuclear targeting sequences between residues 26-29 and 40-43 in the mature secreted peptide is believed to enable translocation of AR into the nuclei (Shoyab *et al.*, 1989). AR has also

been found in the nuclei of terminally differentiated columnar, secretory epithelial cells of colon carcinomas, normal and cancerous ovarian epithelial cells (Saeki *et al.*, 1992; Johnson *et al.*, 1991; Johnson *et al.*, 1992; Kitadai *et al.*, 1993). The growth factor has also been reported to interact with two nuclear proteins of 120 and 205kDa in a number of normal and carcinoma cell lines (Modrell *et al.*, 1992). The differences in nuclear staining of villous ST between our study and that of Lysiak and colleagues (1995), may be due to the different antisera and detection methods utilised but may also reflect temporal differences in AR expression. In a recent study, estrogen was shown to enhance translocation of AR to the nuclei of MCF-7 breast carcinoma cells whereas TPA which is an intermediator of PKC signal transduction pathway was found to promote AR secretion into the media (Martinez-Lacaci *et al.*, 1996). It is possible that estrogen and TPA may play similar roles in AR routing and ultimately in its regulatory effects in the placenta. In the case of the fetal membranes the presence of AR protein has not been described previously.

TGFα immunostaining observed in the chorionic villi, decidua, chorionic trophoblasts and amnion was in agreement with previous findings of Lysiak (1993), Filla (1993) and Faber (1996), but differed from those of Horowitz (1993), who reported that TGFα was absent in the chorionic villi during the third trimester.

AR and TGFα proteins were localised to the same types of cell in the placenta and decidua whereas some differences were observed in the types of cells expressing the growth factors in the fetal membranes. The presence of TGFα and/or AR on the surfaces of CT, ST, decidual cells, chorionic trophoblasts and amniocytes suggest that the two growth factors maybe involved in growth and functional regulation. Both of these growth factors are ligands of the EGFR and the receptor protein is known to be present on the surfaces of these cells types (Rao *et al.*, 1984; Ladines-Llave *et al.*, 1991). Interaction of AR and TGFα with the EGFR may elicit a number of autocrine, paracrine and possibly juxtacrine regulatory loops within these tissues. Juxtacrine regulation by activation of EGFR receptors on adjacent cells by TGFα precursors have been demonstrated in vitro using CHO and BHK cells (Brachman *et al.*, 1989; Wong *et al.*, 1989). Although it has not been clearly demonstrated that the membrane bound AR precursors activate the

EGFR, secretory species between 55-60kDa that are thought to correspond to the AR precursor have been shown to stimulate the growth of MCF-10A cells by interacting with the EGFR (Martinez-Lacaci *et al.*, 1996). Further the association of AR with the cell surface antigen CD9 on human keratinocytes was demonstrated to potentiate juxtacrine stimulation by the membrane anchored growth factor (Inui *et al.*, 1997).

It is quite likely that at least some of the functions exerted by these two growth factors in the gestational tissues maybe redundant. In support of this view, in vitro studies using placental trophoblast cells from the first trimester have shown that AR and TGFα exert autocrine regulation increasing the rate of proliferation (Lysiak *et al.*, 1995; Li & Zhang 1997). Alternatively TGFα and AR maybe involved in selective regulatory effects. Findings that support this view are that the EGFR on the placental ST and CT membranes possess binding sites with different affinities (Carson *et al.*, 1983), that AR can act as an inhibitor proliferation of cells where TGFα acts as a stimulator (Johnson & Wong 1994), interaction of the AR with the EGFR may require heparin sulphate proteoglycans and/or glucose amine glycans and that binding of glycans lowers or removes the mitogenic activity of AR (Schuger *et al.*, 1996; Thorne & Plowman 1994; Matinez-Lacaci *et al.*, 1996).

Our findings also reveal that in vitro CT and ST cells do synthesise AR and TGFα but that differences exist in their temporal synthesis. Densitometric analysis showed that TGFα was present at similar levels per microgram of total protein in CT and ST cells whereas AR was consistently higher in the ST cells compared with CT cells. Immunocytochemical findings also paralleled the densitometric analyses. These findings supports the view that there may be differences in their regulatory activities. For example AR may play a role in the differentiation of CT into ST cells and their functioning whereas TGFα probably exert effects on both cell types. The 32kDa TGFα protein was also found in the media conditioned by both CT and ST cells which suggests that it is a potential autocrine and/or paracrine regulator of both cell types. A previous study also found TGFα was synthesised and secreted by cultured CT cells isolated from the second trimester but does not indicate if it is the precursor or processed protein (Filla *et al.*, 1993). The mature and meso-TGFα peptides may also be present in the conditioned

media however may not have been detected in our study as we did not concentrate the conditioned media. Enzymes designated pro-TGFα-Iase and pro-TGFα-IIase are thought to release the mature 6kDa peptide by successive cleavages of the Ala^{39}-Val^{40} and Ala^{89}-Val^{90} peptide bonds whereas preferential cleavage at Ala^{89}-Val^{90} is thought to release the meso-TGF species that range between 18-21kDa (Pandiella & Massague 1991a, b). A TGFα isoform 28.5kDa which is comparable in size to the species detected in our studies has been found in media conditioned by human alveolar macrophages (Madtes *et al.*, 1988) and larger forms of 40, 42kDa detected in conditioned media from transformed rat embryo cells (Linsley *et al.*, 1985). The manner in which these species are released have not been determined.

Secreted forms of AR were not detected by immunoprecipitation in our studies and this may be due to the fact that the conditioned media was not concentrated. The manner in which AR species of variable size are released remains uncertain, however studies have shown that the heparin binding domain of AR found in the N-terminal region is necessary for the secretion of AR protein (Thorne & Plowman 1994).

In terms of their effects, TGFα has been shown to increase inhibin synthesis in cultured CT cells in the presence of hPL (Qu & Thomas 1993). We are unaware of any published reports that have investigated the effects that AR may be exerting in cultured placental CT and ST cells isolated from placentae late in gestation.

In conclusion these findings show that the growth factors AR and TGFα are co-expressed in a number of important cell types that perform vital functions during pregnancy and parturition. It is very likely that these growth factors are involved in functional regulation. Further studies are required to investigate the roles exerted by these growth factors towards the end of gestation.

CHAPTER 6

Effects of TGFα on Proliferation, Differentiation and Progesterone Synthesis by Cytotrophoblast Cells *In Vitro*

CT cells isolated from villous cotyledons close to term proliferate, aggregate and differentiate into multinucleated ST in vitro. The effects of TGFα on the proliferation and aggregation of CT cells are investigated in the first part of this study.

Progesterone is one of the principal steroid hormones produced by the CT and ST cells of the placenta and in vivo is necessary for establishing and maintaining pregnancy. The second part of this study examines the effects of TGFα on the expression of 3βHSD mRNA;(3βHSD enzyme converts pregnenolone to progesterone) and progesterone synthesis by CT cells in vitro.

6.1 Introduction

The growth and differentiation of the CT into the ST cells is an important pre-requisite of normal placental function as the ST is the major site of feto-maternal exchanges and placental hormone synthesis. Studies *in vitro* have shown that CT isolated from the first, third trimesters divides spontaneously and aggregate into dense clusters (Daniels-McQueen *et al.*, 1986; Richards *et al.*, 1994), and that the aggregated CT cells fuse to form the multinucleated ST cells (Kliman *et al.*, 1986; Loke & Burland 1988). Another view is that ST cells are formed by endomitosis of CT cells and not by aggregation and cellular fusion (Sarto & Stubblefield 1982).

Treatment of CT cells isolated from the first and second trimesters with nanogram quantities of TGFα have been shown to increase the rate of proliferation (Li & Zhang 1997; Filla *et al.*, 1993, Lysiak et al., 1993). However whether TGFα exerts any effect on the rate of proliferation of CT cells isolated from the third trimester is uncertain. The structurally related protein EGF, which interacts with the same receptor as TGFα, has been shown to exert no effect on the rate of proliferation of CT isolated from placenta close to term (Qu *et al.*, 1992).

In the case of differentiation, TGFα has been found to inhibit the formation of multicellular aggregates by EVT cells isolated from the second trimester (Lysiak *et al.*, 1993). EGF on the other hand was shown to increase the rate of differentiation of CT cells from the third trimester (Morrish *et al.*, 1987). It remains uncertain if TGFα exerts any regulatory control on the differentiation of CT during the latter stages of pregnancy.

Progesterone is one of the main steroid hormones produced by the human placenta and by 8 weeks gestation placental progesterone synthesis surpasses that of the corpus luteum (Csapo & Pulkkinen 1978; Simpson & MacDonald 1981). In women, progesterone is necessary for the establishment and maintenance of pregnancy, has immunosuppressive properties that may contribute to the tolerance of the fetal allograft, maintains the quiescent state of the myometrium and together with estrogen enlarges the uterine smooth muscle cells (Heap & Flint 1988).

Previous studies have shown that the placenta is unable to synthesise cholesterol which is a substrate that is required for progesterone synthesis. Cholesterol that is bound to low density lipoproteins found in the maternal circulation has been shown to enter the placenta and used as the main substrate for progesterone synthesis (Hellig *et al.*, 1970). During progesterone synthesis the cholesterol side chain is cleaved by the placental mitochondrial enzyme P-450scc to yield pregnenolone (Meigs & Ryan 1968; Mason & Boyd 1971). Thereafter pregnenolone is partially isomerised to progesterone by the enzyme 3βHSD found on mitochondria, microsomes and cytoplasm of CT and ST cells (Thomas 1988).

In vitro progesterone is actively synthesised and secreted by placental organ cultures and CT cells, hence organ and CT cultures have been widely used to investigate the factors that regulates progesterone synthesis in the placenta. Previous studies have shown that hCG, LDL-cholesterol, pregnenolone, intermediates of the estrogen synthesis such as DHA and DHAS, the hormone LHRH, cAMP, phorbol esters, intermediates or activators of PKC, PKA signal transduction pathways, $Ca^{2}+$ ions, calmodulin and β-adrenergic receptor agonists regulate placental progesterone synthesis *in vitro* (Winkel *et al.*, 1980; Branchaud *et al.*, 1983; Ritvos *et al.*, 1988; Albrecht & Pepe 1990).

However there have been very few reports on the effects exerted by growth factors on placental progesterone production. Nestler & Williams (1987), reported that IGF-1 increased the activity of the 3βHSD enzyme. There have been conflicting reports on the effects exerted by EGF on placental progesterone synthesis. Bahn and co-workers (1980), found that EGF increased progesterone synthesis in cultured JEG-3 choriocarcinoma cells while Ilekis & Benveniste (1985), reported that progesterone production was not altered in JEG-3 cells treated with EGF. Although there have been no reports on the influence of TGFα on placental progesterone production it has been shown to stimulate progesterone synthesis in cultured rat corpora luteum cells (Tekpetey *et al.*, 1995b). Increased progesterone synthesis was also reported in rat ovarian granulosa cells incubated with TGFα and a further 10 fold increase was observed when these cells were treated with TGFα and FSH (Yeh *et al.*, 1993).

During placental estrogen steroidogenesis, both TGFα and EGF have been shown to increase the rate of transcription of 17βHSD type I mRNA; the enzyme 17βHSD interconverts estrone to estradiol in the placenta (Lewintre *et al.*, 1994). Whether TGFα exerts any regulatory control over the mRNA expression of enzymes involved in placental progesterone synthesis however is uncertain.

This study was conducted to gain a better understanding of the effects that may be exerted by TGFα in villous CT cells isolated from term placenta. The aims of the study were:

- to investigate the effects exerted by TGFα on the proliferation and differentiation of CT cells isolated from villous cotyledons between 38-40 weeks pregnancy and
- to investigate the effects of TGFα on progesterone synthesis and expression of 3βHSD mRNA in cultured CT cells isolated from villous cotyledons close to term.

6.2 Materials and methods

Villous CT cells were isolated from term placenta (delivered by Caesarean section at 38-40 weeks gestation) as described in Chapter 2 section 2.2.1. Cells were characterised by immunocytochemical staining against cytokeratin 8 and 18 as described in Chapter 2 section 2.2.3.

To determine the effects of TGFα on proliferation of villous CT cells, the cells were seeded at a density of $0.5x10^6$ cells/ml in EMEM containing 10% FCS and cultured as described in Chapter 2, section 2.2.4 (n=8). Cells were treated with 10ng TGFα/ml and cultured for 3, 6, 12 and 18h. For each time interval, quadruplicate wells were treated while another four wells served as untreated controls in each replicate. Additional cells were treated with 20 and 50ng TGFα/ml and also incubated for the 3, 6, 12 and 18h to determine the effects of different TGFα concentrations. After the incubation period cells were lifted by trypsinisation and counted using a haemocytometer. The mean, ±SEM were calculated and differences between controls and treatments were assessed by the Students *t* test. Details of the treatment, trypsinisation and analysis are given in Chapter 2, section 2.9.1

In order to study the effects of TGFα on the aggregation, villous CT cells were seeded at a density of 10^5 cells/ml in EMEM containing 10% FCS and cultured for 12h (n=6). Media was aspirated at replaced with fresh media containing 10% FCS and 10ng/ml TGFα in treated cultures and fresh media and 10% FCS only in untreated controls. Cells were grown for 6, 12 and 18h post treatment, media was removed and cells stained with haematoxylin. For each replicate 4 wells were treated while another 4 wells served as untreated controls. The number of nuclei distributed between the mononucleated and aggregated cells were counted under 400x magnification; 12 microscopic fields were counted for each set of quadruplicate control or treated cultures. The percentage of nuclei distributed among aggregated and mono-nucleated cells was calculated. Additional cultures were also treated with 20 and 50ng TGFα/ml in order to assess the effects of varying concentration of TGFα on aggregation of CT cells. The mean, ±SEM were calculated and differences between controls and treatments were assessed by the Students *t* test. Details of the treatment, staining and analysis are given in Chapter 2, section 2.9.2.

To investigate the effects of TGFα on progesterone synthesis, CT cells were diluted to $0.75x10^6$ cells per ml in EMEM with 10% FCS. $3x10^6$ cells were placed in scintillation vials and treated with 60ng TGFα per vial while control cultures received no growth factor treatment. Cells were cultured 0min, 15min, 30min, 1, 2, 3, 6, 12, 18h post treatment in liquid culture on a shaking water bath. An 10μl aliquot was used to determine the cell numbers. Cells were then lysed in the culture media and the progesterone concentration in the CT cells and conditioned media was measured by RIA (n=6). The experiment was repeated using a higher TGFα dosage of 30ng per $0.75x10^6$ cells per ml. Details of the culture, treatment and progesterone RIA are given in Chapter 2 sections 2.10.1 and 2.10.2.

The expression of 3βHSD mRNA was investigated using semi-quantitative RT-PCR analysis. CT cells were seeded and cultured as described in Chapter 2, section 2.10.1. Treated cultures received 15ng/ TGFα per $0.75x10^6$ cells per ml (n=5). Cells isolated from 2 patients also received 6μg of neutralising TGFα per ml in addition to the growth factor treatment. Cells were harvested after 0min, 15min, 30min, 1, 2, and 3h and total RNA was isolated from treated and control cultures. Semi-quantitative RT-PCR was

carried out using 3βHSD specific reverse and forward primers and GAPDH mRNA as an internal standard. The amount of 3βHSD mRNA relative to GAPDH mRNA in the treated and control cultures were assessed by laser densitometric scanning of the RT-PCR generated amplicons from the treatments and controls. Details of the 3βHSD reverse and forward primers and RT-PCR are given in Chapter 2, section 2.10.3.

6.3 Results

6.3.1 Appearance and characterisation of villous cytotrophoblast cells

Cells isolated from the villous cotyledons were rounded in appearance and ranged between 10-13μm in diameter. The CT cells had a large centrally located nucleus with a granular appearance. The majority of cells exhibited strong immunocytochemical staining against cytokeratin 8 and 18. As assessed by the immunocytochemical staining the purity of the preparations ranged between 92.5 and 96.2%. Macrophages and leucocytes could sometimes be identified as contaminant cells.

6.3.2 Effect of TGFα on proliferation of cytotrophoblast cells

The total number of cells per ml was found to increase with incubation time in both treated and control cultures after an initial seeding density of $0.5x10^6$ cells per ml. The cells numbers had doubled after 6h in culture in both control and cultures treated with 10ng TGFα per ml. Cell numbers continued to increase up to 18h but at a lesser rate increasing between 30-41% in number between 6 and 18h. After 18 in culture cells showed visible signs of aggregation, making it difficult to estimate the total number of mono-nucleated cells following trypsinisation. However there was no significant difference in total cell numbers between controls and cells treated with 10ng per 10^5 cells/ml at T=0h and cultured for 3, 6, 12 and 18h ($P>0.05$, Student's *t* test, Fig. 6.1). Increasing the concentrations of TGFα to 20ng and 50ng per $0.5x10^6$ cells / ml was found to have no significant effect on the total number of cells per ml after 3, 6, 12 and 18h post treatment.

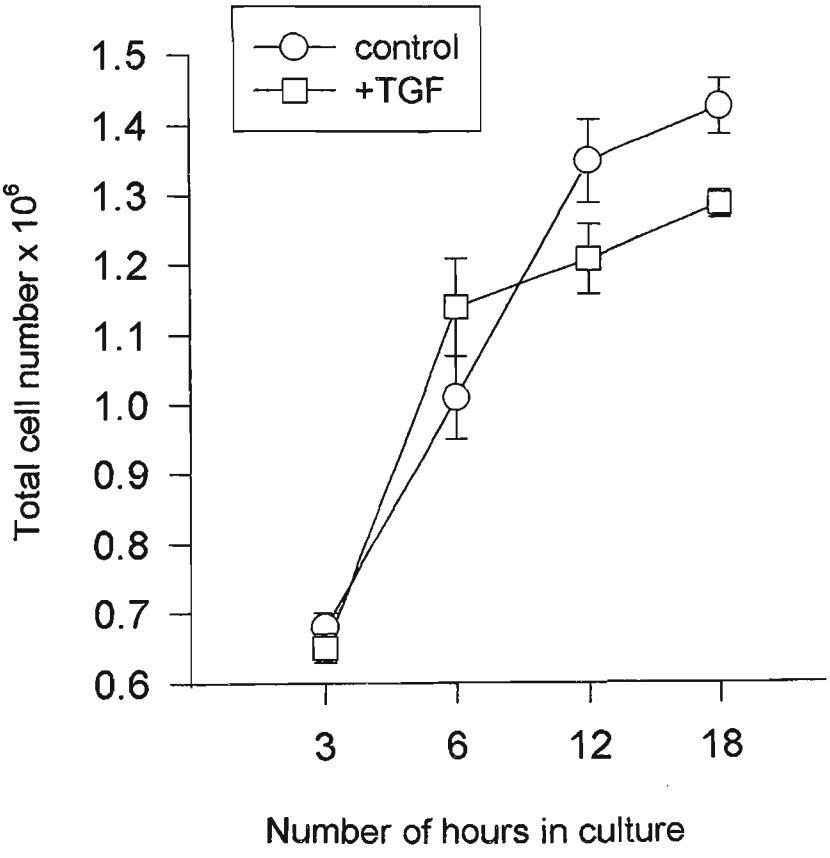


FIG. 6.1 Effect of TGFα on the total number. Villous CT cells seeded at a density of $0.5x10^5$ cells per ml and grown for varying periods of time with or without the addition of TGFα (n=8). For each replicate, cells in four wells were treated with 10ng TGFα per 10^5 cells / ml at T=0h, while another four wells were left untreated. Cells were cultured for 3, 6, 12 and 18h. Media was removed, cells lifted with trypsin and counted. Counts shown represent the mean, ±SEM. No significant differences were found between sets of controls and treatments ($P>0.05$, Student's *t* test).

6.3.3 Effect of TGFα on differentiation of cytotrophoblast cells

Microscopic examination of cells soon after seeding at a density of 10^5 cells per ml showed that between 2-2.7% of the cells were multinucleated. The percent of nuclei distributed in cell aggregates increased marginally to 4.5-4.7% after 12h in culture prior to the commencement of the experiment. After 6h the percent of nuclei in aggregates varied between 19-23%. The percentage of nuclei in cell aggregates continued to increase with incubation period reaching 66-72% after 18h post treatment. during the course of the experiment compared with mono-nucleated cells in both treated and control cultures indicating that CT were differentiating spontaneously to ST. However there was no significant difference in the percentage distribution of nuclei in the aggregated cells between controls and cells treated with 10ng TGFα per ml after 6, 12 or 18h ($P>0.05$, Students *t* test, Fig. 6.2). By 21h post treatment 86-88% of nuclei were found in aggregates in both control and treated cultures.

Increasing the dosage of TGFα to 20 and 50ng per ml was found to have no significant difference on the percentage distribution of nuclei compared with the control cultures after 6, 12 or 18h.

6.3.4. Effect of TGFα on progesterone synthesis by cytotrophoblast cells

Immediately after seeding the mean progesterone concentration in the control and cultures treated with 15ng TGFα/ml per $0.75x10^6$ cells was 0.65ng/ml. Between 0.25-1h in culture the progesterone concentrations showed a minimal increase and reaching mean value of 0.81ng/ml. The progesterone concentrations gradually increased in both control and treated cultures reaching 2.04 ±0.05 for controls and 1.85 ±0.07 ng/ml per 10^6 cells in the treated cultures. Further increases were detected in cultures maintained for 6, 12 and 18h and by 18h the progesterone concentrations had reached almost 13ng/ml per 10^6 cells. At 18h the cells were visible aggregated making it difficult to estimate the total cells number. Throughout the experimental period no significant differences in total progesterone concentrations per 10^6 cells were found between paired treatments and controls (Student's *t* test $P>0.05$, Fig. 6.3).

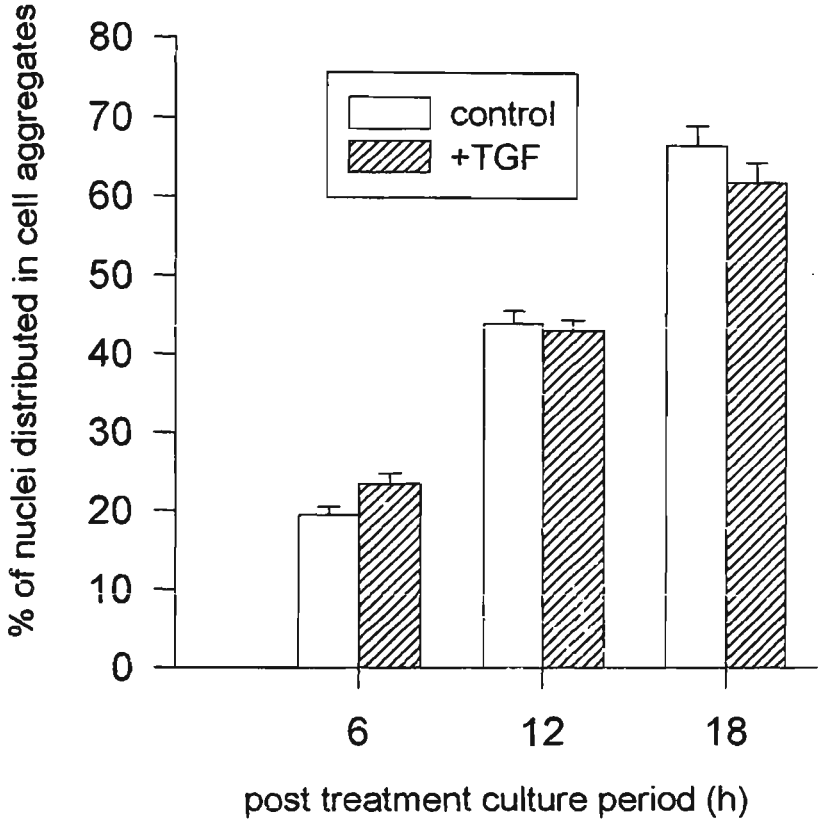


FIG. 6.2 Percentage of nuclei distributed in cell aggregates. CT cells were seeded at a density of 10^5 cells/ml, cultured for 12h and media gently aspirated (n=6). For each replicate CT cells in 4 wells were treated with 10ng/ml TGFα in fresh media, 4 untreated wells served as controls. Cells were cultured for a further 6, 12 or 18h post treatment and media removed. Cells were stained with haematoxylin and the number of nuclei distributed between mono-nucleated cells and cell aggregates were counted. The percent of nuclei distributed in cell aggregates are shown as the mean, ± SEM. No significant differences between paired controls and treatments were found; ($P>0.05$) by the Students *t* test.

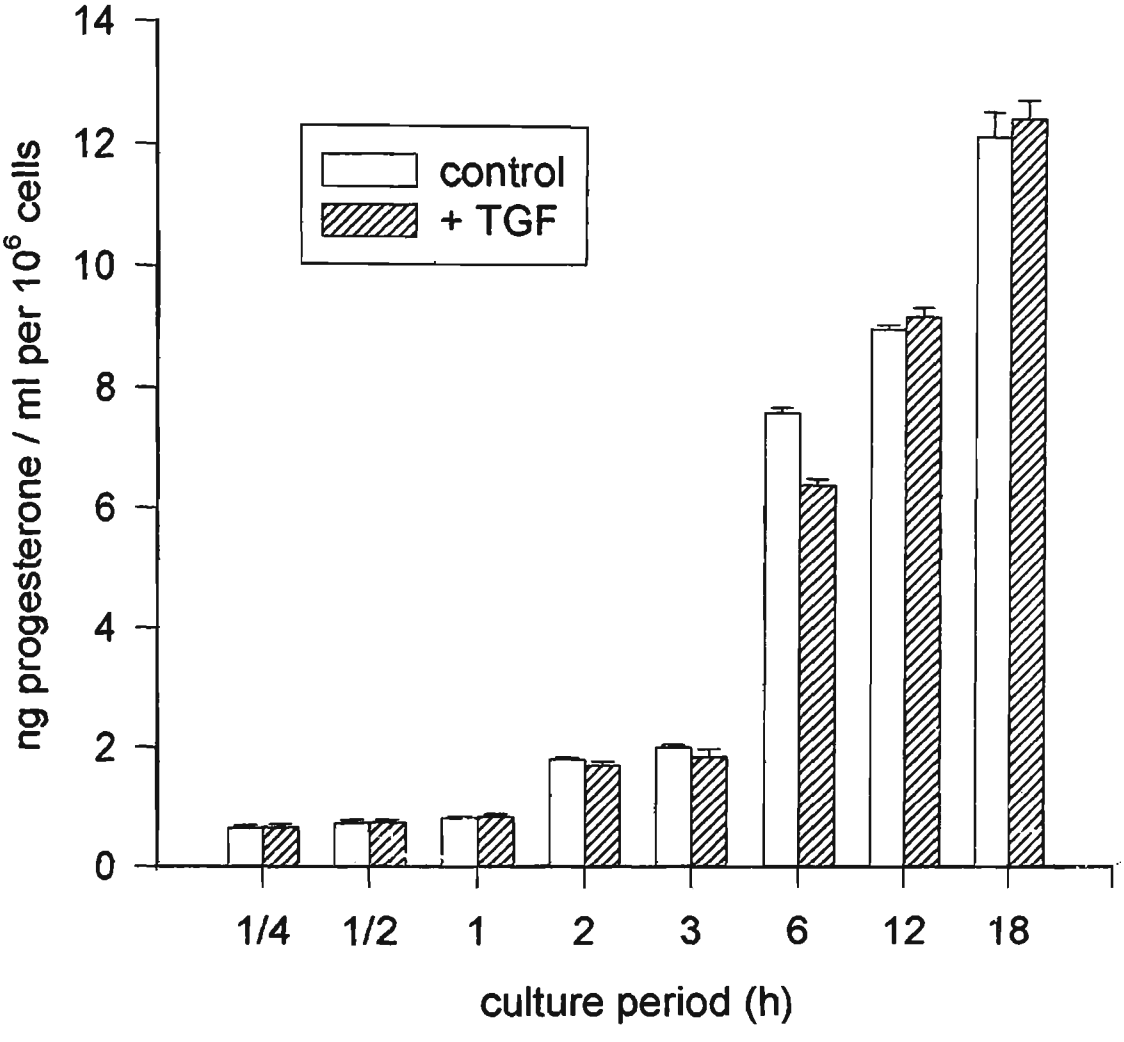


FIG. 6.3 Effect of TGFα treatment of progesterone concentration. CT cells were treated with 15ng TGFα / ml per $0.75x10^6$ cells and cultured for varying time intervals. The amount of progesterone in the cells and conditioned media was measured by RIA and number of cells estimated by total cell counts. Data shown represents the mean, ±SEM (n=6). No significant differences between paired treatments and controls were found; ($p>0.05$) by the Student's *t* test

Increasing the concentrations of TGFα to 30ng/ml per $0.75x10^6$ cells did not cause significant differences in the concentrations of progesterone in the treated and control cultures during the experimental period.

The mean inter assay and intra assay coefficients of variation were 5.8 and 7.9 respectively.

6.2.4 Effect of TGFα treatment on 3βHSD mRNA expression

Formaldehyde agarose gel electrophoresis of total RNA prepared from treated and control CT cells after different incubation periods had 28S:18S rRNA band intensity, size ratios ≥1.56. A single cDNA fragment, 531bp in size was detected by agarose gel electrophoresis following RT-PCR analysis of GAPDH mRNA in the treated and control total RNA samples isolated after different incubation periods.

Using the 3βHSD specific reverse and forward primers, a single band 549bp is size was observed on agarose gels from 0.5μg, 1.0μg human term placental total RNA samples following RT-PCR. The estimated size of 549bp was identical to the predicted size for the reverse and forward primer combination. The 549bp band was also found in the reaction mixes containing 1μg total RNA from CT cells (controls, T=0h) as a faint band and as a more prominent band in the reaction mixes containing 1.5μg RNA from CT cells. Therefore for the semi-quantitative analysis of 3βHSD mRNA following treatment with TGFα, 1.5μg of total RNA was used to set up the reaction mixes. Visible cDNA bands were absent in the three negative control reactions that consisted of yeast tRNA, genomic DNA and reaction mix minus reverse transcriptase (Fig. 6.4).

The nucleotide sequence of the 549bp cDNA fragment matched the base sequence of 3βHSD type 1 cDNA published by The and co-workers (1989), confirming that the cDNA was a part of the 3βHSD gene. The percentage of base mismatches over a stretch of 508 bases that were compared with the nucleotide sequence published by The and co-workers (1989), was found to be < 0.8 (Fig. 6.5).

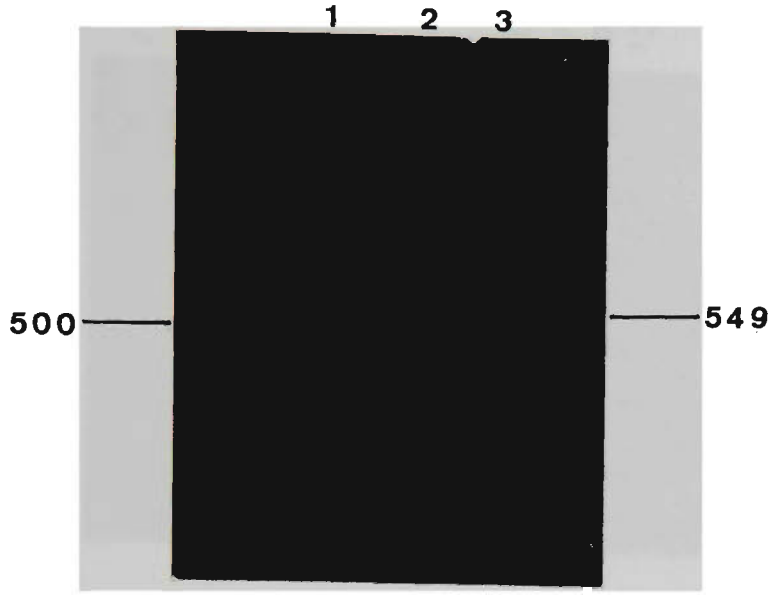


FIG. 6.4 RT-PCR analysis of total RNA using 3βHSD specific forward and reverse primers. 1/10th volume of reaction mixes were resolved on a 1.4% EtBr stained agarose gel. Lane 2 - human term placenta starting with 1.0μg total RNA, lane 3 - CT cells (control, T=0h) starting with 1.5μg total RNA. Lane 1 - 100 bp DNA ladder, spiked band = 500bp.

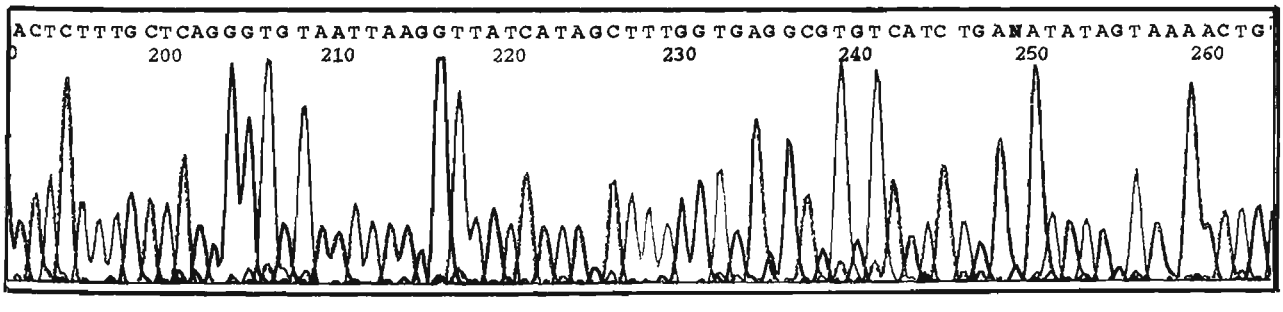
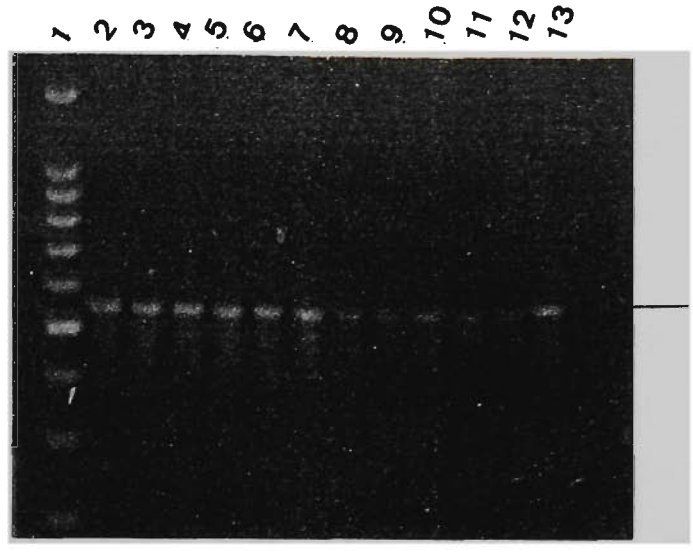
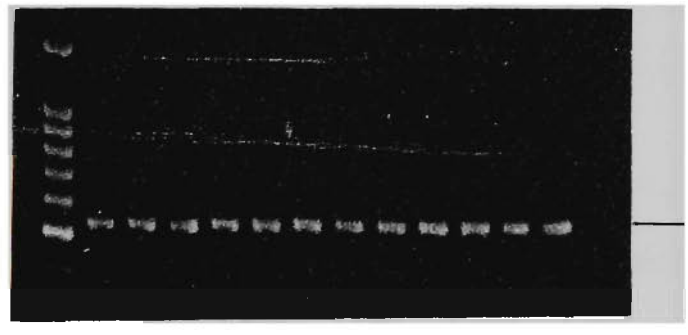
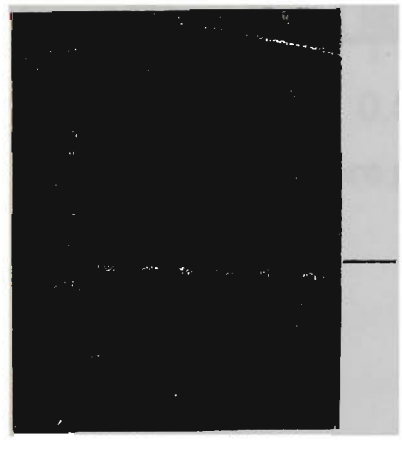


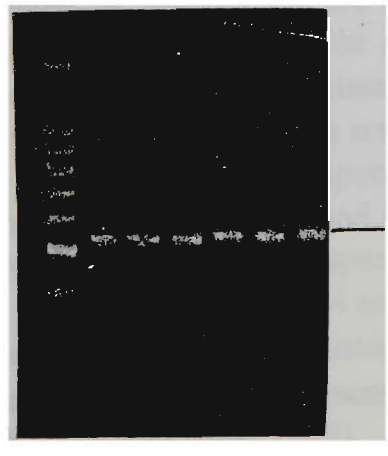
FIG. 6.5 Nucleotide sequence trace of 549bp cDNA amplified by the forward and reverse 3βHSD primers and total RNA from CT cells (control, T=0h). The bases shown completely matched the nucleotide sequence in exon 4 of 3βHSD gene reported by The *et al.*, 1989. Only a part of the trace is shown; complete trace is given in Appendix 13A.


1 2 3 4 5 6 7 8 9 10 11 12 13
3βHSD
549bp


GAPDH
531bp

A-ii


3βHSD
549bp


GAPDH
531bp

B.

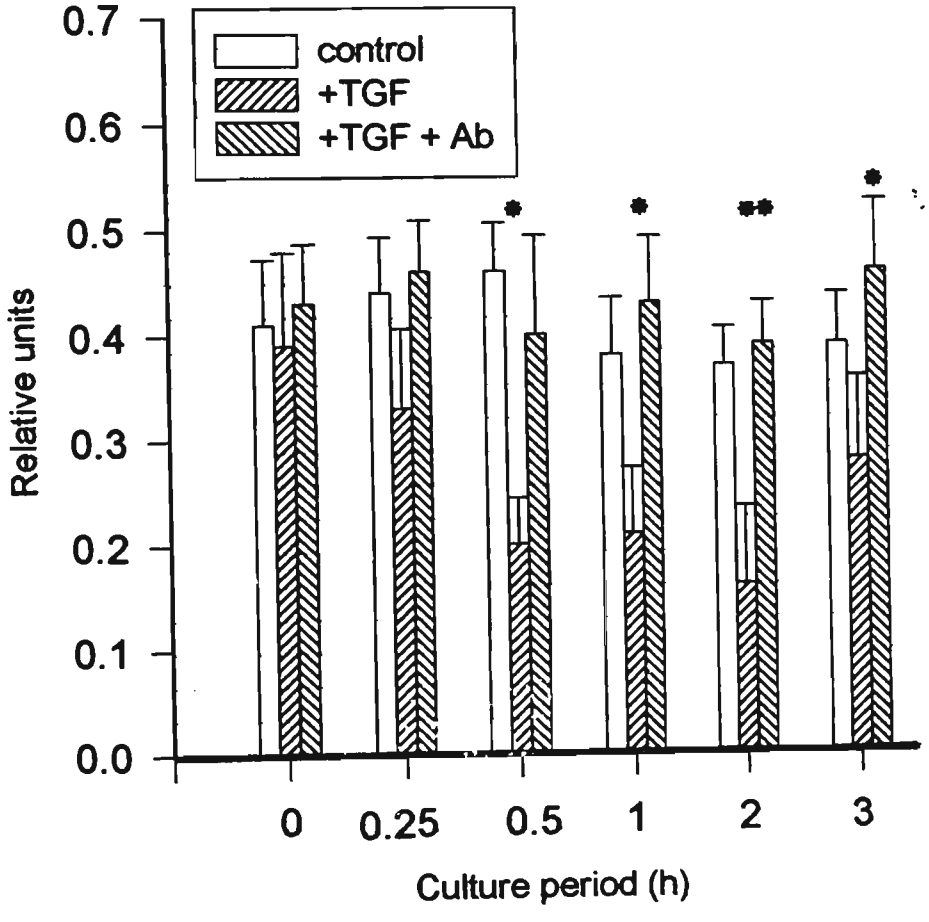


FIG. 6.6 Relative levels of 3βHSD mRNA expression. 1/10th volume of RT-PCR mixes resolved on 1.3% EtBr stained agarose gel. **A-i)** Lanes 2-7 in the upper panel depicts amplicons generated with 3βHSD primers and RNA from 0, 0.25, 0.5, 1, 2, 3h control cultures and lanes 8-13 from CT cells treated with 15ng TGFα/0.75x10^5 cells per ml and cultured for 0, 0.25, 0.5, 1, 2, 3h respectively. **A-ii)** lanes 2-7 depicts amplicons generated from cultures treated with TGFα and 6μg/ml anti-TGFα neutralising antibodies and cultured for 0, 0.25, 0.5, 1, 2, 3h respectively. The respective GAPDH cDNA generated are given in the lower panels of A-i and A-ii. Lane 1, 100bp DNA ladder. **(B)** Laser densitometric analysis of negative photographic images of 3βHSD and GAPDH cDNA resolved on agarose gels. Data represents the mean, ±SEM, n=5 for controls and treatments except antibody treatment (n=2). Significant differences between TGFα treatments and controls were found by the Student's *t* test; * ≡ ($P<0.05$), ** ≡ ($P<0.01$).

Following treatment with 15ng TGFα per ml / $0.75x10^6$ cells, the relative amount of 3βHSD mRNA quantitated by laser densitometry after semi-quantitative RT-PCR was found to decrease within 15min following treatment. After 30min and 1h significant differences were seen in the relative amount of 3βHSD mRNA compared with untreated control cultures ($p>0.05$). After 2h post treatment the relative amount of 3βHSD mRNA declined further compared with levels present after 1h post treatment and were significantly lower than the controls ($p>0.01$). After 3h post treatment the relative levels of 3βHSD mRNA were close to that at the start of the experiment. In the case of the control cultures the amount of 3βHSD cDNA relative to GAPDH cDNA remained static and did not vary significantly during the culture period. In cultures treated with the growth factor plus neutralising anti-TGFα antibody, the decrease in the amount of 3βHSD mRNA from 15min to 2h post-treatment was abolished and the mRNA levels remained static and were comparable with the control cultures. Laser densitometric analysis showed that following treatment with TGFα, the amount of GAPDH cDNA in the treated cultures remained static and was not affected by the treatment. The amount of GAPDH cDNA generated from the control cultures also did not vary with the incubation period (Fig. 6.6).

6.3 Discussion

The findings of this study reveals that although CT cells isolated from villous cotyledons close to term proliferate spontaneously, exogenously applied TGFα does not influence their proliferation. Other investigators have found that CT cells isolated from term placenta proliferate spontaneously and estimated that up to 15% of cultured CT cells are actively dividing or are ready to divide (Kliman *et al.*, 1986). Growth factors such as EGF, which is structurally similar to TGFα and interacts with the same receptor as TGFα has also been reported to exert no measurable effect on the rate of proliferation of CT isolated from term placenta, in experiments where proliferation was measured by total cell number or DNA content (Morrish *et al.*, 1987; Qu *et al.*, 1992). On the other hand, both TGFα and EGF has been shown to increase the rate of proliferation of cultured CT cells isolated from 16 week placenta, passaged 10-20 times and grown in 0.1% BSA (Li &

Zhang 1997) and CT cells isolated from placenta after 8, 9, 12 and 16 weeks gestation grown in serum free medium (Filla *et al.*, 1993 & 1994). Studies by Schreiber and co-workers (1986), have shown that TGFα and EGF have an equivalent ability to stimulate DNA synthesis and proliferation of other epithelial cells such as MCF-7 breast carcinoma cells. In the placenta, TGFα and EGF appear to elicit similar responses on proliferation of cultured villous CT cells isolated from all three trimesters of pregnancy. The extent of the response on CT cells isolated from the three different trimesters also appears to be very similar.

EGF has been shown to enhance the transcription of early response genes c-fos and c-myc in murine BALB/c 3T3 fibroblasts and porcine follicle cells (Ran *et al.*, 1986; Heldin & Westermark 1988). Transcriptional upregulation of c-fos, c-myc can occur via EGFR mediated activation of protein kinase C (Ulrich & Schlesinger 1990; Cross & Dexter 1991). Human term placental CT cells are known to express c-myc and c-fos proteins however, EGFR mediated transcriptional activation of c-myc, c-fos may not be occuring in the senescing placenta. This may explain the inability of TGFα, EGF to stimulate proliferation late in gestation.

Our studies also showed that concentrations of TGFα ranging between 10-50ng per ml did not exert any visible effect on the differentiation of CT cells isolated from placenta close to term. On the other hand studies by Morrish (1987), Qu and colleagues (1992), have shown that the rate of morphological and functional differentiation of cultured CT cells from term placenta increased with addition of EGF in a dose dependant manner. They found that following treatment with EGF the size of syncytial aggregations increased, mitochondrial numbers were higher and that the secretion of hCG, hPL was enhanced (Morrish *et al.*, 1987; Qu *et al.*, 1995). The different effects exerted by EGF and TGFα on differentiation of CT cells could be due to factors such as different binding affinities to the EGFR and modulation of binding to the receptor by other EGF related growth factors. Although TGFα has been shown to interact with the EGFR on human placental membranes (Massague 1983), there have been very few studies that have compared the receptor mediated chain of phosphorylated second messengers generated by the binding to TGFα and EGF to the EGFR on CT or ST cell membranes. In the human

placenta EGF has been shown to phosphorylate a lipocortin related protein and peptides of 25, 35 and 150 kDa (Carpenter *et al.*, 1980; Valentine-Braun *et al.*, 1986; Sheets *et al.*, 1987). Previous studies have also shown that in the human placenta EGFR are not confined to the plasma membrane but have been localised to the endoplasmic reticulum, Golgi apparatus, nuclear membrane and found that EGF can phosphorylate these receptors (Ramani *et al.*, 1986; Cao *et al.*, 1995). The interaction of TGFα with the intracellular receptors has not been described and it is possible that such interactions may contribute to differences in their modes of action.

Our findings also showed that CT cells were actively synthesising progesterone during short term culture but that TGFα exerted no effect on the amount of progesterone produced and secreted by these cells. We believe that this is the first report describing the effect of TGFα on placental progesterone synthesis.

The study was carried without the addition of any exogenous substrates such as LDL or pregnenolone as these substrates have been found to enhance progesterone synthesis in a dose dependant manner *in vitro* (Winkel *et al.*, 1980; Maslar *et al.*, 1990). Short term cultures were used so as to study the effect of the growth factor when the majority of cells were CT in order to minimise effects due to syncytial synthesis of progesterone. The amount of progesterone produced by ST cells has been found to be higher compared with CT cells *in vitro*. In addition increasing concentrations of progesterone has been reported to inhibit the activity of mitochondrial 3βHSD enzyme in the conversion of pregnenolone to progesterone *in vitro* (Das *et al.*, 1985; Rabe *et al.*, 1985).

IGF-I is the only growth factor to date that has been reported to stimulate progesterone synthesis in normal CT cells *in vitro* by increasing the activity of P-450scc enzyme by 19-36% (Nestler & Williams 1987). In the choriocarcinoma cell line JEG-3, EGF has been reported to stimulate progesterone production in the *de novo* pathway where the growth factor has been found to increase the incorporation of labelled acetate into cholesterol (Bahn *et al.*, 1980). In contrast Ilekis & Benveniste (1985), did not observe an increase in progesterone synthesis in JEG-3 cells effects following treatment with EGF.

In other steriodogenic cells however TGFα has been found to play a role in progesterone synthesis. For example a two fold increase in progesterone synthesis in response to 50ng TGFα per ml was observed in rat luteal cells pretreated with media and serum, although no effects were observed in pre-stimulated cells (Tekpetey *et al.*, 1995a). In another study by Tekpetey and colleagues (1995b), TGFα was reported to increase progesterone synthesis in human granulosa lutein cells in a dose dependant manner with a maximal four fold stimulation at a concentration of 50ng/ml and a further synergistic increase observed in the presence of LH. In contrast however TGFα has been reported to lower the stimulatory effects of IGF-1 and IGF-1 plus LH on progesterone synthesis in chicken granulosa cells (Ongabesan & Peddie 1995).

Our findings did reveal however that the relative amount of 3βHSD mRNA is lowered in response to physiological concentrations of TGFα. The inhibition of transcription was almost immediate but transient as the relative amount of 3βHSD mRNA reached steady state levels within 3 hours of treatment with TGFα. The effect was also specific to TGFα and not due to synergistic effects as the decrease in mRNA transcription was arrested in the presence of neutralising anti-TGFα antibodies. Although we observed a lowering of 3βHSD mRNA expression progesterone levels were unaffected during the course of the experiment. An explanation is that the 3βHSD enzyme catalysed conversion of pregnenolone to progesterone may not be a rate limiting step.

There have been no previous reports of the regulation of 3βHSD gene expression by any growth factor in the human placenta. However IGF-I has been shown to increase the expression of 3βHSD in rat granulosa cells and ovarian theca-interstitial cells (Adashi & Resnick 1986; Magoffin & Weitsmann 1993). cAMP, hCG have also been shown to increase the transcription of this gene in porcine granulosa cells in a dose dependant manner (Chedrese *et al.*, 1990a). hCG and cAMP are known to activate the PKA signal transduction pathway increasing the expression of the transcription factor AP-2 (Feige *et al.*, 1992). In porcine granulosa cells treated with hCG or cAMP enchanted 3βHSD mRNA expression probably occurs via the AP-2 inducible region found in the 5' promoter region of the 3βHSD gene (Lachance *et al.*, 1990).

Chedrese and colleagues (1990b), found that the phorbol ester PMA suppressed the increase in 3βHSD mRNA expression induced by PKA activators such as cAMP and hCG in porcine granulosa cells in vitro. They proposed that PKC activation by PMA may interfere with cAMP induced gene expression. In another study using porcine granulosa cells the enhanced expression of 3βHSD mRNA by LH was shown to be lowered significantly by PMA and angiotensin II which is also another PKC activator (Li *et al.*, 1995).

Interaction of TGFα with the EGFR has been found to stimulate the PKC signal transduction pathway. It is therefore possible that the decline in 3βHSD mRNA expression that we detected in CT cells following treatment with TGFα occurs through a mechanism involving PKC interference of the PKA pathway. It is also conceivable that the transient nature of the decline in 3βHSD expression may have been arrested by progesterone and estradiol. Recent studies by Beaudoin and co-workers (1997), have demonstrated that 3βHSD and p450scc gene expression is enhanced in CT and ST cells by progesterone and estradiol. It also remains uncertain whether TGFα plays a role in the regulation of expression of P450scc mRNA and thereby regulates progesterone synthesis in the CT and ST cells of the placenta.

CHAPTER 7

Identification of Upregulated mRNA in Cytotrophoblast Cells Treated with Transforming Growth Factorα

The interaction of TGFα with the EGFR ultimately results in the expression of mRNA's encoding proteins that brings about the cellular responses. In this study efforts were made to identify the mRNA that were being upregulated or transcribed in CT cells treated with TGFα so that the regulatory effects exerted by TGFα in these cells could be better understood.

7.1 Introduction

The human placenta contains several distinct trophoblast populations. Some of these trophoblast cells are proliferative while others represent their differentiated lineages (Boyd & Hamiltion 1970). One of the proliferative trophoblast populations is the villous CT cells that are found below the ST layer in the chorionic villi (Gerbie *et al.*, 1968). An important function of the villous CT is that it differentiates to form the multinucleated syncytial layer which provides an extensive surface for exchange and endocrine function (Patillo 1983). Previous studies have also shown that the villous CT cells produce a large array of growth regulatory substances that are capable of autocrine, paracrine, juxtacrine and endocrine regulation (Lala & Hamilton 1996; Albrecht & Pepe 1990).

TGFα is a known growth regulatory peptide that stimulates the proliferation of epithelial, mesenchymal and endothelial cells (Derynck 1986; Schreiber *et al.*, 1986). The peptide has also been shown to regulate hormone synthesis. For example progesterone synthesis was reported to increase in rat ovarian granulosa cells treated with TGFα wheras placental lactogen 1 synthesis was reported to decrease in response to the growth factor in murine CT cells (Yeh *et al.*, 1993; Yamaguchi *et al.*, 1995).

Immunolocalisation studies have shown that TGFα protein is present on the surfaces of human villous CT cells throughout gestation (Filla *et al.*, 1993; Faber *et al.*, 1996; Lysiak *et al.*, 1993). Previous studies have also shown that TGFα protein is synthesised and secreted by cultured villous CT cells isolated from second and third trimesters of pregnancy (Filla *et al.*, 1993; Chapter 5). Although TGFα has been demonstrated to increase the rate of proliferation of cultured villous CT isolated from the first and second trimesters (Filla *et al.*, 1993), recent data shows that it does not influence the proliferation or aggregation of CT cells isolated from placentae delivered after 38-40 weeks gestation (Chapter 6). In our studies we found that 3βHSD mRNA expression was inhibited in a transient manner in cultured CT cells isolated from third trimester placenta following treatment with TGFα (Chapter 6). Although we are unaware of other studies that have investigated the effects of TGFα on mRNA expression in villous CT cells, TGFα has been shown to enhance gelatinase A and the protease inhibitor TIMP-1 mRNA expression in EVT cells isolated from the first trimester (Lysiak *et al.*, 1994). Proteases such as

gelatinase A and their inhibitors are believed to play important roles in the remodelling and proteolytic degradation of the extra cellular matrix that is essential for haemochorial placentation. TGFα has also been reported to increase 17βHSD type 1 mRNA expression and protein in the choriocarcinoma cell lines JAR, JEG-3 (Lewintre *et al.*, 1994). The enzyme 17βHSD has been shown to catalyse the reversible interconversion of estrone and estradiol (Isomaa *et al.*, 1993).

It is possible that TGFα may be regulating the expression of other mRNA in normal placental villous CT cells isolated from placentae towards the end of pregnancy. The up or down regulation of mRNA expression can be studied using different approaches and techniques. In the past techniques such as Northern hybridization and quantitative RT-PCR have been used extensively to examine changes in mRNA expression. The main drawback of this approach is that the identity of the mRNA species being examined has to be known beforehand and limits the investigation to a few mRNA species at a time. Techniques such as RDA, on the other hand, provide an alternative means of studying gene expression. This method was developed initially to investigate differences between genomes but has been successfully adapted to investigate differences in gene expression (Lisitsyn *et al.*, 1993; Hubank & Schatz 1994). The basis of the RDA technique involves the conversion of mRNA to cDNA using PCR. The cDNA that are common to both populations are removed by subtractive hybridization and the remaining cDNA species that is unique to one of the populations can be identified (Hubank & Schatz 1994). The main advantage of RDA over Northern hybridization and quantitative RT-PCR is that differences in entire populations of mRNA can be examined without assuming pre-knowledge of mRNA species. The technique is therefore being utilised more frequently to study differences in mRNA expression in response to biochemical stimulus at different stages of development. Recently four mRNA species that were upregulated in PCR12 pheochromocytoma cells due to treatment with nerve growth factor but not with EGF were described using RDA (Vician *et al.*, 1997). In other recent studies that have utilised RDA, the cytokine IL-4 was found to induce Fig1 mRNA in murine B cells and six mRNA species were found to be stimulated in NIH-3T3 cells due to the activation of the receptor c-erbB-3 compared with the activation of the structurally related receptor EGFR (Chu & Paul 1997; Edman *et al.*, 1997).

RDA therefore provides a means of identifying mRNA that may be upregulated or transcribed in villous CT cells treated with TGFα. Such an approach may provide further insights into the regulation of mRNA expression by TGFα and provide a greater understanding of the effects exerted by this growth factor in villous CT cells *in vitro*. Therefore this study was undertaken to:

- determine whether there are differences in the mRNA species present in cultured CT cells treated with TGFα compared with untreated control cells
- identify mRNA species that were being upregulated or transcribed in cultured CT cells treated with TGFα by comparing the nucleotide sequences of the mRNA species identified with known data.

7.2 Methods

CT cells were isolated from villous cotyledons removed from placenta delivered by Caesarean section after 38 weeks gestation (n=3). The isolation procedure, morphological appearance and immunological characterisation of CT cells are described in Chapter 2, section 2.2. CT cells that underwent treatment (ie. tester) were dosed with 15ng TGFα per $0.75x10^6$ cell/ml (EMEM with 10% FCS) and grown for 2h in liquid culture in a shaking water bath at 37°C. Total RNA was extracted from tester and untreated control (ie. driver) cells by the spin column procedure. The culture conditions of tester, driver cells and the isolation of total RNA are described in Chapter 2, sections 2.11.1 and 2.3.2.1 respectively. The quality of the total RNA was assessed by spectrophotometry, formaldehyde agarose gel electrophoresis and RT-PCR analysis of GAPDH mRNA as outlined in Chapter 2, sections 2.3.1, 2.3.3 and 2.4.3 respectively. Total RNA from driver and tester were converted to cDNA and cDNA species common to both tester and driver were removed by two successive rounds of subtractive hybridization using the RDA procedure. The cDNA species detected in the tester following subtractive hybridization were cloned into the TA vector and nucleotide sequences were determined. FASTA database searches were carried out to determine the identity of the cloned cDNA. The RDA procedure is given in detail in Chapter 2, section 2.11.

7.3 Results

7.3.1 Morphological appearance and characterisation of CT cells

The cells isolated from the villous cotyledons were rounded in appearance, mono-nucleated and between 12-20μm in diameter. Some of the cells were found to be dividing and after 2h in liquid culture the total number of cells increased from a seeding density of $0.75x10^6$ cells/ml to mean $0.89x10^6$ ±SEM 0.23 and $0.92x10^6$ ± 0.17 cells/ml in the pooled treated, control cultures respectively. hCG was not detected in media conditioned by control or treated cells using an immunofluorescent assay specific for human hCG.

Cells fixed in methanol immediately after isolation and after 2h in liquid culture stained strongly with antibodies directed against cytokeratins 8 and 18 (Fig. 7.1).

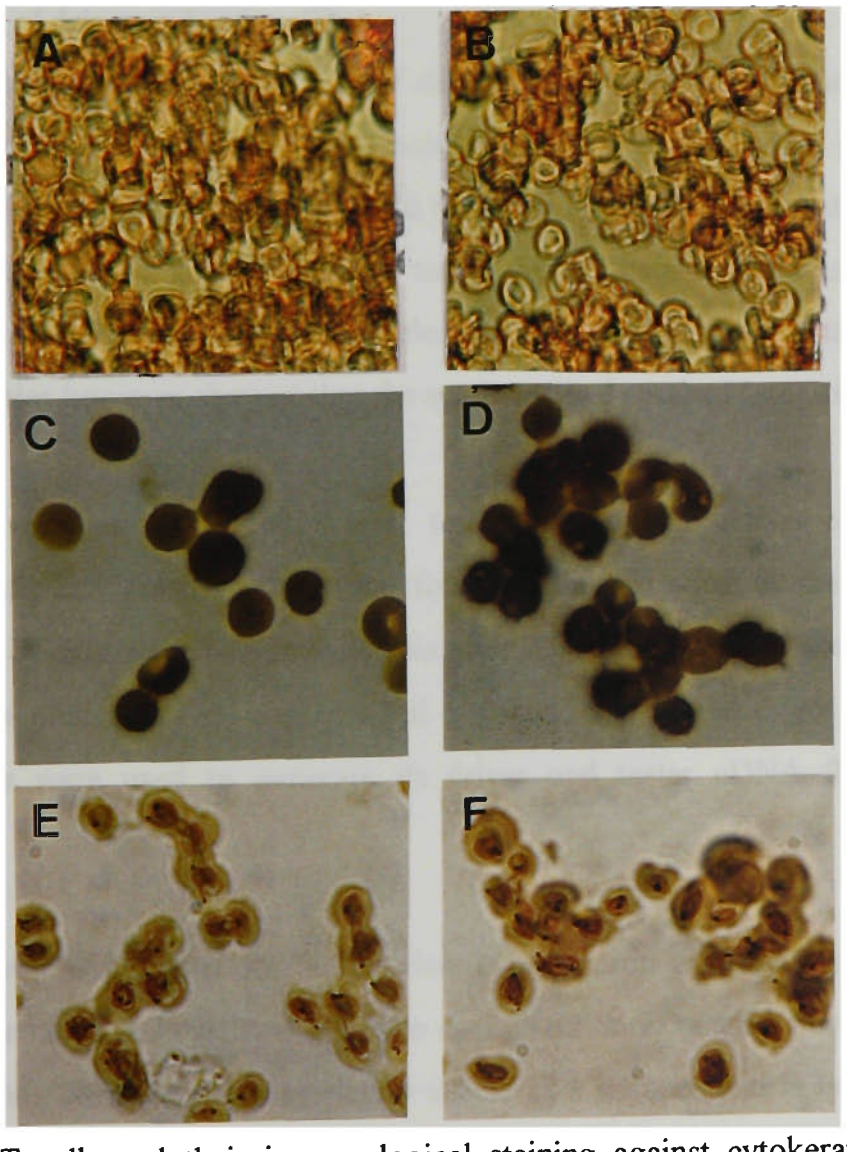


FIG 7.1 CT cells and their immunological staining against cytokeratin. A & B - unstained, freshly isolated and cells cultured for 2h in liquid culture respectively. C & D- freshly isolated and cells cultured for 2h in liquid culture showing strong staining against cytokeratin 8. E & F cells stained with 10% non immune serum only. Magnification = A & B = 250x, C-F = 350x.

7.3.2 Quality assessment of tester and driver RNA preparations

Driver and tester total RNA samples that were used for subtraction experiment had mean A_{260}:A_{280} values of 1.65 and 1.72 respectively. The mean ratios of the size, intensity of the 28S:18S rRNA were found to be 1.8 and 1.7 for driver and tester respectively when total RNA samples were resolved on formaldehyde agarose gels.

A single cDNA fragment 531bp is size was found to be amplified when 1.0μg of tester and driver total RNA was reverse transcribed and cycled 25 times by PCR using primers specific for GAPDH mRNA.

7.3.3 Synthesis of driver and tester cDNA

Preliminary experiments showed that 1μl of diluted driver and 2μl of diluted tester first strand cDNA that was used to synthesise double stranded (ds) cDNA had to be cycled a minimum of 21 times by PCR before it could be visualised on EtBr stained agarose gels. When these samples were amplified further to 23 cycles, tester and driver cDNA were found to be exponentially amplified. Samples analysed by agarose gel electrophoresis after 25 PCR cycles however showed that the syntheses had reached saturation.

Increasing the volumes of diluted driver, tester first strand cDNA to 2 and 3μl respectively during 2nd strand synthesis was found to have no effect on the amount of ds cDNA detected by agarose gel electrophoresis after 23 PCR cycles. Therefore based on the results of the preliminary experiments 2μl of diluted first strand driver, tester cDNA cycled 23 times were used to generate ds driver and tester cDNA for subtractive hybridization.

Driver and tester cDNA used for subtractive hybridization appeared as a smear on agarose gels after 21 and 23 PCR cycles; the size distribution ranging between 0.3-6kb. A cDNA fragment 740bp in size was clearly visible as a diffuse band in both driver and tester cDNA samples after 21 and 23 rounds of PCR (Fig. 7.2-A).

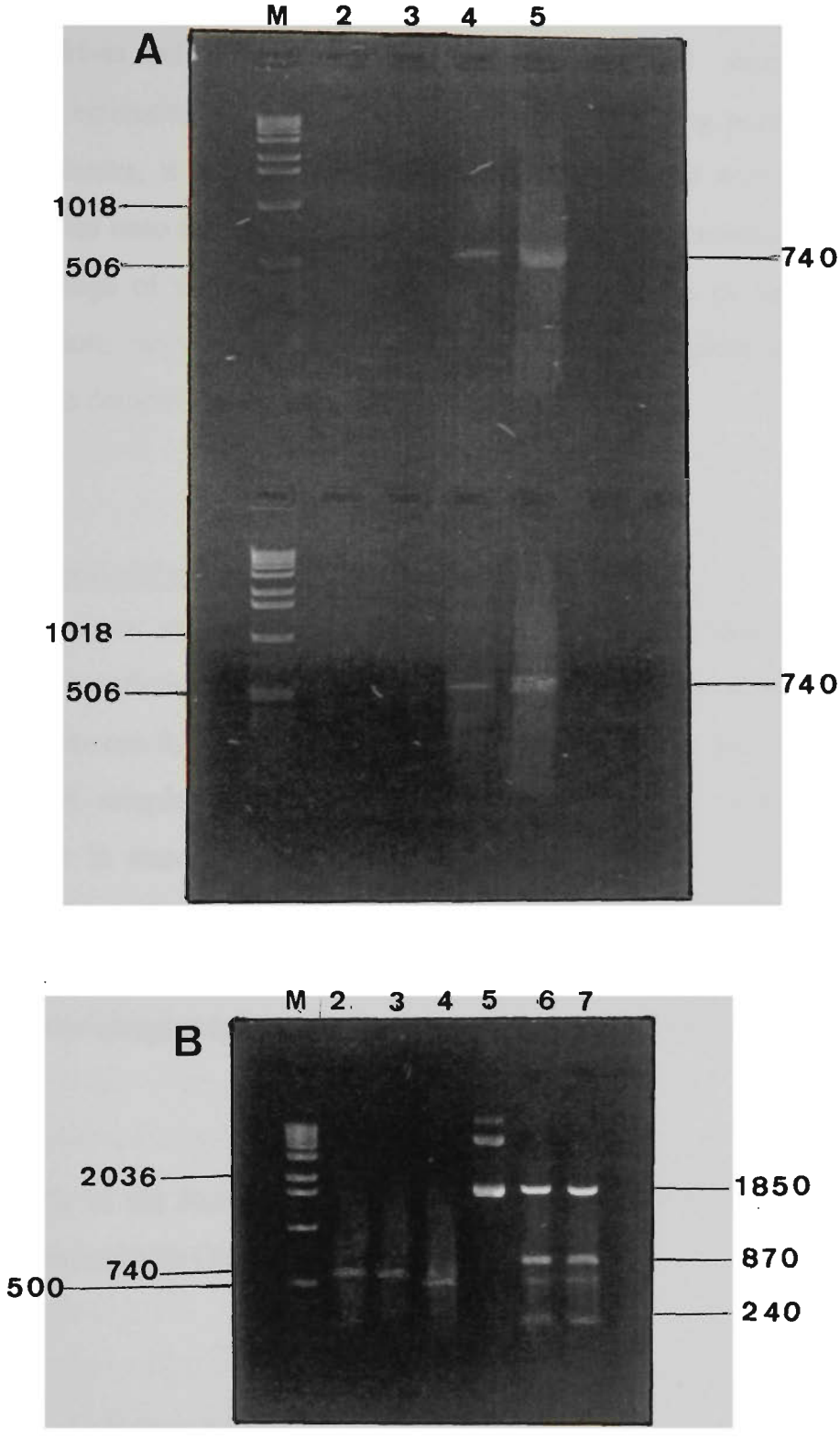


FIG. 7.2 Double stranded cDNA synthesis and *Rsa* 1 digestion. **A)** 5µl volumes of second strand tester (T), driver (D), cDNA syntheses mixes resolved on 1.4% EtBr stained agarose gels after 15, 18, 21 and 23 PCR cycles (lanes 2-5). **B)** Lanes 2-3 contain undigested, tester cDNA samples, 4 = tester cDNA digested with *Rsa* I, 5 = cDNA spiked with plasmid PUC18 DNA before digestion and lanes 6-7 cDNA spiked with plasmid PUC18 DNA after *Rsa* I digestion. M=DNA molecular weight marker; sizes are given in bp.

The yield of ds driver and tester cDNA from 2µl of diluted first strand cDNA after 23 PCR cycles was estimated to be between 600-800ng. Following purification using the Chroma spin columns, it was estimated that 42% of tester and 46% of driver cDNA respectively applied onto the columns were recovered in eluted fractions labelled 320 and 75. The size range of the eluted, purified cDNA appeared to be unaffected by spin column purification, ranged between 0.3-6kb in size and contained the 740bp cDNA fragment that was detected in the unpurified samples.

7.3.4 *Rsa* I digestion of tester and driver cDNA

Column purified driver and tester cDNA samples were found to have been successfully cleaved with the restriction enzyme *Rsa* I. The size distribution of the *Rsa* I digested cDNA ranged between 0.15-3kb whereas the size of the column purified control driver and tester cDNA samples ranged between 0.3-6kb. The size of the 740bp cDNA fragment present in tester and driver samples decreased in to approximately 500bp following digestion with *Rsa* I. Samples of tester and driver cDNA spiked with the plasmid PUC 18 which served as positive controls for *Rsa* I digestion showed that the plasmid DNA was completely cleaved into two fragments of 750 and 1950bp (Fig. 7.2-B).

Between 76-81% of the *Rsa* I digested cDNA was estimated to have been recovered following purification with Qiaex II spin columns.

7.3.5 Ligation of adaptors 1 and 2

Adaptors 1 and 2 were found to have been successfully ligated onto the digested tester cDNA after an overnight incubation at 16°C. This assessment was made on the basis that cDNA samples ligated with both adaptors that served as positive controls for ligation, were successfully amplified using PCR primers 1, 2 that were complimentary to adaptors 1, 2 respectively. Following 30 cycles of PCR the positive control cDNA was clearly visible as a smear on agarose gels and ranged in size between 0.2-2.8kb. Distinct bands were not observed within the smear. Amplified products were not visualised on agarose

gels from tester cDNA samples ligated with adaptor 1 or 2 that served as negative ligation controls after 30 PCR cycles (Fig. 7.3-A).

7.3.6 First and second subtractive hybridizations

The presence or absence of GAPDH cDNA that was used to monitor the success of the subtractive hybridization showed that GAPDH cDNA was present in the unsubtracted tester cDNA. When diluted, unsubtracted, *Rsa* I digested tester cDNA was amplified 20 times by PCR with GAPDH specific primers a 531bp cDNA fragment was visualised on agarose gels. In contrast even after 30 PCR cycles, the 531bp GAPDH cDNA fragment was not detected in the digested tester cDNA samples following the second subtractive hybridization. These findings showed indirectly that the subtraction of common cDNA by excess of driver incorporated during the first and second hybridizations appeared to have succeeded (Fig. 7.3-B).

7.3.7 Primary and nested PCR

Subtracted tester cDNA amplified 30 times with PCR primers 1, 2 appeared as a smear ranging between 0.25-1.5kb in size. Distinct cDNA bands were not observed within this smear. The unsubtracted tester control samples also produced a smear on agarose gels that was similar in size to the subtracted tester samples. Differences were observed however in the concentrations of subtracted tester and control cDNA following PCR. The cDNA concentration in the subtracted tester was estimated to be 2.4x the concentration of the control (Fig. 7.4-A).

Following the nested PCR of subtracted tester cDNA with nested PCR primers 1, 2, seven bands were visible within a background smear after 11 PCR cycles. When the number of PCR cycles was reduced to 9, the bands were less distinct whereas cycling the samples 13 times increased the background to an extent that the cDNA fragments became obscured. The sizes of the seven cDNA bands were estimated to be 655, 580, 520, 400, 310, 220 and 115bp in size. A 655bp band that was identical in size to a fragment found in the subtracted tester was also present in the unsubtracted tester control reaction mix.

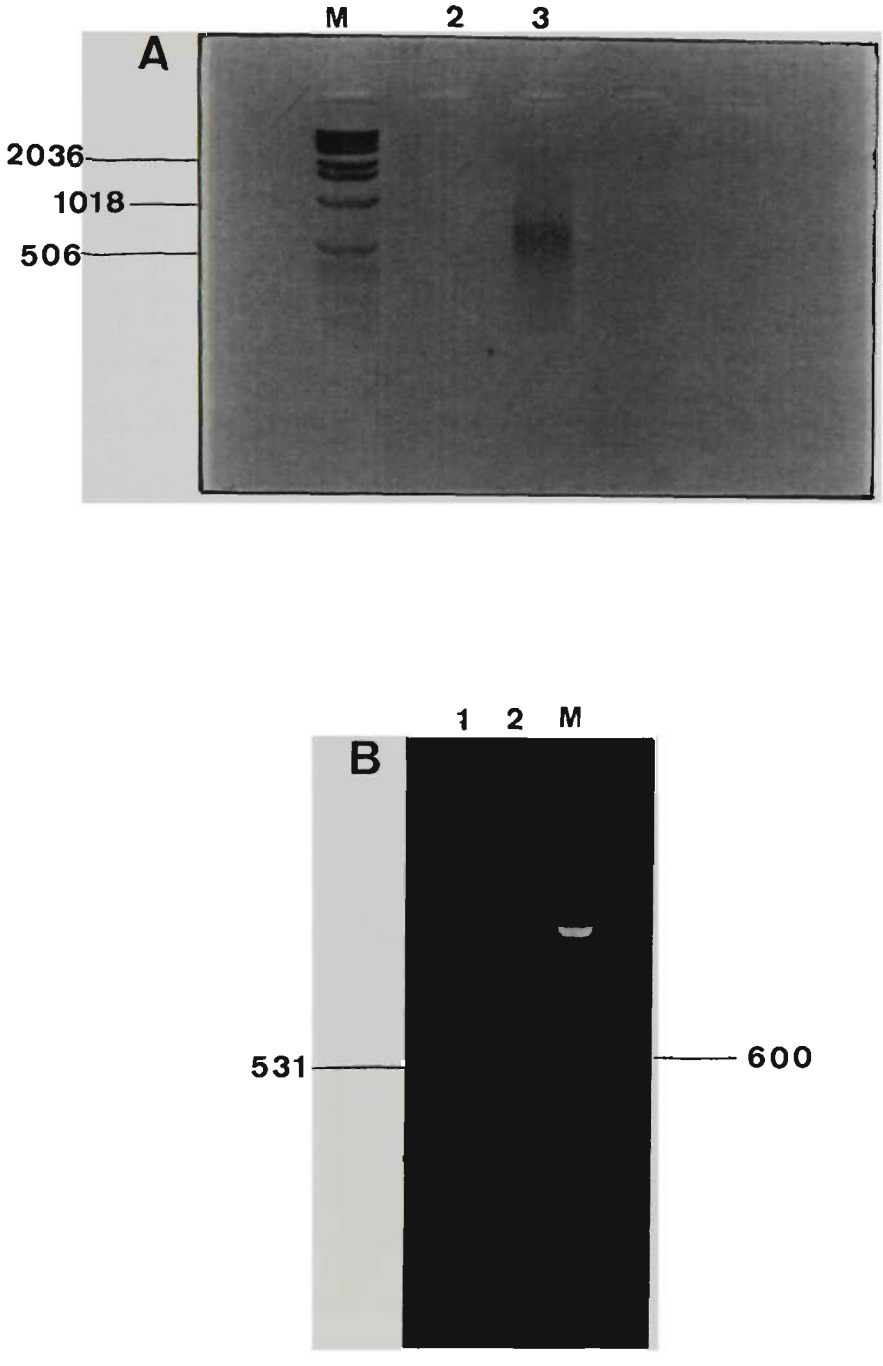


FIG. 7.3 **A)** PCR amplification of *Rsa* I digested tester cDNA. Lane 2 = digested tester cDNA ligated with adaptor 1, lane 3 = digested tester cDNA ligated with adaptors 1 and 2. Both samples were amplifed 30 times with PCR primers 1 and 2. **B)** Amplification of GAPDH cDNA in *Rsa* 1 digested tester. Lane 1=unsubtracted tester, amplified 20 times and lane 2=subtracted tester cDNA amplified 30 times with GAPDH specific primers. M=DNA size marker; sizes are given in bp.

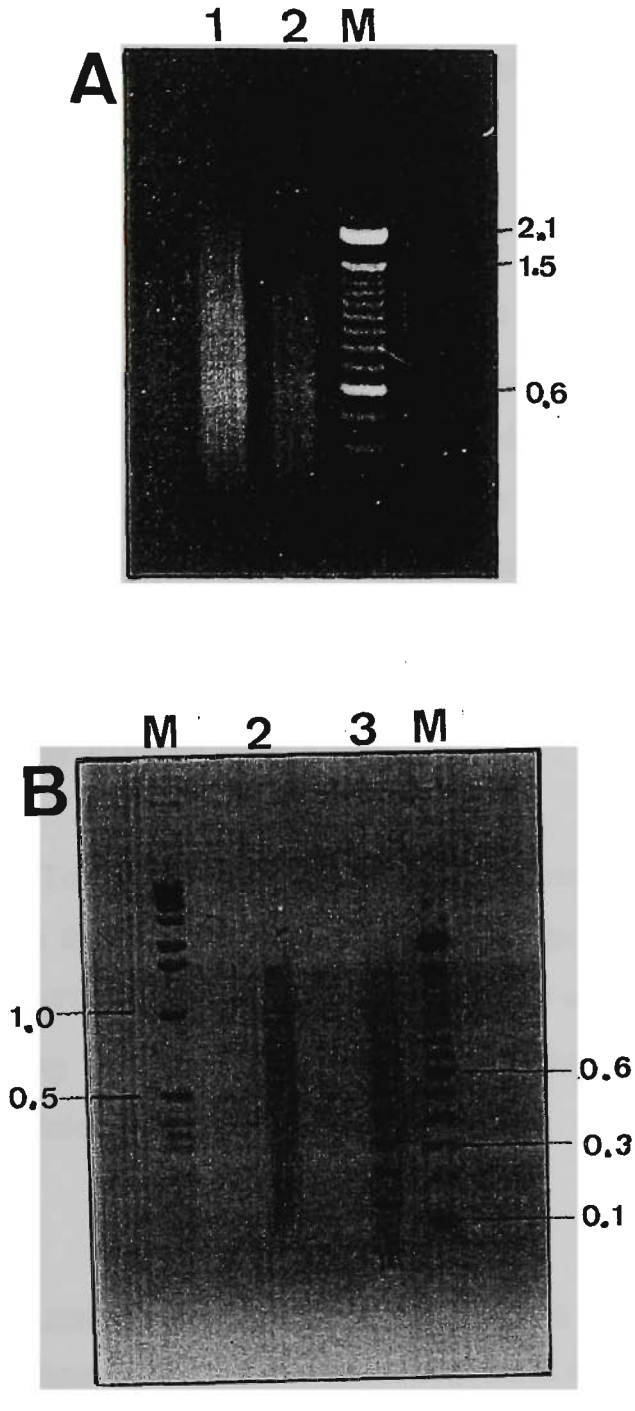


FIG. 7.4 Analysis of cDNA following subtractive hybridizations. **A)** cDNA after 30 cycles of primary PCR with PCR primers 1 and 2. Lane 1=subtracted tester and 2=unsubtracted tester. **B)** cDNA after 11 cycles of nested PCR with nested PCR primers 1 and 2. Lane 2=unsubtracted tester and lane 3=subtracted tester. M=DNA size marker; sizes are given in kb.

also present in the unsubtracted control on agarose gels. The size of the background smear in the control samples and subtracted tester after the 2nd hybridization ranged between 0.2-1.8kb in size when samples were analysed on agarose gels (Fig. 7.4-B).

7.3.8 Post-subtractive PCR amplification and cDNA cloning

The cDNA fragments 655, 580, 520 and 310bp in size that were detected in the subtracted tester after the nested PCR were successfully re-amplified using 20 PCR cycles and nested PCR primers 1, 2. The 440bp fragment detected in the subtracted tester was not amplified by PCR after several attempts. Attempts were not made to amplify the 220 and 115bp fragments that were also detected in the subtracted tester.

The cDNA bands, 655bp in size, detected in the control and subtracted tester were also successfully re-amplified after 20 cycles of PCR (Fig. 7.5-A).

Screening of *E.coli* strain Top 10' transformants by PCR showed that the re-amplified 580, 520, 310bp fragments from the tester had ligated with the TA cloning vector and could be amplified by PCR (Fig. 7.5-B). While several attempts were made to recover the 655bp fragments originating from the subtracted tester and control from transformed *E.coli* cells these attempts failed.

7.3.10 Nucleotide sequence analysis of the cloned cDNA fragments

Using either nested PCR primer 1 or 2 the nucleotide sequences of the 580, 310bp cloned cDNA inserts were obtained through automated DNA sequencing. Neglecting both ends of these two fragments where the nucleotides could not be identified with certainty, the sequences obtained with the forward nested PCR primer 1 were found to be complimentary to the sequences obtained with nested PCR primer 2. This finding showed that the sequences were obtained from double stranded fragments with complimentary base pairing.

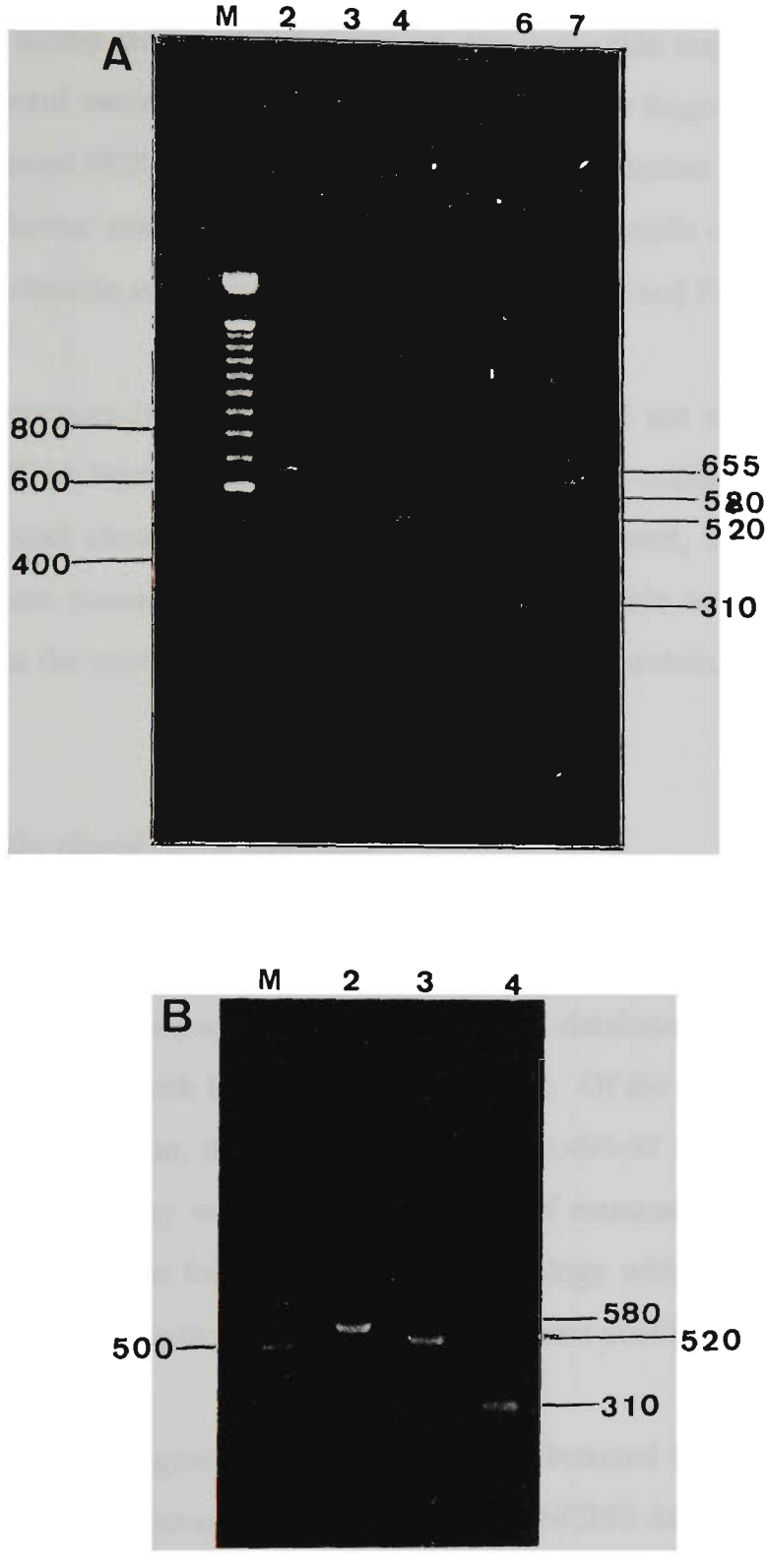


FIG. 7.5 **A)** amplification of subtracted cDNA fragments. cDNA fragments amplified from subtracted tester (lanes 2-4, 6) and unsubtracted tester control sample (lane 7) with nested PCR primers 1 and 2. **B)** amplification of cloned cDNA. Lanes 2-4 contain cloned fragments amplified by 20 rounds of PCR with the nested PCR primers 1 and 2. M=DNA size ladder; sizes are given in kb. Samples are resolved on a 1.4% agarose gel.

In the case of the 520bp cloned cDNA fragment, the nucleotide sequence was obtained using only the forward nested PCR primer 1. When the same fragment was sequenced with the reverse nested PCR primer 2, a poly-A region was detected near the start. This region prevented further analysis due to 'enzyme stuffing'. Details of the cloned inserts and parts of the nucleotide sequence data are given in Table 7.1 and Fig. 7.6 respectively.

The nucleotide sequences obtained from the 3 fragments did not contain transcription initiation sites or rRNA binding regions. The base triplet ATG coding for the amino acid methionine was found close to the 5' termini of each fragment, however due to the absence of upstream consensus sequences it was not possible to determine if these methionines were at the start of translation or were part of the protein.

7.3.11 Identity of the cloned cDNA fragments

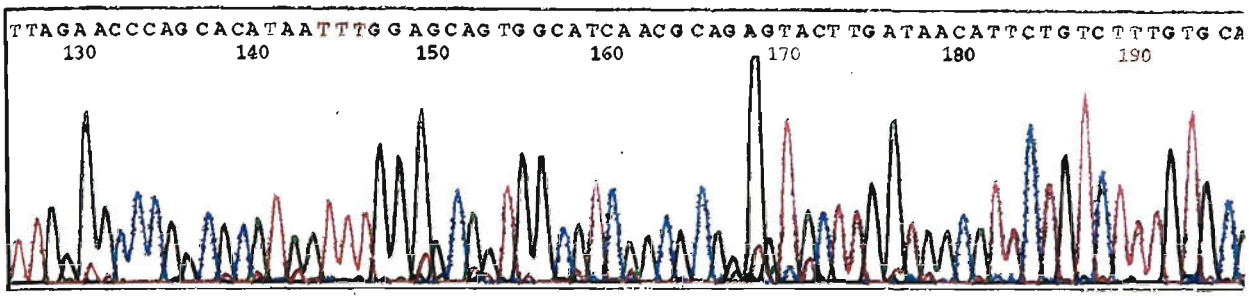
A 87bp region of the 580bp cloned cDNA fragment was found to have 62% homology with the human coagulation factor IX gene when the nucleotide sequences of the cDNA was compared with known sequences using the ANGISS database. This was the highest degree of homology found with known human sequences. Of the 499 bases of this clone that were used for comparison, the greater proportion (ie.499-87 = 312bp) was found to have no significant homology with known sequences of mammalian origin. A shorter stretch of 65 nucleotides was found to have 67% homology with the yeast centromere. Based on these results the identity of the 580bp cloned insert could not be determined.

In the case of the 310bp fragment obtained from the subtracted tester, a stretch of 270 bases were compared with known sequences using the ANGISS database. Regions within a 209 stretch of nucleotides were found to have 74-82% homology to human cDNA clones 141802, 269758 and 334P19, 225D2. The remaining 61 bases analysed did not show any significant homology to known DNA sequences. Therefore based on these results the identity of this fragment could not be determined.

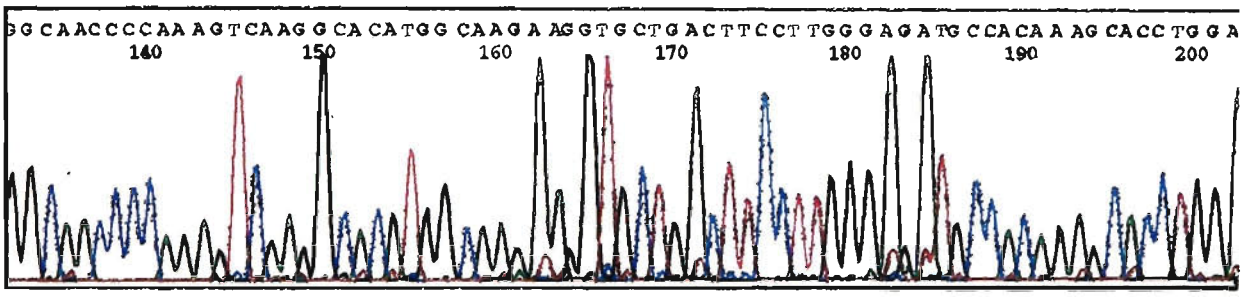
TABLE 7.1 Summary of cDNA fragments analysed

estimated size (bp)	re-amplified by PCR	cloned into TA cloning vector	number of bases obtained by DNA sequencing	number of bases used for ANGISS database comparison
Tester 655	yes	no		
580	yes	yes	531	460
520	yes	yes	504	399
440	no	not attempted		
310	yes	yes	305	270
220	not attempted	not attempted		
115	not attempted	not attempted		
Driver 655	yes	no		

A



B



C

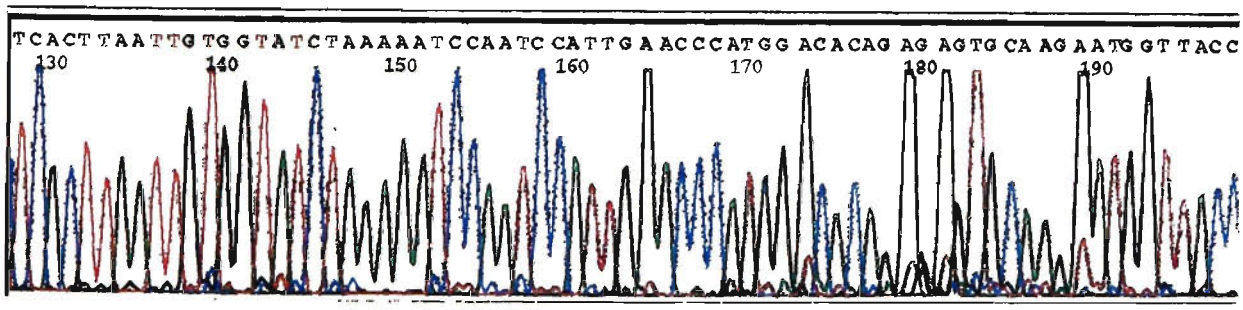


FIG. 7.6 Nucleotide sequences of the cloned cDNA inserts. A part of the nucleotide sequences of the 580, 520 and 310bp fragments obtained through automated DNA sequencing is depicted in panels A, B and C.

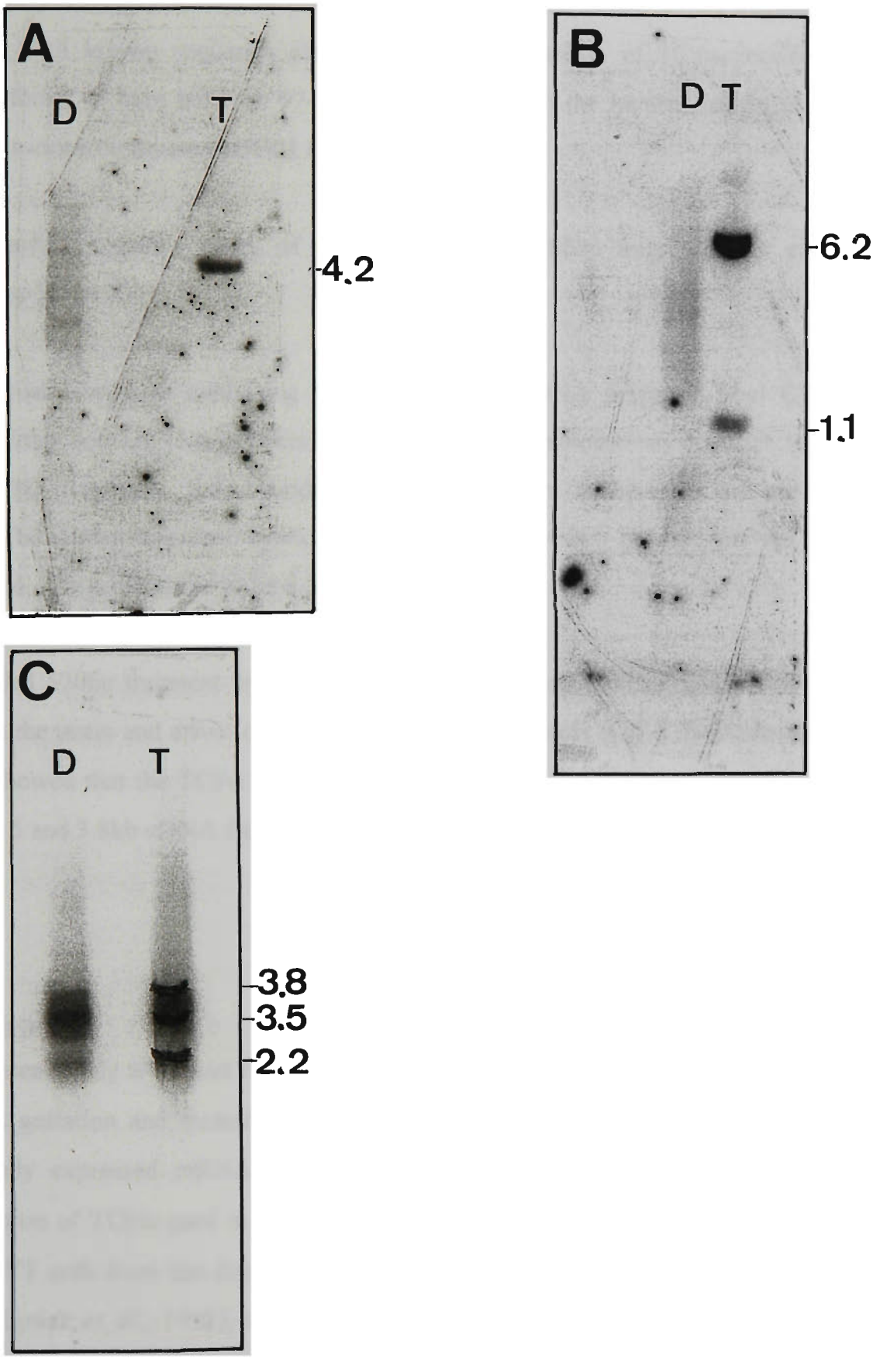


FIG. 7.7 Virtual Northern blot hybridization. Blots containing tester (T) and driver (D) cDNA probed with labelled 310bp fragment (A). Blot probed with 580bp fragment (B) and probed with 520bp fragment (C). Sizes of the hybridization products are given in kb.

A stretch of 399 bases obtained by DNA sequencing from the 520bp cDNA fragment was compared with known sequence data. Except for a stretch of 30 nucleotides, the sequence found to have between 97-100% homology with the human gamma G globin mRNA in a clone designated pHG21 (BLAST search data).

The nucleotide sequence traces of the 310, 520 and 580bp fragments are given in Appendices 14-16.

When virtual Northerns containing tester and driver cDNA prepared from CT cells obtained from another two patients were probed with radioactively labelled 580 and 310bp cDNA fragments, they hybridized to cDNA present in the tester but not in the driver. The 310bp fragment hybridized to a fragment 4.2kb in size and the 580bp fragment to cDNA estimated to be 6.2 and 1.1kb in size.

The labelled 520bp fragment hybridized to cDNA fragments 1.6, 3.5 and 3.8kb in size present in the tester and driver cDNA on virtual Northern blots (Fig. 7.7). Densitometric analysis showed that the TGFα stimulated tester contained 3.4-3.7 times higher levels of the 1.6, 3.5 and 3.8kb cDNA fragments compared with the control driver samples.

7.4 Discussion

In the present study we found that villous CT cells isolated from placenta delivered after 38 weeks gestation and treated with the growth factor TGFα were found to contain differentially expressed mRNA species compared untreated control CT cells. The concentration of TGFα used in our study was 15ng per $0.75x10^6$ cells/ml. In previous studies EVT cells from the first trimester have been treated with 0.1-100ng TGFα/10^4 cells/ml (Lysiak *et al.*, 1993), villous CT from the first trimester dosed with 0.3-30ng TGFα/$2x10^4$ cells/ml (Li & Zhang 1997) or 20ng TGFα/$1.5x10^4$ cells/ml (Filla *et al.*, 1993). In each of these previous studies TGFα was reported to exert a mitogenic response. In our study the cells were seeded at a higher density compared with the earlier

studies but the concentration of TGFα available per 10^4 cells were within the ranges used by Lysiak (1993) and Li and Zhang (1997), but lower than that of Filla (1993).

Using RDA we detected seven cDNA fragments in the TGFα treated tester CT cells that ranged in size between 110-655bp. Another cDNA fragment that was estimated to be 440bp in size could not be isolated from the subtracted reaction mix by PCR for cloning after several attempts. It is possible that what appeared to be a genuine amplicon may have been a PCR artefact. Attempts were not made to amplify the two smallest cDNA bands detected in the subtracted tester estimated to be 115 and 220bp as the fragments were assessed as being too small to generate enough nucleotide sequence data for identification.

An amplicon estimated to be 655bp was detected in both the tester and untreated driver. We were unable to recover the 655bp fragments originating from either tester or driver from transformed E. coli cells thus preventing further analysis. A possible reason why this may have occurred is that the amplicon may not have ligated with the plasmid owing to the presence of overhanging ends.

The nucleotide sequences of three cDNA fragments 580, 520 and 310bp in length were compared using BLAST searches through the ANGISS database. A small stretch of the 580bp fragment displayed 62% homology to the human coagulation factor IX gene, but 77% of the nucleotides compared did not show any significant homology to known mammalian cDNA or genomic sequences. Segments of the 310bp fragment, the longest being a stretch of 145 nucleotides had between 72-84% homology to clones that are currently being sequenced (BLAST search data). One of these clones designated 269758 has been described as having some similarities with the alcohol dehydrogenase mRNA (BLAST search data). In the human liver the enzyme alcohol dehydrogenase has been shown to inter-convert acetaldehyde to alcohol generating either NAD or NADH (Voet & Voet 1990).

On the basis of the results from the BLAST searches, the 310 and 580bp cDNA fragments could not be identified. The inability to identify cDNA found through RDA by

comparing with existing nucleotide sequence data does present a difficulty that previous studies utilising RDA have also encountered (Niwa *et al.*, 1997; Lewis *et al.*, 1997). This drawback prevents further analysis particularly those involving protein expression and functional studies. Such studies may prove valuable in this instance since analysis of virtual Northerns of tester and driver cDNA prepared from CT cells isolated from another two placenta delivered after 38 weeks gestation, showed that these two cDNA hybridized to species present in the tester but not the driver.

On the other hand approximately 95% of the nucleotides of the 520bp cDNA fragment that were compared through BLAST searches were found to have between 97-100% homology to the human γ globin cDNA. This is as somewhat surprising finding since CT cells have not been previously reported to express γ globin mRNA nor have there been previous reports of the regulation of γ globin mRNA expression by TGFα. It is interesting to note however that CT, ST, IT cells and trophoblast cells in anchoring villi from the third trimester placenta and cultured JAR choriocarcinoma cells were found to express erythrpoietin (Conrad *et al.*, 1996).

Gamma globin is a member of the β globin gene family. The β and α globin proteins form the heterodimer haemoglobin (Voet & Voet 1990). In the human and other primates there are several β, α globin genes that are clustered together on two separate loci (Karlsson & Nienhuis 1985; Voet & Voet 1990). The human β locus consists of the genes ε, $^{G}\gamma$, $^{A}\gamma$, ψβ pseudogene and the δ, β genes (Collins & Weissman 1984; Karlsson & Nienhuis 1985). The expression of these genes is developmentally regulated and it has been found that in the human they are expressed in the order found on the locus during erythropoiesis starting with ε globin gene (Weatherall & Clegg 1981). The ε globin gene is expressed in cells derived from yolk sac blood islands between 6-8 weeks post conception followed by $^{G}\gamma$, $^{A}\gamma$ in the fetal liver, spleen and bone marrow cells up to about 6 weeks after birth (Weatherall & Clegg 1981). Soon after birth δ gene is expressed in fetal bone marrow cells for approximately 30 weeks and thereafter is fully supplanted by the β gene (Weatherall & Clegg 1981; Stamatoyannopoulos & Nienhuis 1994).

If we examine the possible mechanisms that may cause the upregulation of γ globin mRNA in CT cells due to TGFα treatment, the most likely explanation is that it is brought about by the activity of TGFα induced transcription factors. There are several regions upstream of the γ globin gene that are believed to be involved in transcriptional regulation. Some of these are the motifs TATA, CACCC and CCAAT (Evans *et al.*, 1990). The TATA region of the γ globin gene is thought to interact with the TFIID transcription factor (Buratowski *et al.*, 1988) while the ubiquitous CP1 protein is believed to recognise the CCAAT region (Gumucio *et al.*, 1988). The CACCC region has been found to interact with the transcription factor Sp1 (Barnhart *et al.*, 1988). However these interactions are not confined to γ globin promoter and are common to numerous other genes. Further, TGFα has not been reported to enhance the transcription of these DNA binding proteins therefore it is unlikely that they bring about the upregulation of the γ globin mRNA expression.

Studies have also shown that there is an enhancer region 10.2-11kb upstream of the ε gene (Evans *et al.*, 1990). This region is believed to be a dominant control region for the entire β locus and it has been reported to enhance regulation up to 300 fold in vitro (Evans *et al.*, 1990). The manner in which the dominant control region influences transcription however is not clearly understood. If TGFα were acting via the dominant control region it is also likely that the other genes of the β locus would also be transcribed, but we did not observe an upregulation in the expression of any other genes of the β locus. Therefore it is unlikely that TGFα influenced the transcription of γ globin gene in the CT cells through the dominant control region.

The human γ globin gene has also been found to contain a DNA consensus sequence TGACTCA that is recognised by the erythroid cell specific factor designated NF-E2 (Evans *et al.*, 1990). It has also been shown that the complex formed between the transcription factors AP-1 and fos proteins recognises the same sequence as NF-E2 (Lee *et al.*, 1987; Angel *et al.*, 1988; Feige *et al.*, 1992). The interaction of growth factors such as TGFα with the EGFR has been shown to enhance the transcription of fos mRNA (Feige *et al.*, 1992). In a recent study it was also reported that stimulation of EGFR by

EGF on NIH-3T3 cells increased the expression of jun mRNA which is translated into AP-1 protein (Edman *et al.*, 1997). Hence it is possible that activation of γ globin mRNA may have occurred through the TGFα induced fos-AP-1 protein complex. The significance of the upregulation of γ globin mRNA by TGFα in CT cells however cannot be explained at the present time.

Since RDA is an extremely sensitive PCR based technique, spurious results can be obtained as a result of a few contaminating cells. Therefore the question whether contaminant cells were the source of γ globin mRNA was examined. Contamination by fetal spleen and bone marrow cells which are known to express γ globin mRNA at 38 weeks gestation, in this culture system would be most unlikely. We isolated CT cells using a modified version of the procedure described by Kliman (1986), and the CT cells were assessed to be up to 97% pure by visual examination and cytokeratin staining. The purity of CT cells isolated by the procedure of Kliman and co-workers (1986), without additional modification have been found to vary between 90-95% (Douglas & King 1989). Previous studies have also shown that fibroblasts and macrophages are most the likely contaminants (Douglas & King 1989; Morrish *et al.*, 1987). In an effort to reduce contaminating fibroblasts and obtain purer CT cell preparations, columns containing the fibroblast antigen CD9 have also been used to remove fibroblasts (Yui *et al.*, 1994). Adoption of such procedures would therefore be beneficial in future RDA studies in order to reduce the numbers of contaminating cells. As there have been no previous reports of γ globin mRNA expression by placental fibroblasts and macrophages we are unable to comment whether these cells may be the source/s of the γ globin mRNA. In situ hybridization studies should be conducted to determine if contaminating cells and/or CT cells express γ globin mRNA.

Another important consideration in the success of RDA are the various intermediate steps of the RDA protocol. If we examine the key steps starting with the cDNA synthesis step the size distribution of the cDNA generated from tester and driver CT cells was similar to that described from β pancreatic cells and human placenta (Niwa *et al.*, 1997; Clontech user manual 1997). Tester and driver cDNA populations having equivalent size distribution before subtraction have been assessed as being an important criterion for the

identification of true differences between cDNA populations (Clontech user manual, 1997).

The restriction enzyme digestion of the cDNA and ligation steps were also assessed as being successful in our study. The smaller restriction enzyme digested cDNA enables efficient hybridization and produces suitably sized fragments that can be amplified efficiently by PCR (Hubank & Schatz 1994). Although we used *Rsa* 1 as the restriction enzyme previous studies have successfully used enzymes such as *Dpn* 1, *Sau* 3A and *Msp* 1 (Hubank & Schatz 1994; Lewis *et al.*, 1997; Edman *et al.*, 1997).

Possibly the most critical steps are the driver tester ratios used and the number of rounds of successive subtractive hybridizations. (Hubank & Schatz 1994; Vician *et al.*, 1997). We and carried out two successive subtractive hybridizations with driver tester ratios recommended in the Clonetech protocol (1997). By altering the driver tester ratios subsets of differentially expressed mRNA have been identified (Hubank & Schatz 1994; Vician *et al.*, 1997). By spiking tester samples with very low copy numbers of known mRNA species an interesting finding was reported by Hubank & Schatz (1994), who showed that an excess of driver can altogether remove low copy mRNA species found in the tester. Although we used two rounds of subtractive hybridization previous studies have described up to four rounds of subtractive hybridization (Vician *et al.*, 1997). It has also been shown that with successive rounds of subtractive hybridization the number of cDNA fragments identified in the tester tends to decrease, however it has been suggested that these cDNA may represent true differences between driver and tester cDNA populations (Hubank & Schatz 1994; O'Neill & Sinclair 1997). The amplicons that we found after subtraction ranged between 0.11-0.65kb which has been reported to be a fairly typical size range for cDNA fragments detected after RDA (Hubank & Schatz 1994). Since the driver tester ratio and the number of rounds of subtractive hybridization have been found to effect the outcome, these two factors should be varied to investigate their effects on the subtracted amplicons found in tester CT cells.

We recovered the subtracted cDNA fragments from the tester by re-amplification of the visible amplicons using PCR. In other studies the entire subtracted cDNA mixture has

been labelled and used as a hybridization probe to screen cDNA libraries (Clontech manual 1997; Herblot *et al.*, 1997). This method generally yields a greater number of clones compared with PCR amplification, hence such analyses should also be carried out to obtain additional differences between the tester and driver.

It should also be borne in mind that RDA is one of the PCR based methods that allows the study of differentially expressed mRNA. A parallel technique is DD-RT-PCR. The main advantage of DD-RT-PCR over RDA is that a number of cDNA populations can be compared enabling study of temporal changes or changes brought about by different treatments. On the other hand the main advantages of RDA compared with DD-RT-PCR is that it eliminates the visual comparison of hundreds of cDNA fragments resolved on polyacrylamide gels in order to identify differences in mRNA expression. Secondly the reaction kinetics have been optimised in RDA to enrich low abundance or rare mRNA so that high and low abundant species are equalised before subtraction thus making it more likely that the low/rare species will be detected (Hubank & Schatz 1994). In a recent study using DD-RT-PCR, invasive CT cells from first trimester placenta were found to express integrin-β1, ATP synthetase U6 which were absent in term CT cells (Huch *et al.*, 1998). Although RDA and DD-RT-PCR are superseding subtractive hybridization this method also yields useful data. For example Morrish and co-workers (1997), found calreticulin, HSP 27 and adrenomedullin were among some of the mRNA species detected in the in differentiated villous CT cells isolated from the third trimester which were absent in CT cells prior to differentiation. Therefore studies using these methods particularly DD-RT-PCR where CT cells could be cultured for varying time intervals following TGFα treatment to investigate temporal changes in mRNA expression should also be carried out. Another useful aspect would be to study the downregulation of mRNA by TGFα using RDA in order to gain a better understanding of the effects exerted by this growth factor.

CHAPTER 8

General discussion and future directions

The experiments described in this thesis have investigated the expression of a number of EGF related growth factors and their receptors in gestational tissue and CT cells. It has also investigated the effects exerted by TGFα on cultured villous CT cells that were isolated from placenta delivered after 38-40 weeks gestation.

The results presented in Chapter 3 shows that the mRNA's of a number of EGF related growth factors such as AR, TGFα, CR, HRG and HB-EGF are present in the placenta, chorion-adherent decidua and amnion during the third trimester. BTC mRNA was found only in the villous cotyledons of the placenta. This study also revealed that the mRNA of the EGFR related receptors c-erb-B2, c-erb-B3 and c-erb-B4 were also present in the gestational tissue towards the end of the third trimester.

In order to identify the cells that were expressing some of the EGF related growth factors in situ hybridization studies were conducted. The results presented in Chapter 4 showed that there were similarities in the distribution of AR, HB-EGF mRNA and CR, HRG mRNA in the fetal membranes. In the placental villi AR, HB-EGF, HRG and CR mRNA were localised to the ST cell layer. Although the initial study revealed that BTC mRNA was present in the placenta; studies were not carried out to localise BTC mRNA expression in the cells of the placental villi. TGFα mRNA was also detected in the initial study in the gestational tissue however, TGFα mRNA expression was not localised by in situ hybridization in this study as there have been previous studies that have examined TGFα mRNA expression in situ. The first study also showed that the EGFR related receptor mRNA were also present in the gestational tissue at term. Further studies could be conducted to identify the cells that may be expressing BTC mRNA in the placental villi and the EGFR related receptor mRNA in the fetal membranes and placental villi. Such studies may provide a clearer picture of the target cells where interaction of growth factors such as HRG and BTC and their receptors might occur within the gestational tissue. It is uncertain why these tissues express mRNA of several members of the EGF family. In vitro studies may provide clues to selective advantages, redundancy or opposing functions in these tissues.

Previous studies have not demonstrated the presence of AR protein in the fetal membranes or term placenta. It was uncertain if similar cell types in the gestational tissue co-express TGFα and AR protein and the relative amounts of TGFα, AR protein present in the gestational tissue towards the end of pregnancy. Using antisera directed against AR and TGFα, results given in Chapter 5 shows that immunoreactive TGFα and AR proteins are present together in several cell types such as the ST, CT cells in the placental villi and decidua. These findings indicate that both TGFα and AR are produced endogenously in the gestational tissue and probably exert autocrine and paracrine regulatory controls over several different cell types in these tissues. The cells expressing HB-EGF, BTC, CR, HRG and c-erb-B2, c-erb-B3 and c-erb-B4 proteins in the gestational tissue was not determined in this study and further studies should be conducted to determine if these proteins are also synthesised endogenously in these tissues. If functional growth factor proteins were detected treatment of dispersed cells from the fetal membranes and placenta with each of these EGF related growth factors could lead to a better understanding of the autocrine and or paracrine regulation exerted by these growth factors.

Studies involving the mRNA expression of the EGF/EGFR related growth factors and receptors in cultured villous CT cells showed that growth factors such as CR, AR and TGFα mRNA were present in both CT and ST cells, whereas HRG and HB-EGF mRNA was present only in the CT cells. There were differences however between the findings of the in situ hybridization experiments using tissue samples of placental villi and cultured cells (Chapter 3 & 4). For example HRG and HB-EGF mRNA were localised to the ST cell layer using in situ hybridization but were absent in cultured ST cells.

Using the same antibodies that were used to localise AR and TGFα protein expression in the gestational tissue samples, AR and TGFα protein expression was studied in cultured CT and ST cells (Chapter 5). These findings showed that TGFα, AR protein was present in both CT and ST cells but that AR protein was present in significantly higher amounts in ST cells. Studies *in vitro* were carried out to investigate some of the effects that TGFα may be exerting in villous CT cells isolated from placenta collected towards the end of

gestation (Chapters 6 & 7), however effects exerted by TGFα on ST cells and AR on villous CT or ST cells remains to be investigated.

The findings presented in Chapter 6 shows that TGFα does not appear to have any influence on cell division or on the aggregation of CT cells which is required for syncytialisation *in vitro*. The concentrations of TGFα used in the study were similar to doses that have been used to elicit a mitogenic response in CT and EVT cells isolated from placenta obtained from the first and second trimesters. The growth factor was found to elicit a transient inhibition of the mRNA of the enzyme 3βHSD which converts pregnenolone to progesterone in CT cells. TGFα has also been shown to regulate 3βHSD expression in corpora luetein and granulosa cells in studies that were carried out by other workers. Whether TGFα exerts regulatory effects on 3βHSD mRNA expression in CT cells isolated from earlier trimesters and in ST cells which also synthesises progesterone remains to be investigated. Studies by others have also shown that IGF-1 enhances the expression of P450scc mRNA (enzyme which converts LDL-cholesterol to pregnenolone). Whether TGFα exerts any effect on the expression of this gene could also be investigated.

In order to investigate whether TGFα may be upregulating mRNA expression in CT cells, subtractive hybridization studies were carried out to identify such mRNA species using RDA. Although the findings presented in Chapter 7 shows that up to eight different cDNA fragments were present in the subtracted tester, the identity of only one cDNA fragment was verified. By varying the conditions in the subtractive hybridization protocol it may be possible to identify further differences. In this experiment only mRNA species that were being upregulated were identified however, RDA could also be used to investigate mRNA that were being down regulated in CT cells in response to treatment with TGFα.

BIBLIOGRAPHY

Aboagye-Mathiesen G, Zdravkovic M, Toth FD and Ebbesen P (1997). Effects of human trophoblast-induced interferons on the expression of proto-oncogenes c-fms/CSF-1R, EGF-R and c-erbB2 in invasive and non-invasive trophoblast. Placenta **18:** 155-161.

Abramovich DR, Dacke CG, Elcock C and Page KR (1987). Calcium transport across the isolated dually perfused placental lobule. Journal of Phsiology **382:** 397-410.

Adamson ED (1990). Developmental activities of the epidermal growth factor receptor. Current Topics in Developmental Biology **24:** 1-29.

Adashi EY and Resnick CE (1986). Antagonistic interactions of transforming growth factors in the regulation of granulosa cell differentiation. Endocrinology **119:** 1879-1881.

Ahmed NA and Pearson Murphy BE (1988). The effects of various hormones on human chorionic gonadotropin production in early and late placental explant cultures. American Journal of Obstetrics and Gynecology **159:** 1220-1227.

Albrecht ED and Pepe GJ (1990). Placental steroid hormone biosynthesis in primate pregnancy. Endocrine Reviews **11:** 124-150.

Alsat E, Mirlesse V, Fondacci C, Dodeur M and Evain-Brion D (1990). Parathyroid hormone increases epidermal growth factor receptors in cultured human trophoblastic cells from early and term placenta. Journal of Clinical Endocrinology and Metabolism **73:** 288-295.

Alsat E, Haziza J and Evain-Brion D (1993). Increase in epidermal growth factor receptor and its messenger ribonucleic acid levels with differentiation of human trophoblast cells in culture. Journal of Cellular Physiology **154:** 122-128.

Ameniya K, Kurachi H, Adachi H, Morishige K-I, Adachi K, Imai T and Miyake A (1994). Involvement of epidermal growth factor (EGF)/EGF receptor autocrine and paracrine mechanism in human trophoblast cells: functional differentiation *in vitro*. Journal of Endocrinology **143:** 291-301.

Angel P, Allegretto EA, Okino ST, Hattori K and Boyle WJ (1988). Oncogene jun encodes a sequence specific trans activator similar to AP-1. Nature **332:** 166-171.

Anthony M and Klugman KP (1997). Quantitative RT-PCR for human fas (CD95) expression. Biotechniques **22:** 442-446.

Arcari P, Martinelli R and Salvatore F (1984) The complete sequence of a full length cDNA for human liver glyceraldehyde-3-phosphate dehydrogenase: evidence for multiple mRNA species. Nucleic Acids Research **12:** 9179-9189.

Babalola GB and Schultz RM (1995). Modulation of gene expression in the periimplantation mouse embryo by TGF-α and TGF-β. Molecular Reproduction and Development **41:** 133-139.

Bacus SS, Gudcov AV, Zelnick CR, Chin D, Stern R, Stancovski I, Peles E, Ben-Baruch N, Farbstein H, Lupa R *et al.*, (1993). Neu differentiation factor (heregulin) Induces expression of intercellular adhesion molecule 1: implications for mammary tumors. Cancer Research **53:** 5251-5261.

Bahn RS, Speeg Jr KV, Ascoli M and Rabin D (1980). Epidermal growth factor stimulates production of progesterone in cultured human choriocarcinoma cells. Endocrinology **107:** 2121-2123.

Barnea ER, Feldman D, Kaplan M and Morrish DW (1990). The dual effect of epidermal growth factor upon human chorionic gonadotropin secretion by the first trimester placenta *in vitro*. Journal of Clinical Endocrinology and Metabolism **71:** 923-928.

Barnhart KM, Kim CG, Banerjeri SS and Sheffery M (1988). Purification and characterisation of an erythriod cell-specific factor that binds the murine alpha and beta-globin genes. Molecular and Cellular Biology **9:** 2606-2614.

Bass KE, Morrish D, Roth I, Bhardwaj D, Taylor R, Zhou Y and Fisher SJ (1994). Human cytotrophoblast is up-regulated by epidermal growth factor evidence that paracrine factors modify this process. Developmental Biology **164:** 550-561.

Bax CMR, Ryder TA, Mobberley MA, Tyms AS, Taylor DL and Bloxam DL (1989). Ultrastructural changes and immunocytochemical analysis of human placental trophoblast during short-term culture. Placenta. **10:** 179-194.

Beaudoin C, Blomquist CH, Bonenfant M and Tremblay Y (1997). Expression of the genes for 3β-hydroxysteroid dehydrogenase type 1 and cytochrome P450scc during syncytium formation by human placental cytotrophoblast cells in culture and the regulation by progesterone and estradiol. Journal of Endocrinology **154:** 379-387.

Beerli RR, Graus-Porta D, Woods-Cook K, Chen X, Yarden Y and Hynes NE (1995). Neu differentiation factor activation of ErbB-3 and Erb-B4 is cell specific and displays a differential requirement for Erb-B2. Molecular and Cellular Biology **15:** 6496-6505.

Bennett PR, Geoffrey MD, Chamberlain VP, Patel L, Elder MG and Myatt L (1990). Mechanisms of parturition: the transfer of prostoglandin E2 and 5-hydroxyeicosatetraenoic acid across fetal membranes. American Journal of Obstetrics and Gynecology **162:** 683-687.

Benirschke K and Kaufmann P (1995). The pathology of human placenta. Berlin: Springer-Verlag.

Benveniste R, Speeg KV, Carpenter G, Cohen S, Lindner J and Rabinowitz D (1978). Epidermal growth factor stimulates secretion of human chorionic gonadotropin by cultured human choriocarcinoma cells. Jounal of Clinical Endocrinology and Metabolism **46:** 169-172.

Benyo DF, Miles TM and Conrad KP (1997). Hypoxia stimulates cytokine production by villous explants from the human placenta. Journal of Clinical Endocrinology and Metabolism **82:** 1582-1588.

Bhaumick B and Bala RM (1991). Epidermal growth factor enhancement of insulin-like growth factor-I-induced down-regulation of insulin-like growth factor I receptor in cultured placental trophoblast cells. Hormone Research **35:** 246-251.

Bhaumick B, George D and Bala RM (1992). Potentiation of epidermal growth factor-induced differentiation of cultured human placental cells by insulin-like growth factor-I. Journal of Clinical Endocrinology and Metabolism **74:** 1005-1011.

Bissonnette F, Cook C, Geoghegan T, Steffen M, Henry J, Yussman MA and Schultz G (1991). Transforming growth factor-α and epidermal growth factor messenger ribonucleic acid and protein levels in human placentas from early, mid and late gestation. American Journal of Obstetrics and Gynecology **166:** 192-199.

Blay J and Hollenberg MD (1989). The nature and function of polypeptide growth factor receptors in the human placenta. Journal of Developmental Physiology **12:** 237-248.

Blotnick S, Peoples GE, Freeman MR, Eberlein TJ and Klagsbrun M (1994). T lymphocytes synthesize and export heparin-binding epidermal growth factor-like growth factor and basic fibroblast growth factor, mitogens for vascular cells and fibroblasts: Differential production and release of CD4+ and CD8+ T cells. Cell Biology **91:** 2890-2894.

Bloxam DL, Bax CMR and Bax BE (1997a). Culture of syncytiotrophoblast for the study of human placental transfer. Part 1: Isolation and purification of cytotrophoblast. Placenta **18:** 93-98.

Bloxam DL, Bax BE and Bax CMR (1997b). Culture of syncytiotrophoblast for the study of human placental transfer. Part II: Production, culture and use of syncytiotrophoblast. Placenta **18:** 99-108.

Bourne G (1962). The human amnion and chorion. Lloyd-Luke Ltd.

Boyd JD and Hamilton WJ (1970). The human placenta. MA Heffer and Sons.

Brachmann R, Lindquist PB, Nagashima M, Kohr W, Lipari T, Napier M and Derynck R (1989). Transmembrane TGF-α precursors activate EGF/TGF-α receptors. Cell **56:** 691-700.

Branchaud CL, Goodyer CG and Lipowski S (1983). Progesterone and estrogen production by placental monolayer cultures: effect of dehydroepiandrosterone and luteinizing hormone-releasing hormone. The Journal of Endocrinology and Metabolism **56:** 761-766.

Brandt *et al.*, (1994). Identification and biological charaterization of an epidermal growth factor-ralated protein: cripto-1. The Journal of Biological Chemistry **24:** 17320-17328.

Bringman TS, Lindquist PB and Derynck R (1987). Different transforming growth factor-α species are derived from a glycosylated and palmitoylated transmembrane precursor. Cell **48:** 429-440.

Brown MJ, Cook CL, Henry JL and Schultz GS (1987). Levels of epidermal growth factor in third-trimester and term human placentas: elevated binding in term placentas of male fetuses. American Journal of Obstetrics Gynecology **156:** 716-720.

Bulmer JN (1992). Immune aspects of pathology of the placental bed contributing to pregnancy pathology. Bailliare's Clinical Obstetrics and Gynecology **6:** 461-488.

Buratowski S, Hahn S, Sharp PA and Guarente L (1988). Function of yeast TATA element-binding protein in a mamalian transcription system. Nature **334:** 37-42.

Burgess TS, Ross SL, Qian Y, Brankow D and Hu S (1995). Biosynthetic processing of *neu* differentiation factor. Journal of Biological Chemistry. **270:** 19188-19196.

Butterworth BH and Loke YW (1985). Immunocytochemical identification of cytotrophoblast from other mononuclear cell populations isolated from first-trimester human chorionic villi. Journal of Cell Science **76:** 189-197.

Cameron B, Gown AM and Tamimi HK (1994). Expression of c-erb-B2 oncogene product in persistent trophoblastic disease. American Journal of Obstetrics and Gynecology **170:** 1616-1622.

Cao H, Lei ZM, Bian L and Rao V (1995). Functional nuclear epidermal growth factors receptors in human choriocarcinoma JEG-3 cells and normal human placenta. Endocrinology **136:** 3163-3172.

Carpenter G, Poliner L and King Jr L (1980). Protein phosphorylation in human placenta stimulation by epidermal growth factor. Molecular and Cellular Endocrinology **18:** 189-199.

Carson SA, Chase R, Ulep E, Scommegna A and Benveniste R (1983). Ontogenesis and characteristics of epidermal growth factor receptors in human placenta. American Journal of Obstetrics Gynecology. **147:** 932-939.

Carver RS, Sliwkowski MX, Sitaric S and Russell WE (1996). Insulin regulates heregulin binding and ErbB3 expression in rat hepatocytes. Journal of Biological Chemistry **271:** 13491-13496.

Cedard L, Alsat E, Urtasun MJ and Varangot J (1970). Studies on the mode of action of luteinzing hormone and chorionic gonadotropin estrogenic biosynthesis and glycogenolysis by human placenta perfused *in vitro*. Steroids **16:** 361-370.

Challis JRG and Olson DM (1988). Parturition. The Physiology of Reproduction. Editors Knobil E and Neill J, Raven Press, New York.

Chedrese PJ, Luu-The V, Labrie F, Juorio AV and Murphy BD (1990a). Evidence for the regulation of 3β hydroxysteroid dehydrogenase messenger RNA by human chorionic gonadotrophin in luteinized porcine granulosa cells. Endocrinology **126**: 2228-2230.

Chedrese PJ, Zhang D, Luu-The V, Labrie F, Juorio AV and Murphy BD (1990b). Regulation of mRNA expression of 3β-hydroxy-5-ene steroid dehydrogenase in porcine granulosa cells in culture: a role for the protein kinase-C pathway. Molecular Endocrinology **4:** 1532-1538.

Chegini N and Rao ChV (1985). Epidermal growth factor binding to human amnion, chorion, decidua and placenta from mid- and term pregnancy: quantitative light microscopic autoradiographic studies. Journal of Clinical Endocrinology & Metabolism **61:** 529-535.

Chen C, Kurachi H, Fujita Y, Terakawa N, Miyake A and Tanizawa O (1988). Changes in epidermal growth factor receptor and its messenger ribonucleic acid levels in human placenta and isolated trophoblast cells during pregnancy. Journal of Clinical Endocrinology and Metabolism **67:** 1171-1177.

Chia CM, Winston RML and Handyside AH (1995). EGF, TGF-α and EGFR expression in human preimplantation embyros. Development **121:** 299-307.

Chien EK, Tokuyama Y, Rouard M, Phillippe M and Bell GI (1997). Identification of gestationally regulated genes in rat myometrium by use of messenger ribonucleic acid differential display. American Journal of Obstetrics and Gynecology **177:** 645-652.

Chomczynski P and Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry **162:** 156-159.

Chu CC and Paul WE (1997). Fig 1, an interleukin 4-induced mouse B cell gene isolated by cDNA representational difference analysis. Proceedings of the National Academy of Sciences, USA **94:** 2507-2512.

Chung HK, McLimans WF, Horoszewicz J and Hreshchyshyn MM (1969). *In vitro* studies of human trophoblast. American Journal of Obstetrics and Gynecology **104:** 945-952.

Ciardiello F, Kim N, Saeki T, Dono R, Persico MG, Plowman GD, Garrigues J, Radke S, Todaro GJ and Salomon DS (1991). Differential expression of epidermal growth factor-related proteins in human colorectal tumors. Proceedings of the National Academy of Sciences, USA **88:** 7792-7796.

Ciccodicola A, Dono R, Obisi S, Simeone A, Zoll M and Persico MG (1989). Molecular characterization of a gene of the "EGF family" expressed in undifferentiated human NTERA2 teratocarcinoma cells. EMBO **8:** 1987-1991.

Clontech PCR select cDNA subtraction kit user manual (1997).

Coffey R *et al.*, (1987). Production and auto-production of transforming growth factor-α in human keratinocytes. Nature **328:** 817-820.

Cohen S and Elliott GA (1963). Journal of Investigations Dermatology **40:** 1-5.

Cohen BD, Green JM, Foy L and Fell HP (1996). HER4-mediated biological and biochemical properties in NIH3T3 cells. Evidence for HER1-HER4 heterodimers. Journal of Biological Chemistry **271:** 4813-4818.

Collins S & Weissman (1984). Molecular genetics of human hemoglobin. Progress in Nucleic Acid Research **31:** 315-462.

Conrad KP, Benyo DF, Westerhausen-Larsen A and Miles TM (1996). Expression of erythropoietin by human placenta. FASEB **10:** 760-766.

Contractor SF, Eaton BM, Firth JA and Bauman KF (1984). A comparison of the effects of different perfusion regimes on the structure of the isolated human placental lobule. Cell Tissue Research **237:** 609-617.

Cook PW, Mattox PA, Keeble WW, Pittlekow MR, Plowman GD, Shoyab M, Adelman JP and Shipley GD (1991). A heparin sulphate-regulated human keratinocyte autocrine factor is similar or identical to amphiregulin. Mollecular and Cellular Biology **11:** 2547-2557.

Cross M and Dexter TM (1991). Growth factors in development, transformation and tumorigenesis. Cell **64:** 271-280.

Csapo AI and Pulkkinen M (1978). Indispensability of the human corpus luteum in the maintenance of early pregnancy luteectomy evidence. Obstetrical and Gynecological Survey **33:** 69-81.

Daneau I, Ethier JF, Lussier JG and Silversides DW (1996). Porcine SRY gene locus and genital ridge expression. Biology of Reproduction **55:** 47-53.

Daniels-McQueen S, Krichevsky A and Biome I (1986). Isolation and characterization of human cytotrophoblast cells. Trophoblast Research **7:** 423-445.

Dardik A, Smith RM and Schultz RM (1992). Colocalization of transforming growth factor-α and a functional epidermal growth factor receptor (EGFR) to the inner cell mass and preferential localization of the EGFR on the basolateral surface of the trophectoderm in the mouse blastocyst. Developmental Biology **154:** 396-409.

Dart LL, Smith DM, Meyers CA, Sporn MB and Frolik CA (1985). Transforming growth factors from a human tumor cell: Characterization of transforming growth factor β and identification of high molecular weight transforming growth factor α. Biochemistry. **24:** 5925-5931.

Das NP, Khan-Dawood FS and Dawood MY (1985). Effects of steroid hormones and gonadotropins on in vitro placental conversion of pregnenolone to progesterone. Journal of Steroid Biochemistry **23:** 517-522.

Das SK, Wang X, Paria BC, Damm D, Abraham JA, Klagsbrun M, Andrews GK and Dey SK (1994). Heparin-binding EGF-like growth factor gene is induced in the mouse uterus temporally by the blastocyst solely at the site of its apposition: a possible ligand for interaction with blastocyst EGF-receptor in implantation. Development **120:** 1071-1083.

Das SK, Chakraborthy I, Paria BC, Wang X-N, Plowman G and Dey SK (1995). Amphiregulin is an implantation-specific and progesterone-regulated gene in the mouse uterus. Molecular Endocrinology **9:** 691-705.

DePalo L and Das M (1988). Epidermal growth factor-induced stimulation of epidermal growth factor-receptor synthesis in human cytotrophoblasts and A431 carcinoma cells. Cancer Research **48:** 1105-1109.

Derynck R, Roberts AB, Winkler ME, Chen EY and Goeddel DV (1984). Human transforming growth factor-α: precursor structure and expression in E. coli. Cell **38:** 287-297.

Derynck R (1988). Transforming growth factor α. Cell. **54:** 593-595.

Dickson RB, Bates SE, McManaway ME and Lippman ME (1986). Cancer Research **46:** 1707-1713.

Dluz SM, Higashiyama S, Damm D, Abraham JA and Klagsbrun M (1993). Heparin-binding epidermal growth factor-like growth factor expression in cultured fetal human vascular smooth muscle cells. Induction of mRNA levels and secretion of active mitogen. Journal of Biological Chemistry **268:** 18330-18334.

Dono R, Scalera L, Pacifico F, Acampora D, Persico MG and Simeone A (1993). The murine cripto gene: expression during mesoderm induction and early heart morphogenesis. Development **118:** 1157-1168.

Dougall WC, Qian X, Peterson NC, Miller MJ, Samanta A and Greene MI (1994). The *neu*-oncogene: signal transduction pathways, transformation mechanisms and evolving therapies. Oncogene **9: 2109-2123.**

Douglas GC and King BF (1989). Isolation of pure villous cytotrophoblast from term human placenta using immunomagnetic microspheres. Journal of Immunological Methods **119:** 259-268.

Douglas GC and King BF (1990). Differentiation of human trophoblast cells *in vitro* as revealed by immunocytochemical staining of desmoplakin and nuclei. Journal of Cell Science **96:** 131-141.

Eaton BM, Richard RC and Contractor SF (1982). Uptake and metabolism of epidermal growth factor in perfused human placenta. Journal of Developmental Physiology **9:** 69.

Edman CF, Prigent SA, Schipper A and Feramisco JR (1997). Identification of ErbB3-stimulated genes using modified representational difference analysis. Biochemistry Journal **323:** 113-118.

Ethier SP, Kokeny KE, Ridings JW and Dilts CA (1996). erbB family receptor expression and growth regulation in a newly isolated human breast cancer cell line. Cancer Research **56:** 899-907.

Elias JM et al (1989). Sensitivity and detection efficiency of the peroxidase antiperoxidase (PAP) avidin-biotin peroxidase complex (ABC) and peroxidase-labeled avidin-biotin (LAB) methods. American Journal of Clinical Pathology **92:** 62-67.

El-Roeiy A and Myers SA (1990). The relationship of the immune system to hypertension and interuterine growth retardation. Immunology and Allergy Clinics of North America **10:** 109-117.

Evain-Brion D (1992). Growth factors and trophoblast differentiation. Trophoblast Research **6:** 1-18.

Evans T, Felsenfeld G and Reitman M (1990). Control of globin gene transcription Annual Review of Cell Biology **6:** 95-124.

Faber BM, Metz SA and Chegini N (1996). Immunolocalization of eicosanoid enzymes and growth factors in human myometrium and fetoplacental tissues in failed labor inductions. Obstetrics and Gynecology **88:** 174-179.

Farmer DR and Nelson DM (1992). A fibrin matrix modulates the proliferation, hormone secretion and morphologic differentiation of cultured human placental trophoblast. Placenta **13:** 1630167.

Feige JJ, Vilgrain I and Chambaz EM (1992). Extracellular signals and transduction mechanisms in target cells. Trophoblast Research **6:** 39-54.

Fen Z, Dhadly MS, Yoshizumi M, Hilkert RJ, Quertermous T, Eddy RL, Shows TB and Lee M (1993). Structural organization and chromosomal assignment of the gene encoding the human heparin-binding epidermal growth factor-like growth factor/diptheria toxin receptor. Biochemistry **32:** 7932-7938.

Filla MS, Zhang CX and Kaul KL (1993). A potential transforming growth factor α/epidermal growth factor receptor autocrine circuit in placental cytotrophoblasts. Cell Growth & Differentiation 387-393.

Filla MS and Kaul KL (1997). Relative expression of epidermal growth factor receptor in placental cytotrophoblasts and choriocarcinoma cell lines. Placenta **18:** 17-27.

Fisher DA and Lakshmanan (1990). Metabolism and effects of epidermal growth factor and related growth factors in mammals. Endocrine Reviews **11:** 418-439.

Forsyth IA, Taylor JA, Keable S, Turvey A and Lennard S (1997). Expression of amphiregulin in the sheep mammary gland. Molecular and Cellular Endocrinology **126:** 41-48.

Gardener RM, Lingham RB and Stancel GM (1987). Contractions of the isolated uterus stimulated by epidermal growth factor. FASEB Journal **1:** 224-228.

Genbacev O, Schubach SA and Miller RK (1992). Villous culture of first trimester human placenta-model to study extravillous trophoblast (EVT) differentiation. Placenta **13:** 439-461.

Gentry LE, Twardzik DR, Lim GJ, Ranchalis JE and Lee DC (1987). Expression and characterisation of transforming growth factor α precursor protein in transfected mammalian Cells. Molecular and Cellular Biology **7:** 1585-1591.

Gerbie AB, Hathaway HH and Brewer JI (1968). Autoradiographic analysis of normal trophoblast proliferation. American Journal of Obstetrics and Gynecology **100:** 640-648.

Goustin AS, Betsholtz C, Pfeifer-Ohisson S, Persson H, Rydnert J, Bywater M, Holmgren G, Heldin C, Westermark B and Ohisson R (1985). Coexpression of the *sis* and *myc* proto-oncogenes in developing human placenta suggests autocrine control of trophoblast growth. Cell **41:** 301-312.

Graham CH and Lala PK (1992). Mechanisms of placental invasion of the uterus and their control. Biochemistry and Cell Biology **70:** 867-874.

Graham CH, McCrae KR and Lala PK (1993). Molecular mechanisms controlling trophoblast invasion of the uterus. Trophoblast Research **7:** 237-250.

Gumucio DL, Rood KL, Gray TA, Riordan MF, Sartor CI and Collins FS (1988). Nuclear proteins that bind the human gamma-globin gene promoter: alterations in binding produced by point mutations associated with hereditary persistance of fetal hemoglobin. Mollecular Cell Biology **8:** 5310-5322.

Haining REB, Schofield JP, Jones DSC, Rajput-Williams J and Smith SK (1991). Identification of mRNA for epidermal growth factor and transforming growth factor-α present in low copy number in human endometrium and decidua using reverse transcriptase-polymerase chain reaction. Journal of Molecular Endocrinology **6:** 207-214.

Hakala-Ala-Pietila TH, Koistinen RA, Salonen RK and Seppala MT (1993). Elevated second-trimester amniotic fluid concentration of insulin-like growth factor binding protein-1 in fetal growth retardation. American Journal of Obstetrics and Gynecology **169:** 35-39.

Hall PF (1986). Cytochrome P-450 and the regulation of steroid synthesis. Steroids **48:** 133-146.

Hames BD and Rickwood D (eds.) (1990). Gel electophoresis of proteins: a practical approach, 2nd edition Oxford University Press, New York.

Han VKM, Hunter ES, Pratt RM, Zendegui JG and Lee DC (1987). Expression of rat transforming growth factor alpha mRNA during development ocurs predominantly in the maternal decidua. Molecular and Cellular Biology **7:** 2335-2343.

Hashimoto K *et al.*, (1994). Heparin-binding epidermal growth factor-like growth factor is an autocrine growth factor for human keratinocytes. The Journal of Biological Chemistry **269:** 20060-20066.

Heap RB and Flint APF (1988). Pregnancy: Hormonal control of reproduction. Editors Austin CR and Short RV. Cambridge University Press.

Heldin N-E and Westermark B (1988). Epidermal growth factor, but not thyrotropin, stimulates the expression of c-fos and c-myc messenger ribonucleic acid in porcine thyroid follicle cells in primary culture. Endocrinology **122:** 1042-1046.

Hellig H, Gattereau D, Lefebvre Y and Bolte E (1970). Steroid production from plasma cholosterol. I. Conversion of plasma cholesterol to placental progesterone in humans. Journal of Clinical Endocrinology and Metabolism **30:** 624-631.

Herblot S, Vekris A, Rouzaut A, Najeme F, De Miguel C, Bezian JH and Bonnet J (1997). Selection of down-regulated sequences along the monocytic differentiation of leukemic HL60 cells. Federation of European Biochemical Societies **414:** 146-152.

Higashiyama S, Abraham JA, Miller J, Fiddes JC and Klagsbrun M (1991). A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. Science **251:** 936-939.

Higashiyama S, Abraham JA and Klagsbrun M (1993). Heparin binding EGF-like growth factor stimulation of smooth muscle cell migration: dependance of interactions with cell surface heparan sulphate. Journal of Cell Biology **122:** 933-940.

Hofmann GE, Scott RT, Bergh PA and Deligdisch L (1991). Immunohistochemical localisation of epidermal growth factor in human endometrium, decidua and placenta. Journal of Clinical Endocrinology and Metabolism **73:** 882-887.

Hofmann GE, Drews MR, Scott TR, Navot D, Heller D and Deligdisch L (1992). Epidermal growth factor and its receptor in human implantation trophoblast: immunohistochemical evidence for autocrine/paracrine function. Journal of Clinical Endocrinology and Metabolism **74:** 981-988.

Hofmann GE, Horowitz GA, Scott RT and Navot D (1993). Transforming growth factor-α in human implantation trophoblast: immunohistochemical evidence for autocrine/paracrine function. Journal of Clinical Endocrinology and Metabolism **76:** 781-785.

Hollenberg MD (1989). Growth factors, their receptors and development. American Journal of Medical Genetics **34:** 35-42.

Holmes WE *et al.*, (1992). Identification of heregulin a specific activator of p185erbB2. Science **256:** 1205-1210.

Horowitz GM, Scott Jr RT, Drews MR, Navot D and Hofman GE (1993). Immunohistochemical localization of transforming growth factor-α in human endometrium, decidua and trophoblast. Journal of Clinical Endocrinology and Metabolism **76:** 786-792.

Hubank M and Schatz DG (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA. Nucleic Acids Research **22:** 5640-5648.

Huch G, Hohn HP and Denker HW (1998). Identification of differentially expressed genes in human trophoblast cells by differential-display RT-PCR. Placenta **19:** 557-567.

Huot RI, Foidart JM and Stromberg K (1979). Effects of culture conditions on the synthesis of human chorionic gonadotrophin by placental organ cultures. In Vitro **15:** 497-502.

Huot RI, Foidart JM, Nardone RM and Stromberg K (1981). Differential modulation of human chorionic gonadotropin secretion by epidermal growth factor in normal and malignant placental cultures. Journal of Clinical Endocrinology and Metabolism **53:** 1059-1063.

Hustin J, Jauniaux E and Schaaps JP (1990). Histological study of the materno-embryonic interface in spontaneous abortion. Placenta **11:** 477-486.

Ilekis J and Benveniste R (1985). Effects of epidermal growth factor, phorbol myristate acetate, and arachidonic acid on choriogonadotropin secretion by cultured human choriocarcinoma cells. Endocrinology **116:** 2400-2409.

Ilekis JV, Stark BC and Scoccia B (1995). Possible role of variant RNA transcripts in the regulation of epidermal growth factor receptor expression in human placenta. Molecular Reproduction and Development **41:** 149-156.

Inui S, Higashiyama S, Hashimoto K, Higashiyama M, Yoshikawa K and Taniguchi (1997). Possible role of co-expression of CD9 with membrane-anchored heparin-binding EGF-like growth factor and amphiregulin in cultured human keratinocyte growth. Journal of Cellular Physiology **171:** 291-298.

Irving JA, Lysiak jj, Graham CH, Hearn S, Han VKM and Lala PK (1995). Characteristics of trophoblast cells migrating from first trimester chorionic villus explants and propagated in culture. Placenta **16:** 413-433.

Isomaa VV, Ghersevich SA, Maentausta OK, Peltoketo EH, Poutanen MH and Vihko RK (1993). Steroid biosynthetic enzymes: 17β-hydroxysteroid dehydrogenase. Annals of Medicine **25:** 91-97.

Johnson GR, Saeki T, Auersperg N, Gordon AW, Shoyab M, Salomon DS and Stromberg K (1991). Response to an expression of amphiregulin by ovarian carcinoma and normal ovarian surface epithelial cells: Nuclear localization of endogenous amphiregulin. Biomedical and Biophysical Research Communications **180:** 481-488.

Johnson GR, Saeki T, Gordon AW, Shoyab M, Salomon DS and Stromberg K (1992). Autocrine action of amphiregulin in a colon carcinoma cell line and immunocytochemical localization of amphiregulin in human colon. Journal of Cell Biology **118:** 741-751.

Johnson GR, KannanB, Shoyab M and Stromberg K (1993a). Ampheregulin induces tyrosine phosphorylation of the epidermal growth factor receptor and $p185^{erbB2}$. Journal of Biological Chemistry **268:** 2924-2931.

Johnson GR, Prigent SA, Gullik WJ and Stromberg K (1993b). Characterization of high and low molecular weight forms of amphiregulin that differ in glycosylation and peptide core length. Journal of Biological Chemistry **268:** 18835-18843.

Johnson GR and Wong L (1994). Heparan sulphate is essential to amphiregulin-induced mitogenic signalling by the epidermal growth factor receptor. Journal of Biological Chemistry **269:** 27149-27154.

Johnson SE, Rothstein JL and Knowles BB (1994). Expression of epidermal growth factor family gene members in early mouse development. Developmental Dynamics **201:** 216-226.

Jokhi PP, King A, Gardner L and Loke YW (1994). Reciprocal expression of epidermal growth factor receptor (EGF-R) and c-erbB2 by non-invasive and invasive human trophoblast populations. Cytokine **6:** 433-442.

Jones SA, Brooks AN and Challis JRG (1989). Steroids modulate corticotropin-releasing hormone production in human fetal membranes and placenta. Journal of Clinical Endocrinology and Metabolism **68:** 825-830.

Kannan S *et al.*, (1997). Cripto enhances the tyrosine phosphorylation of Shc and activates mitogen-activated protein kinase (MAPK) in mammary epithelial cells. Journal of Biological Chemistry **272:** 3330-3335.

Kao LC, Caltabiano S, Wu S, Strauss 111 JF and Kliman HJ (1988). The human villous cytotrophoblast: interactions with extracellular matrix proteins, endocrine function and cytoplasmic differentiation in the absence of syncytium formation. Developmental Biology **130:**693-702.

Karlsson S and Nienhuis AW (1985). Annual Review of Biochemistry **54:** 1089-1112.

Kasugai M, Kato H, Iriyama H, Kato M, Ninagawa T and Tomoda Y (1987). The roles of Ca^{2+} and adenosine 3', 5'- monophosphate in the regulation of progesterone production by human placental tissue. Journal of Clinical Endocrinology and Metabolism **65:** 122-126.

KatoY and Braunstein GD (1989). Discordant secretion of placental protein hormones in differentiating trophoblasts *in vitro*. Journal of Clinical Endocrinology and Metabolism **68:** 814-820.

Kawagoe K, Akiyama J, Kawamoto T, Morishita Y and Mori S (1990). Immunohistochemical demonstration of epidermal growth factor (EGF) receptors in normal human placental villi. Placenta **11:** 7-15.

Kennedy TG, Brown KD and Vaughan TJ (1994). Expression of the genes for the epidermal growth factor receptor and its ligands in porcine oviduct and endometrium. Biology of Reproduction **50:** 751-756.

Kim GY, Besner GE, Steffen CL, Mccarthy DW, Downing MT, Luquette MH, Abad MS and Brigstock DR (1995). Purification of heparin-binding epidermal growth factor-like growth factor from pig uterine luminal flushings and its production by endometrial tissues. Biology of Reproduction **52:** 561-571.

Kitadai Y, Yasui W, Yokozaki H, Kuniyasu H, Ayhan A, Haruma K, Kajiyama G, Johnson GR and Tahara E (1993). Expression of amphiregulin, a novel gene of the epidermal growth factor family in human gastric carcinomas. Japanese Journal of Cancer Research **84:** 879-884.

Kliman HJ, Nestler JE, Sermasi E, Sanger JM and Strauss III JF (1986). Purification, characterisation and in vitro differentiation of cytotrophoblasts from human term placentae. Endocrinology **118:** 1567-1581.

Kohler M, Bauknecht T, Grimm M, Birmelin G, Kommoss F and Wagner E (1992). Epidermal growth factor receptor and transforming growth factor alpha expressions in human ovarian carcinomas. European Journal of Cancer **28A:** 1432-1437.

Koida SS and Torres MT (1965). Distribution of 3-β- hydroxysteroid dehydrogenase and Δ^5-3-oxosteroid isomerase in homogenate fractions of human term placenta. Biochemical and Biophysical Acta **105:** 115-120.

Kraus MH, Issing W, Miki T, Popescu NC and Aaronson SA (1989). Isolation and characterization of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: evidence for over-expression in a subset of human mammory tumors. Proceedings of The National Academy of Sciences, USA **86:** 9193-9197.

Kung W, *et al.*, (1994). Isolation of amphiregulin-like growth factor secreted by estrogen receptor-negative MDA-MB-231 human breast cancer cells that stimulates estrogen receptor-positive cells. Biochemical and Biophysical Research Communications **3:** 1357-1365.

Lachance Y, Luu-The V, Labrie C, Simard J, Dumont M, de Launoit Y, Guerin S, Leblanc G and Labrie F (1990). Characterisation of human 3β hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 -isomerase gene and its expression in mammalian cells. Journal of Biological Chemistry **265:** 20469-20475.

Ladines-Llave CA, Maruo T, Manalo AS and Mochizuki M (1991). Cytologic localization of epidermal growth factor and its receptor in developing human placenta varies over the course of pregnancy. American Journal of Obstetrics Gynecology **165:** 1377-1382.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680-685.

Lai WH and Guyda HJ (1984). Characterization and regulation of epidermal growth factor receptors in human placental cell cultures. Journal of Clinical Endocrinology and Metabolism **58:** 344-352.

Lai WH, Guyda HJ and Bergeron JJM (1986). Binding and internalization of epidermal growth factor in human term placental cell in culture. Endocrinology **118:** 413-423.

Lala PK and Hamilton GS (1996). Growth factors, proteases and protease inhibitors in the maternal-fetal dialogue. Placenta **17:** 545-555.

Lamb E, Mancuso S, Dell'Acqua S, Wigvist N and Diczfalusy E (1967). Studies on the metabolism of C-19 steroids in the human foeto-placental unit. Acta Endocrinology **55:** 267-277.

Lee W, Mitchell P and Tjian R (1987). Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell **49:** 741-752.

Lewintre EJ, Orava M and Vihko R (1994). Regulation of 17β-hydroxysteroid dehydrogenase type by epidermal growth factor and transforming growth factor-α in choriocarcinima cells. Endocrinology **135:** 2629-2634.

Lewis BC, Shim H, Li Q, Wu CS, Lee LA, Maity A and Dang CV (1997). Identification of putative c-myc-responsive genes: charaterization of rcl, a novel growth-related gene. Molecular and Cellular Biology **17:** 4967-4978.

Lewis GD, Lofgren JA, McMurtrey AE, Nuijens A, Fendly BM, Bauer KD and Sliwkowski MX (1996). Growth regulation of human breast and ovarian tumor cells heregulin: evidence for the requirement of ErbB2 as a critical component in mediating heregulin responsiveness. Cancer Research **56:** 1457-1465.

Li RH and Zhuang LZ (1997). The effects of growth factors on human normal placental cytotrophoblast cell proliferation. Human Reproduction **12:** 830-834.

Lim H, Dey SK and Das SK (1997). Differential expression of the erbB2 gene in the periimplantation mouse uterus: potential mediator of signaling by epidermal growth factor-like growth factors. Endocrinology **138:** 1328-1337.

Linsley PS, Hargreaves WR, Twardzik DR and Todaro GJ (1985). Detection of larger polypeptides structurally and functionally related type I transforming growth factor. Proceedings of the National Academy of Sciences, USA **82:** 356-360.

Lisitsyn N, Lisitsyn N and Wigler M (1993). Cloning the difference between two complex genomes. Science **259:** 946-951.

Loke YW and Whyte A (1983). Biology trophoblast. Elsevier, New York.

Loke TW and Burland K (1988). Human trophoblast cells cultured in modified medium and supported by extracellular matrix. Placenta **9:** 173-182.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951). Protein measurement with the folin phenol reagent. Journal of Biological Chemistry **193:** 265-275.

Luetteke NC, Michalopoulos GK, Teixido J, Gilmore R, Massague J and Lee DC (1988). Characterization of high molecular weight transforming growth factor α produced by rat hepatocellulor carcinoma cells. Biochemistry **27:** 6487-6494.

Lundin-Schiller and Mitchell MD (1991). Regulation of chorion laeve prostaglandin E2 production by epidermal growth factor, protein kinase c activation and calcium. Placenta **12:** 597-603.

Lysiak JJ, Han VKM and Lala PK (1993). Localization of transforming growth factorα in the human placenta and decidua: role in trophoblast growth. Biology of Reproduction **49:** 885-894.

Lysiak JJ, Connelly IH, Khoo NKS, Stetler-Stevenson W and Lala PK (1994). Role of transforming growth factor-α and epidermal growth factor (EGF) on proliferation and invasion by first trimster human trophoblast. Trophoblast Research **8:** 455-467.

Lysiak JJ, Johnson GR and Lala PK (1995). Localisation of amphiregulin in the human placenta and decidua throughout gestation: role in trophoblast growth. Placenta **16:** 359-366.

Madtes DK, Raines EW, Sakariassen KS, Assoian RK, Sporn MB, Bell GI and Ross R (1988). Induction of transforming growth factor-α in activated human alveolar macrophages. Cell **53:** 285-293.

Magid M, Nanney LB, Stoscheck CM and King LE (1985) Epidermal growth factor binding and receptor distribution in term human placenta. Placenta **6:** 519-526.

Magoffin DA and Weitsmann SR (1993). Insulin like growth facotr 1 stimulates the expression of 3βdehydrogenase messenger ribonucleic acid in ovarian theca cells. Biology of Reproduction **48:** 1166-1173.

Mallasine A, Besse C, Roche A, Alsat E, Rebourcet R, Mondon F and Cedard L (1987). Ultrastructural localization visualization of the internalization of low density lipoprotein by human placental cells. Histochemistry **87:** 457-464.

Marquardt H, Hunkapiller MW, Hood LE and Todaro GJ (1984). Rat transforming growth factor type 1: structure and relation to epidermal growth factor. Science **223:** 1079-1082.

Martinez-Lacaci I, Johnson GR, Salomon DS and Dickson RB (1996). Characterization of novel amphiregulin-related molecule in 12-O-tetradecanoylphorbol-13-acetate-treated breast cancer cells. Jounal of Cellular Physiology **169:** 497-508.

Maruo T and Mochizuki M (1987a). Immunohistochemical localization of epidermal growth factor receptor and myc oncogene product in human placenta: implication for trophoblast proliferation and differentiation. American Journal of Obstetrics and Gynecology **156:** 721-727.

Maruo T, Matsuo H, Oishi T, Hayashi M, Nishino R and Mochizuki M (1987b). Induction of differentiated trophoblast function by epidermal growth factor: relation of immunohistochemically detected cellular epidermal growth factor receptor levels. Journal of Clinical Endocrinology and Metabolism **64:** 744-750.

Maruo T, Matsuo H, Murata K and Mochizuki M (1992). Gestational age-dependent dual action of epidermal growth factor on human placenta early in gestation. Journal of Clinical Endocrinology and Metabolism **75:** 1362-1367.

Maruo T, Matsuo H, Otani T and Mochizuki M (1995). Role of epidermal growth factor (EGF) and its receptor in the development of the human placenta. Reproduction Fertility and Development. **7:** 1465-1470.

Maslar IA, Hess DL, Buckmaster JG, Lazur JJ, Stanczyk FZ and Novy MJ (1990). Steroid production by early pregnancy human placental villi in culture. Placenta **11:** 277-288.

Mason JI and Boyd GS (1971). The chromosomal side chain cleavage enzyme system in mitochrondira of human term plecenta. European Journal of Biochemistry **21:** 308-321.

Massague J (1983). Epidermal growth factor like transforming growth factor. Journal of Biological Chemistry **22:** 13606-13613.

Massague J and Pandiella A (1993). Membrane-anchored growth factors. Annual Review of Biochemistry **62:** 515-541.

McKay I and Leigh I (1993). Growth factors a practical approach. Oxford University Press.

Medan H, Meilke S, Wuttke W and Kuhn W (1997). Elevated serum levels of the c-erbB-2 encoded oncoprotein fragment in cases of pure preeclampsia and HELLP syndrome. Journal of Obstetrics and Gynecology Research **23:**213-217.

Meigs RA and Ryan (1968). Cytochrome P-450 and steroid biosynthesis in the human placenta. Biochemical and Biophysical Acta **165:** 476-482.

Michiels L, Van Leuven F, Van Den Oord JJ, De Wolf-Peeters C and Delabie J (1998). Representational difference analysis using minute quantities of DNA. Nucleic Acid Research **26:** 3608-3610.

Mirlesse V, Alsat E, Fondacci C and Evain-Brion D (1990). Epidermal growth factor receptors in cultured human trophoblast cells from first- and third-trimester placentas. Hormone Research **34:** 234-239.

Modrell B, McDonald VL and Shoyab M (1992). The interaction of amphiregulin with nuclei and putative nuclear localization sequence binding proteins. Growth Factors **7:** 305-314.

Moharam A, Aleem F, Farmakides G, Schulman H, Maghzangy M, Rashed A and Konstantinou (1992). Urinary and amniotic epidermal growth factor during normal and abnormal pregnancies. A comparison based upon umbilical doppler velocimetry. Gynecological Endocrinology **6:** 287-292.

Moise J, Ilekis J, Scommegna A and Beveniste R (1986). The effect of dehydroepiandrosterone sulfate on *de novo* and low-density lipoprotein-stimulated progesterone secretion by human choriocarcinoma JEG-3 cell. American Journal of Obstetrics and Gynecology **154:** 1080-1085.

Moore KL (1989). Before we are born. W.B.Saunders Company.

Morrish DW, Bhardwaj D, Dabbagh LK, Marusyk H and Siy O (1987). Epidermal growth factor induces differentiation and secretion of human chorionic gonadotropin and placental lactogen in normal human placenta. Journal of Clinical Endocrinology and Metabolism **65:** 1282-1290.

Morrish DW, Linetsky E, Bhardwaj D, Li H, Dakour J, Marsh RG, Paterson MC and Godbout R (1996). Identification by subtractive hybridization of a spectrum of novel and unexpected genes associated with in vitro differentiation of human cytotrophoblast cells. Placenta **17:**431-441.

Morrish DW, Dakour J, Li H, Xiao J, Miller R, Sherburne R, Berdan RC and Guilbert LJ (1997). *In vitro* cultured human term cytotrophoblast: a model for normal primary epithelial cells demonstrating a spontaneous differentiation programme that requires EGF for extensive development of syncytium. Placenta **18:** 577-585.

Mueller H, Kueng W, Schoumacher F, Herzer S and Eppenberger U (1995). Selective regulation of steroid receptor expression MCF-7 brest cancer cells by a novel member of the heregulin family. Biochemical and Biophysical Research Communications **3: 1271-1278.**

Muhlhauser J, Crescimanno C, Kaufmann P, Hofler H, Zaccheo D and Castellucci (1993). Differentiation and proliferation patterns in human trophoblast revealed by c-erbB-2 oncogene product and EGF-R1. Journal of Histochemistry and Cytochemistry **41:** 165-173.

Muttukrishna S *et al.*, (1997). Activin A and inhibin A as a possible endocrine markers for pre-eclampsia. Lancet **349:** 1285-1288.

Nakano T, Raines EW, Abraham JA, Wenzel FG, Higashiyama S, Klagsbrun M and Ross R (1993). Glucocorticoid inhibits thrombin-induced expression of platelet-derived

growth factor A-chain and heparin-binding epidermal growth factor-like growth factor in human aortic smooth muscle cells. Journal of Biological Chemistry **268:** 22941-22947.

Nestler JE and Williams T (1987). Modulation of aromatase and P450 cholesterol side-chain cleavage enzyme activities of human placental cytotrophoblasts by insulin and insulin-like growth factor I. Endocrinology **121:** 1845-1851.

Niikura H, Sasano H, Kaga K, Sata S and Yajima A (1996). Expression of epidermal growth factor family proteins and epidermal growth factor receptor in human endometrium. Human Pathology **27:** 282-289.

Niwa H, Harrison LC, DeAaizpurua HJ and Cram DS (1997). Identification of pancreatic β cell-related genes by representational difference analysis. Endocrinology **138:** 1419-1426.

Normanno N, Kim N, Wen D, Smith K, Harris AL, Plowman G, Colletta G, Ciardiello F and Salomom DS (1995). Expression of messenger RNA for amphiregulin, heregulin and cripto-1, three new members of the epidermal growth factor family, in human breast carcinomas. Breast Cancer Research **35:** 293-297.

O'Neill MJ and Sinclair AH (1997). Isolation of rare transcripts by representational difference analysis. Nucleic Acid Research **25:** 2681-2682.

Ohlsson R (1989). Growth factors, protooncogenes and human placental development. Cell Differentiation and Development **28:** 1-16.

Onagbesan OM and Peddie MJ (1995). Effects of insulin like growth factor I and interactions with transforming growth factorα and LH on proliferation of chicken granulosa cells and production of progesterone in culture. Journal of Reproduction and Fertility **104**: 259-265.

Pandiella A and Massague J (1991a). Cleavage of the membrane precursor for transforming growth factor α is a regulated process. Proceedings of The National Academy of Sciences, USA **88:** 1726-1730.

Pandiella A and Massague J (1991b). Multiple signals activate cleavage of the membrane transforming growth factor α precursor. Journal of Biological Chemistry **266:** 5769-5773.

Panico L *et al.*, (1996). Differential immunohistochemical detection of transforming growth factor alpha, amphiregulin and CRIPTO in human normal and malignant breast tissues. International Journal of Cancer **65:** 51-56.

Panigel M (1972). Morphological evaluation of perfused tissues. Acta Endocrinology **158:** 74-94.

Pattillo Ra, Gey GO, Delfs E and Mattingly RF (1968). In vitro identification of the trophoblastic stem cell of the human villous placenta. American Journal of Obstetrics and Gynecology **100:** 582.

Penchow JD (1994). Hybridization histochemisty 8.6.1-8.6.36. Laboratory Histopathology, editors Wood AE & Ellis RC. Churchill Livingstone.

Perry JA (1981). The mammalian fetal membranes. Journal of Reproduction and Fertility **62:** 321-335.

Peterson GL (1977). A simplification of the protein assay method Lowry *et al.*, which is more generally applicable. Analytical Biochemistry **83:** 346-356.

Pfeifer-Ohisson S, Goustin AS, Rydnert J, Wahistorm T, Bjersing L, Stehelin D and Ohisson R (1984). Spatial and temporal pattern of cellular *myc* oncogene expression in developing human placenta: Implications for embryonic cell proliferation. Cell **38:** 585-596.

Pijnenborg R (1990). Trophoblast invasion and placentation in the human: morphological aspects. Trophoblast Research **4:** 33-47.

Plouzek CA, Leslie KK, Stephens JK and Chou JY (1993). Differential gene expression in the amnion, chorion and trophoblast of the human placenta. Placenta **14:** 277-285.

Plowman GD, Green JM, McDonald VL, Neubauer MG, Disteche CM, Todaro GJ and Shoyab M (1990a). The amphiregulin gene encodes a novel epidermal growth factor-related protein with tumor-inhibitory activity. Molecular and Cellular Biology **10:** 1969-1981.

Plowman GD, Whitney GS, Neubauer MG, Green JM, McDonald VL, Todaro GJ and Shoyab M (1990b). Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. Proceedings of The National Academy of Sciences, USA **87:** 4905-4909.

Plowman GD, Green JM, Culouscou J, Carlton GW, Rothwell VM and Buckley S (1993a). Heregulin induces tyrosine phosphorylation of HER4/p180*erb*B4. Nature **2:** 473-475.

Plowman GD, Culouscou J, Whitney GS, Green JM, Carlton GW, Foy L, Neubauer MG and Shoyab M (1993b). Ligand-specific activation of HER4/p180^{erb4}, a fourth member of the epidermal growth factor receptor family. Biochemistry **90:** 1746-1750.

Prigent SA, Lemoine NR, Hughes CM, Plowman GD, Selden C and Gullick WJ (1992a). Expression of the c-erbB-3 protein in normal human adult and fetal tissues. Oncogene **7:** 1273-1278.

Prigent SA and Lemoine NR (1992b). The type 1 (EGFR-related) family of growth factor receptors and their ligands. Progress in Growth Factor Research **4:** 1-24.

Qu A, Brulet C and Thomas K. (1992). Effect of epidermal growth factor on inhibin secretion in human placental cell culture. Endocrinology **131:** 2173-2181.

Qu J and Thomas K (1993). Regulation of inhibin secretion in human placental cell culture by epidermal growth factor, transforming growth factors and activin. Jounal of Clinical Endocrinology and Metabolism **7:** 925-931.

Raab G, Kover K, Paria BC, Dey SK, Ezzell RM and Klagsbrun M (1996). Mouse preimplantation blastocysts adhere to cells expressing the transmembrane form of heparin-binding EGF-like growth factor. Development **122:** 637-645.

Rabe T, Kielsel L and Runnebaum B (1985). Regulation of human placental progesterone synthesis *in vitro* by naturally occuring steriods. Journal of Steroid Biochemistry **22:** 657-664.

Ramani N, Chegini N, Rao CHV, Woost PG and Schultz GS (1986). The presence of epidermal growth factor sites in the intracellular organelles of term human placenta. Journal of Cell Science **84:** 19-40.

Ran W, Dean M, Levine RA, Henkle C and Campisi J (1986). Induction of c-fos and c-myc mRNA by epidermal growth factor or calcium ionophore is cAMP dependent. Proceedings of the National Academy of Sciences, USA **83:** 8216-8220.

Rao Ch.V, Carman Jr FR, Chegini N and Schultz GS (1984). Binding sites for epidermal growth factor in human fetal membranes. Journal of Clinical Endocrinology and Metabolism **58:** 1034-1042.

Rappolee DA, Brenner CA, Schultz R, Mark D, Werb Z (1988). Developmental expression of PDGF, TGF-α , and TGF-β genes in preimplantation mouse embryos. Science **241:**1823-1825.

Redman CWG (1991). Pre-eclampsia and the placenta. Placenta **12:** 301-308.

Renfree MB (1982). Implantation and placentation. Embryonic and fetal development. Editors Austin CR and Short RV. Cambridge University Press.

Reynolds Jr FH, Todaro GJ, Fryling C and Stephenson JR (1981). Human tansforming growth factors induce tyrosine phosphorylation of EGF receptors. Nature **292:** 259-292.

Rheaume E, Lachance Y, Zhao H-F, Breton N, Dumont M, de Launoit Y, Trudel C, Luu-The V, Simard J and Labrie F (1991). Structure and expression of a new complementary DNA encoding the almost exclusive 3β hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 -isomerase in human adrenals and gonads. Molecular Endocrinology **5:** 1147-1157.

Richards RG, Hartman SM and Handwerger S (1994). Human cytotrophoblast cells cultured in maternal serum progress to a differentiated syncytial phenotype expressing both human chorionic gonadotropin and human placental lactogen. Endocrinology **135:** 321-329.

Riese II DJ, Kim ED, Elenius K, Buckley S, Klagsbrun M, Plowman GD and Stern DF (1996a). The epidermal growth factor receptor couples transforming growth factor - α , heparin-binding epidermal growth factor-like factor, and amphiregulin to Neu, ErbB-3, and ErbB-4. The Journal of Biological Chemistry **271:** 20047-20052.

Riese DJ, Bermingham Y, van-Raaij TM, Buckley S, Plowman GD and Stern DF (1996b). Betacellulin activates the epidermal growth factor receptor and erbB-4, and induces cellular response patterns distinct from those stimulated by epidermal growth factor or neuregulin-beta. Oncogene **12:** 345-353.

Ringler GE and Strauss 111 JF (1990). In vitro systems for the study of human placental endocrine function. Endocrine Reviews **11:** 105-123.

Ritvos O, Butzov R, Jalkanen J, Stenman UH, Huhtaniemi I and Ranta T (1988). Differential regulation of hCG and progesterone secretion by cholera toxin and phorbol ester in human cytotrophoblasts. Mollecular and Cellular Endocrinology **56:** 165-169.

Rodway M, Zhou F, Benoit J, Yuen BH and Leung PCK (1988). Differential effects of 8-Bromo-Cyclic AMP on human chorionic gonadotropin (hCG), progesterone and estrogen production by term placental cells. Life Sciences **43:** 1451-1458.

Romero R, Wu YK, Oyarzun E, Hobbins JC and Mitchell MD (1989). A potential role for epidermal growth factor / ∝-transforming growth factor in human parturition. European Journal of Obstetrics & Gynecology and Reproductive Biology **33:** 55-60.

Roulier S, Rochette-Egly C, Rebut-Bonneton C, Porquet D and Evain-brion D (1994). Nuclear retinoic acid receptor characterization in cultured human trophoblast cells: effect of retinoic acid on epidermal growth factor receptor expression. Molecular and Cellular Endocrinology **105:** 165-173.

Saeki T, Stromberg K, Qi CF, Gullick WJ, Tahara E, Normanno N, Ciardiello F, Kenny N, Johnson GR and Solomon DS (1992). Differential immunohistochemical detection of amphiregulin and cripto in human normal colon and colorectal tumors. Cancer Research **52:** 3467-3473.

Sambrook J, Fritsch EF and Maniatis T (1989). Molecular cloning: a laboratory manual. Editor Nolan C. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

Sanger F, Nicklen S and Coulson AR (1977). DNA sequencing with chain terminating inhibitors. Proceedings of The National Academy of Sciences, USA **74:** 5463.

Sarto GE, Stubblefield PA and Therman E (1982). Endomitosis in human trophoblast. human genetics **62:** 228-232.

Sasada R, Ono Y, Taniyama Y, Shing Y, Folkman J and Igarashi K (1993). Cloning and expression of cDNA encoding human betacellulin, a new member of the EGF family. Biomedical and Biophysical Research Communucations **190:** 1173-1179.

Schagger H and Von Jagow G (1987). Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Analytical Biochemistry **166:** 368-379.

Schomon B, Hartmann M, Jones CJ and Desoye G (1991). Insulin and glucose do not affect the glycogen content in isolated and cultured trophoblast cells of human term placenta. Journal of Clinical Endocrinology and Metabolism **73:** 888-893.

Schreiber AB, Winkler ME and Derynck R (1986). Transforming growth factor-α: a more potent angiogenic mediator than epidermal growth factor. Science **242:** 1250-1253.

Schuger L, Johnson GR, Gilbride K, Plowman GD and Mandel R (1996). Amphiregulin in lung branching morphogenesis: interaction with heparan sulfate proteoglycan modulates cell proliferation. Development **122:** 1759-1967.

Sheets EE, Giugni TD, Coates GG, Schlaepfer DD and Haigler HT (1987). Epidermal growth factor dependent phosphorylation of a 35-kilodalton protein in placental membranes. Biochemistry **26:** 1164-1172.

Shing Y, Christofori G, Hanahan D, Ono Y, Sasada R, Igarashi K and Folkman J (1993). Betacellulin: a mitogen from pancreatic β cell tumors. Science **259:** 1604-1607.

Shoyab M, McDonald VL, Bradley JG and Todard GJ. (1988). Amphiregulin: a bifunctional growth-modulating glycoprotein produced by the phorbol 12-myristate 13-acetate-treated human breast adenocarcinoma cell line MCF-7. Proceedings of the National Academy of Sciences, USA **85:** 6528-6532.

Shoyab M, Plowman GD, McDonald VL, Bradley JG and Todaro GJ (1989). Struture and function of human amphiregulin: a member of the epidermal growth factor family. Science **243:** 1074-1076.

Siebert PD, Chenchik A, Kellog DE, Lukyanov KA and Lukyanov SA (1995). An improved method for walking in uncloned genomic DNA. Nucleic Acids Research **23:** 1087-1088.

Simpson ER and MacDonald PC (1981). Endocrine physiology of the placenta. Annual Reveiew of Physiology **43:** 163-188.

Sliwkowski MX, Schaefer G, Akita RW, Lofgren JA, Fitzpatrick VD, Nuijens A, Fendlt BM, Cerione RA, Vandlen RL and Carraway KL (1994). Coexpression of erbB2 and erbB3 proteins reconstitutes a high affintity receptor for heregulin. Journal of Biological Chemistry **269:** 14661-14665.

Sporn MB and Roberts AB (1988). Peptide growth factors are multifunctional. Nature **332:** 217-219.

Stamatoyannopoulos G and Nienhurs AW (1994). The molecular basis of blood disesses. W.B.Saunders, Philadelphia.

Steinborn A, Geisse M and Kaufmann M (1998). Expression of cytokine receptors in the placenta in term and preterm labour. Placenta **19:** 165-170.

Stromberg K, Collins IV TJ, Gordon AW, Jackson CL and Johnson GR (1992). Transforming growth factor-α acts as an autocrine growth factor in ovarian carcinoma cell lines. Cancer Research **52:** 341-347.

Tahara M, Tasaka K, Masumoto N, Adachi K, Adachi H, Ikebuchi Tahara M, Tasaka K, Masumoto N, Adachi K, Adachi H, Ikebuchi , Kurachi H and Miyake A (1995). Expression of messenger ribonucleic acid for epidermal growth factor (EGF), transforming growth factor -α(TGFα), and EGF receptor in human amnion cells: possible role of TGFα in prostaglandin E2 synthesis and cell proliferation. Journal of Clinical Endocrinology and Metabolism **80:** 138-146.

Tal M, King CR, Kraus MH, Ullrich A, Schlessinger J and Givol D (1987). Human HER2 (neu) promoter: evidence for multiple mechanisms for transcriptional initiation. Molecular and Cellular Biology **7:** 2597-2601.

Tavare JM, Diggle TA and Denton RM (1987). Epidermal growth factor, but not insulin, stimulates tyrosine phosphorylation of an endogenous protein of Mr 95,000 in triton extracts of human placental syncytiotrophoblast membranes. Biochemical Journal **244:** 769-774.

Taylor CM, Stevens H, Anthony FW and Wheeler T (1997). Influence of hypoxia on vascular endothelical growth factor and chorionic gonadotrophin production in the trophoblast-derived cell lines: JEG, JAr and BeWo. Placenta **18:** 451-458.

Teixido J, Gilmore R, Lee DC and Massague J (1987). Integral membrane glycoprotein properties of the prohormone pro-transforming growth factor-α. Nature **326:** 883-885.

Teixido J and Massague J (1988). Structural properties of a soluble bioactive precursor for transforming growth factor -α. Journal of Biological Chemistry **263:** 3924-3929.

Tekpetey FR, Singh B, Barbe G and Armstrong DT (1995a). Localisation of epidermal growth factor (EGF) receptor in the rat corpus luteum and EGF and transforming growth factor -α stimulation of luteal cell steroidgenesis in vitro. Molecular and Cellular Endocrinology **110:** 95-102.

Tekpetey FR, Daniel SAJ and Yuzpe A (1995b). Epidermal growth factor (EGF) receptor localization in cultured human granulosa lutein cells and the stimulation of progesterone production by EGF and transforming growth factor α (TGFr-α). Journal of Assisted Reproduction and Genetics **12:** 720-727.

Telgedy G, Weeks KW, Lerner U, Stakemann G and Diczfalusy E (1970). Acetate and cholesterol matabolism in the human foetoplacental unit at midgestation. 1. Synthesis of cholesterol. Acta Endocrinology (Copenhagen) **63:** 91-104.

Temizer DH, Yoshizumi M, Parrella MA, Susanni EE, Quertermous T and Lee ME (1992). Induction of heparin-binding epidermal growth factor-like growth factor mRNA by phorbol ester and angiotensin II in rat aortic smooth muscle cells. Journal of Biological Chemistry **267:** 24892-24896.

The VL, Lachance Y, Labrie C, Leblanc G, Thomas JL, Strickler RC and Labrie F (1989). Full length cDNA structure and deduced amino acid sequence of human 3β-Hydroxy-5-Ene steroid dehydrogenase. Molecular Endocrinology **3:** 1310-1312.

Thomas JL, Berko EA, Faustino A, Myers RP and Strickler (1988). Human placental 3β-hydroxy-5-ene-steroid dehydrogenase and steroid 5→4-ene-isomerase: purification from microsomes, substrate kinetics and inhibition by product steroids. Journal of Steroid Biochemistry **31:** 785-793.

Thorne BA and Plowman GD (1994). The heparin-binding domain of amphiregulin necessitates the precursor pro-region for growth factor secretion. Molecular and Cellular Biology **14:** 1635-1646.

Tulchinsky D, Hobel CJ, Yeager E and Marshall JR (1972). Plasma estrone, estradiol, estriol, progesterone and 17-hydroxyprogesterone in human pregnancy. I. Normal pregnancy. American Journal of Obstetrics and Gynecology **112:** 1095-1100.

Tzahar E *et al.*, (1994). ErbB-3 and ErbB-4 function as the respective low and high affinity receptors of all neu differentiation factor/heregulin isoforms. Journal of Biological Chemistry **269:** 25226-25233.

Ullrich A and Schlessinger J (1990). Signal transduction by receptors with tyrosine kinase activity. Cell **61:** 203-212.

Valentine-Braun KA, Northup JK and Hollenberg MD (1986). Epidermal growth factor (urogastrone)-mediated phosphorylation of a 35-kDa substrate in human placental membranes: Relationship to the β subunit of the guanine nucleotide regulatory complex. Proceedings of the National Academy of Sciences, USA **83:** 236-240.

Varangot J, Cedard L and Yannotti (1965). Perfusion of the human placenta in vitro. Study of the biosynthesis of estrogens. American Journal of Obstetrics and Gynecology **92:** 534-543.

Vaughan TJ, Pascall JC and Brown KD (1992). Tissue distribution of mRNA for heparin-binding epidermal growth factor. Biochemistry Journal **287:** 681-684.

Vician L, Basconcillo R and Herschman HR (1997). Identification of genes preferentially induced by nerve growth factor versus epidermal growth factor in PC12 pheochromocytoma cells by means of representational difference analysis. Journal of Neuroscience Research **50:** 32-43.

Voet D and Voet JG (1990). Biochemistry, Chapter 33. John Wiley & Sons.

Wang X, Das SK, Damm D, Klagsbrun M, Abraham A and Dey SK (1994). Differential regulation of heparin-binding epidermal growth factor-like growth factor in the adult ovariectomized mouse uterus by progesterone and estrogen. Endocrinology **135:** 1264-1271.

Wallasch C, Weiss FU, Niederfellner G, Jallal B, Issing W and Ulrich A (1995). Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodemerization with HER3. EMBO Journal **14:** 4267-4275.

Warren JC and Timberlake CE (1962). Steroid sulphatase in the human placenta. Journal of Clinical Endocrinology **22:** 1148-1151.

Watanabe T, Shintani A, Nakata M, Shing Y, Folkman J Igarashi K and Sasada R (1994). Recombinant human betacellulin. Journal of Biological Chemistry **269:** 9966-9973.

Watanabe T, Fukaya T, Yajima A and Sasano H (1997). Expression of epidermal growth factor (EGF) family and expression of epidermal growth factor receptor (EGRF) in human chorionic villi. Trophoblast Research **9:** 41-52.

Weatherall DJ and Clegg JB (1981). The thallasssaemia syndromes. Blackwell Scientific Publications.

Wen D et al., (1992). Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. Cell **69:** 559-572.

Wilcox JN and Derynck R (1988). Developmental expression of transforming growth factors alpha and beta in mouse fetus. Molecular and Cellular Biology **8:** 3415-3422.

Wilson EA and Jawad MJ (1980). Luteinizing hormone-releasing hormone suppression of human placental progesterone production. Fertility and Sterility **33:** 91-93.

Wilson EA, Jawad MJ and Vernon MW (1984). Effect of epidermal growth factor on hormone secretion by term placenta in organ culture. American Journal of Obstetrics and Gynecololgy **149:** 579-580.

Winkel CA, Snyder JM, MacDonald PC and Simpson ER (1980). Regulation of cholesterol and progesterone synthesis in human placental cells in culture by serum lipoproteins. Endocrinology **106:** 1054-60.

Wong ST, Winchell LF, Mccune BK, Earp HS, Teixido J, Massague J, Herman B and Lee DC (1989). The TGF-α precursor expressed on the cell surface binds to the EGF receptor on adjacent cells, leading to signal transduction. Cell **56:** 495-506.

Wunsch DM, Anderson LD, Pepe GJ and Albrecht ED (1986). Regulation of progesterone formation by human placental cells in culture. Endocrinology **119:** 998-1003.

Yagel S, Casper RF, Powell W, Parhar RS and Lala PK (1989). Characterization of pure human first-trimster cytotrophoblast cells in long-term culture: Growth pattern, markers, and hormone production. American Journal of Obstetrics Gynecology. **160:** 938-945.

Yamaguchi M, Endo OH, Thordarson G, Kensinger R and Talamantes F (1992). Epidermal growth factor stimulates mouse placental lactogen I but inhibits mouse placental

lactogen II secretion in vitro. Proceedings of the National Academy of Sciences, USA **89:** 11396-11400.

Yamaguchi M, Ogren L, Kurachi H, Hirota K, Imai T and Talamantes F (1995). Opposite effects of transforming growth factor α and epidermal growth factor on mouse placental lactogen I secretion. Physiology **92:** 2830-2834.

Yasui W, Ji Z-Q, Kuniyasu H, Ayhan A, Yokozaki H, Ito H and Tahara E (1992). Expression of transforming growth factor alpha in human tissues: immunohistochemical study and Northern Blot analysis. Virchows Achives A Pathological Anatomy and Histopathology **421:**513-519.

Yeger H, Lines LD, Wong P and Silver MM (1989). Enzymatic isolation of human trophoblast and culture on various substrates: comparison of first trimester with term trophoblast. Placenta **10:** 137-151.

Yeh J, Lee GY and Anderson E (1993). Presence of transforming growth factor-alpha messenger ribonucleic acid (mRNA) and absence of epidermal growth factor mRNA in rat ovarian granulosa cells, and the effects of these factors on sreroidogenesis in vitro. Biology of Reproduction **48:** 1071-1081.

Yui J, Garcia-Lloret M, Brown AJ, Berdan RC, Morrish DW, Wegmann TG and Guilbert LJ (1994). Functional, long-term cultures of human term trophoblasts purified by column-elimination of CD9 expressing cells. Placenta **15:** 231-246.

Zhang Z, Funk C, Roy D, Glasser S and Mulholland J (1994a). Heparin-binding epidermal growth factor-like growth factor is differentially regulated by progesterone and estradiol in rat uterine epithelial and stromal cells. Endocrinology **134:** 1089-1094.

Zhang Z, Funk C, Glasser SR and Mulholland J (1994b). Progesterone regulation of heparin-binding epidermal growth factor-like growth factor gene expression during sensitization and decidualization in the rat uterus: effects of the antiprogestin, ZK 98.299. Endocrinology **135:** 1256-1263.

Mercy Hospital For Women

Explanation and Consent Form

Title: Paracrine Interactions in the Human Placenta

Investigators: Dr. M. Towstoless and Dr. R. Fairclough

We seek healthy pregnant volunteers who are willing to provide us with samples of the placenta (afterbirth) and attached membranes prior to their normal disposal.

These tissues (placenta and attached membranes) are usually removed from the womb at the time of birth by your doctor and disposed of through the Hospital's system. The collection of these tissue samples for this research project will not interfere with the delivery of your baby nor cause problems with any future pregnancies you may desire.

A wide range of chemical compounds (such as hormones and related substances) are thought to play a vital role in the growth, development and maintenance of the fetus, the placenta (afterbirth) and attached membranes, and in the initiation of labor. These compounds are produced by the placenta and attached membranes and often exert their effects locally on neighboring cells.

In this study we seek to understand the role of these compounds in normal pregnancy and labour.

Mercy Hospital for Women

Consent form: Paracrine Interactions in the Human Placenta

Would you feel comfortable about disclosing whether you smoked cigarettes during your pregnancy?
Yes/No

Have you smoked cigarettes while you have been pregnant?
Yes/No

Did you smoke throughout your pregnancy?
Yes/No

How many days/week did you smoke?.....

How many cigarettes/day (on average) did you smoke?.....

I.....agree to participate in the research study entitled "Paracrine Interaction in the Human Placenta".

I have read and understood the attached information sheet and .....has discussed the study with me so I believe that I am fully informed on the matter.

I also understand that I am free to with draw from the study at any time and that my treatment at the hospital will not be affected if I do.

....
Patients name (print) Patients signature Date

....
Witnesses name (print) Witnesses signature Date

w/(plac) :

gest. age :

216

Appendix 2a Amino acid sequence alignments of members of the EGF family

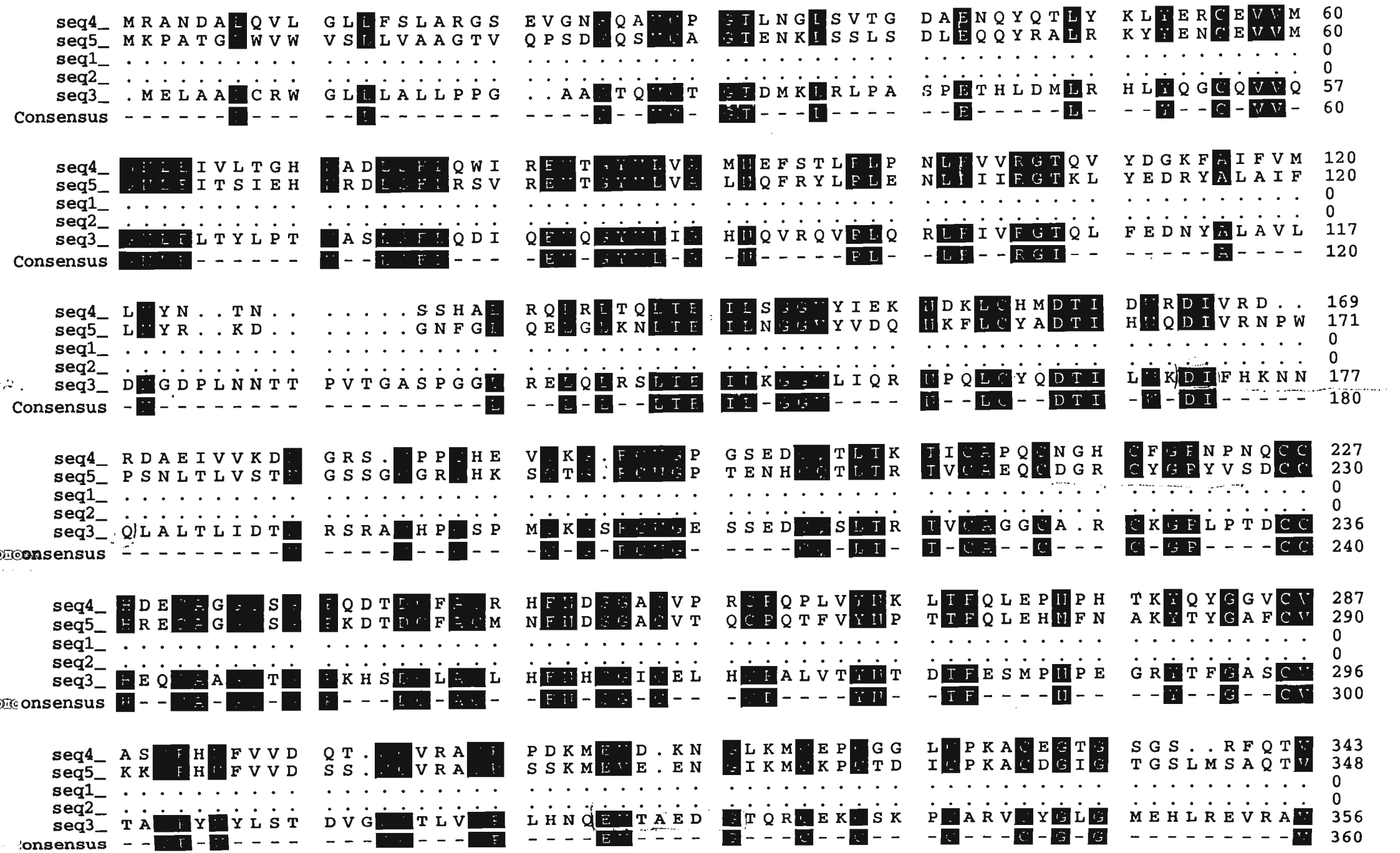
PRETTYBOX of: humplacprot2.msf(*) September 15, 1996 09:24:32.43

```
           p35070 MDRAARCSGA SSLPLLLALA LG...LVILH CVVADGNSTR SPETNGLLCG DPEENCAATT 57
              tgf2 .....MVPSA GQLAL...FA LG...IVLAA C.QALENSTS P......LSA DPPVAAAVV. 41
              egf2 GGYVCRCSEG YQGDGIHCLD IDEC.QLGVH SCGENASCTN TEGGYTCMCA GRLSEPGLIC 951
           humanar VSEMPSSSEP SSGADYDYSE EYDN.EPQIP GYIVDDS... VRVEQVVKPP QNKTESENTS 121
           a43273 V..ISKLGND SASANITIVE SNEI.ITGMP ASTEGAYVSS ESPIRISVST EGANTSSSTS 170
        crio_human ..MDCRKMAR FSYSVIWIMA ISKVFELGLV AGLGHQEFAR PSRGYLAFRD DSIWPQEEPA 58
         Consensus ---------- ---------- ---------- ---------- ---------- ---------- 960

           p35070 TQSKRKGHF. .......... ..SRCPKQYK HYCIK.GRCR FVVAEQTPS. ..CVCDEGYI 100
              tgf2 ......SHF. .......... ..NDCPDSHT QFCFH.GTCR FLVQEDKPA. ..CVCHSGYV 78
              egf2 PDSTPPPHLR EDDHHYSVRN SDSECPLSHD GYCLHDGVCM YIEALDKYA. ..CNCVVGYI 1008
           humanar DKPKRKKKGG KNGKNRRNRK KKNPCNAEFQ NFCIH.GECK YIEHLEAVT. ..CKCQQEYF 177
           a43273 TSTTGTSHLV K.......... ....CAEKEK TFCVNGGECF MVKDLSNPSR YLCKCQPGFT 217
        crio_human IRPRSSQRVP PMGIQ..... ...HSKELNR TCCLNGGTCM LGS....... .FCACPPSFY 102
         Consensus -------H-- ---------- ----C----- --C---G-C- ---------- --C-C--GY- 1020

           p35070 GARCERVDL. .FYLRG...D RGQILVICLI AVMVVFIILV IGVCTCCHPL RKRRKRKK.. 153
              tgf2 GARCEHADL. .LAVVAASQK KQAITALVVV SIVALAVLII TCVLIHCCQV RKHCEWCRAL 136
              egf2 GERCQYRDLK WWELRHAGHG QQQKVIVVAV CVVVLVMLLL LSLWGAHY.Y RTQKLLSKNP 1067
           humanar GERCGEKSMK THSMIDSSLS KIALAAIAAF ....MSAVIL TAVAVITVQL RRQYVR.... 229
           a43273 GARCTENVPM KVQNQEKAEE LYQKRVLTIT GICIALLVVG IMCVVAYCKT KKQRKKLHDR 277
        crio_human GRNCEHDVRK ENCGSVPHDT WLPKKCSLCK CWHGQLRCFP QAFLPGCDGL VMDEHLVASR 162
         Consensus G-RC-------- ---------- ---------- ---------- ---------- R--------- 1080

           p35070 .....KEEEM ETLGKDITPI NEDIEETNIA .......... .......... .......... 178
              tgf2 IC...RHEKP SALLKGRTAC CH..SETVV. .......... .......... .......... 160
              egf2 KN...PYEES SRDVRSRRPA DTEDGMSSCP QPWFVVIKEH QDLKNGGQPV AGEDGQAADG 1124
           humanar .....KYEGE AEERKKLRQE NGNVHAIA.. .......... .......... .......... 252
           a43273 LRQSLRSERN NMMNIANGPH HPNPPPENVQ LVNQYVSKNV ISSEHIVERE AETSFSTSHY 337
        crio_human TPELPPSART TTFMLVGICL SIQSYY.... .......... .......... .......... 188
         Consensus -------E-- ---------- ---------- ---------- ---------- ---------- 1140

           p35070 .......... .......... .......... .......... .......... .......... 178
              tgf2 .......... .......... .......... .......... .......... .......... 160
              egf2 SMQPTSWRQE PQLCGMGTEQ GCWIPVSSDK GSCPQVMERS FHMPSYGTQT LEGGVEKPHS 1184
           humanar .......... .......... .......... .......... .......... .......... 252
           a43273 TSTAHHSTTV TQTPSHSWSN GHTESILSES HSVIVMSSVE NSRHSSPTGG PRGRLNGTGG 397
        crio_human .......... .......... .......... .......... .......... .......... 188
         Consensus -------- ---------- ---------- ---------- ---------- ---------- 1200
```

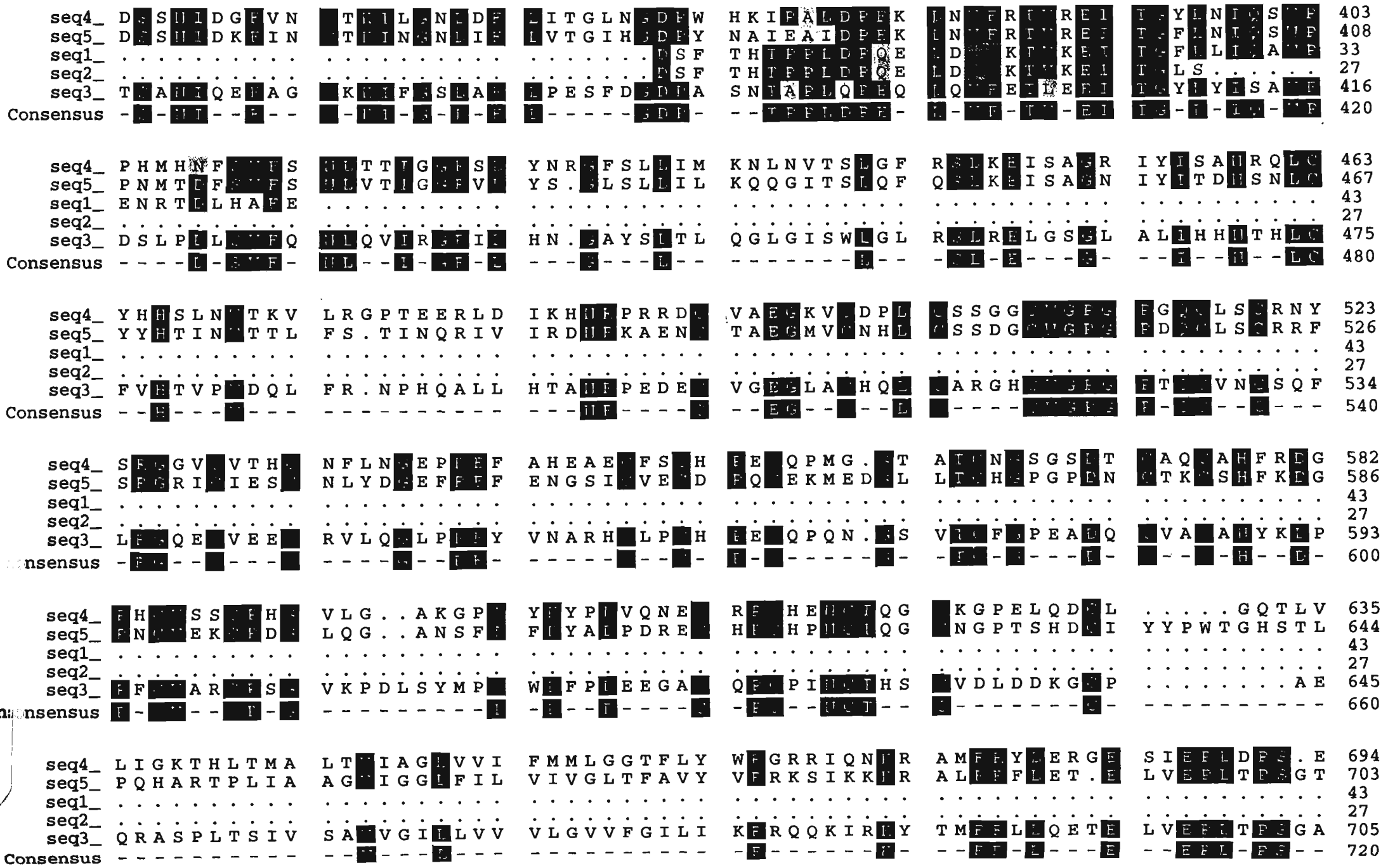
p35070=BTC, tgf2=TGFα, egf2=EGF, humanar=AR, a43273=HRG and crio-human=CR

```
          p35070  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  178
            tgf2  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  160
            egf2  L L S A N P L W Q Q  R A L D P P H Q M E  L T Q . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  1207
         humanar  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  252
          a43273  P R E C N S F L R H  A R E T P D S Y R D  S P H S E R Y V S A  M T T P A R M S P V  D F H T P S S P K S  P P S E M S P P V S  457
        crio_human  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  188
         Consensus  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  1260

          p35070  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  178
            tgf2  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  160
            egf2  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  1207
         humanar  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  252
          a43273  S M T V S M P S M A  V S P F M E E E R P  L L L V T P P R L R  E K K F D H H P Q Q  F S S F H H N P A H  D S N S L P A S P L  517
        crio_human  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  188
         Consensus  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  1320

          p35070  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  178
            tgf2  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  160
            egf2  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  1207
         humanar  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  252
          a43273  R I V E D E E Y E T  T Q E Y E P A Q E P  V K K L A N S R R A  K R T K P N G H I A  N R L E V D S N T S  S Q S S N S E S E T  577
        crio_human  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  188
         Consensus  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  1380

          p35070  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  178
            tgf2  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  160
            egf2  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  1207
         humanar  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  252
          a43273  E D E R V G E D T P  F L G I Q N P L A A  S L E A T P A F R L  A D S R T N P A G R  F S T Q E E I Q A R  L S S V I A N Q D P  637
        crio_human  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  188
         Consensus  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  - - - - - - - - - -  1440

          p35070  . . . 178
            tgf2  . . . 160
            egf2  . . . 1207
         humanar  . . . 252
          a43273  I A V 640
        crio_human  . . . 188
         Consensus  - - - 1443
```

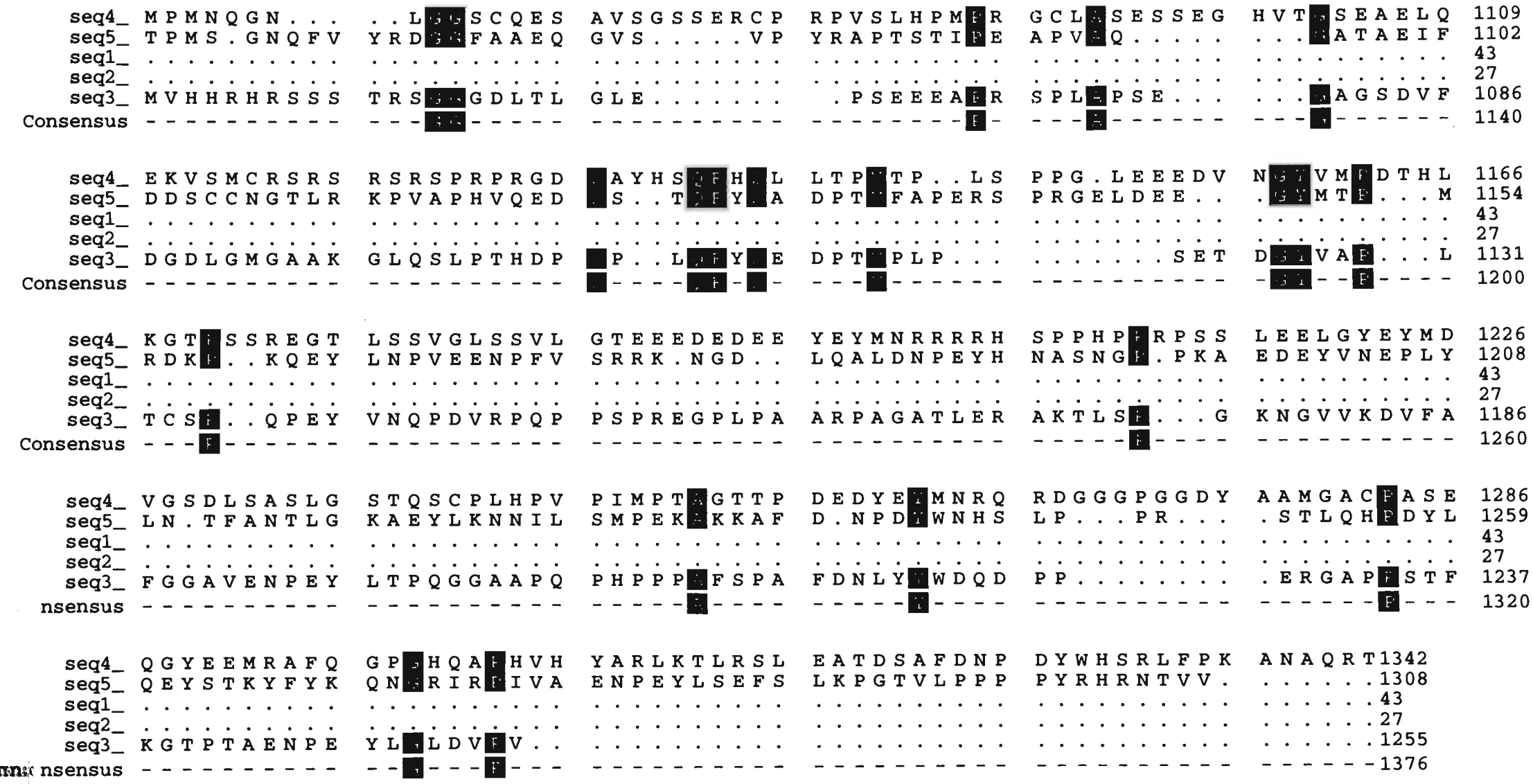
Appendix 2b Amino acid sequence alignments of the members of the EGFR family



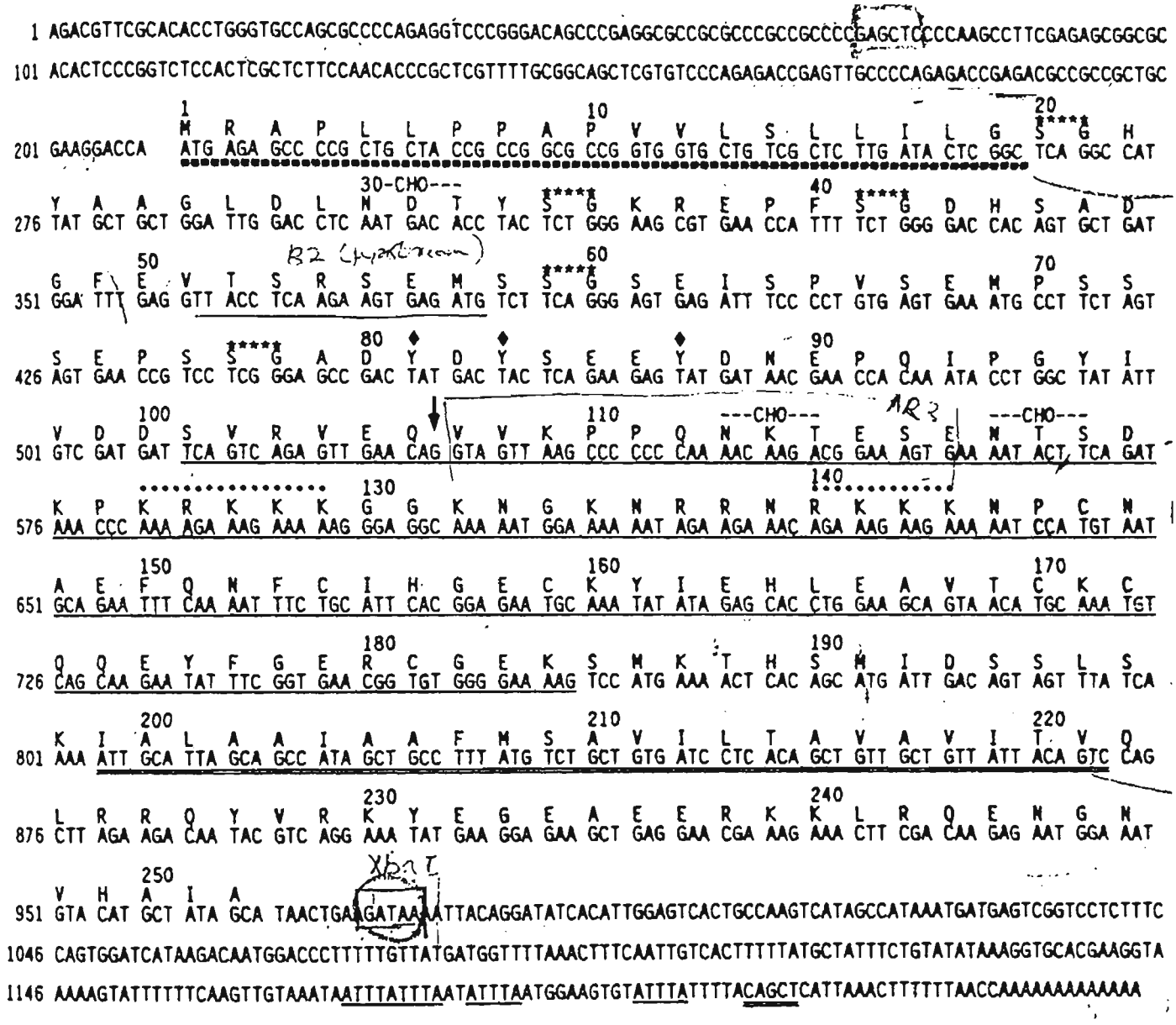
seq2=EGFR, seq3=c-erb-B2, seq4=c-erb-B3 and seq5=c-erb-B4



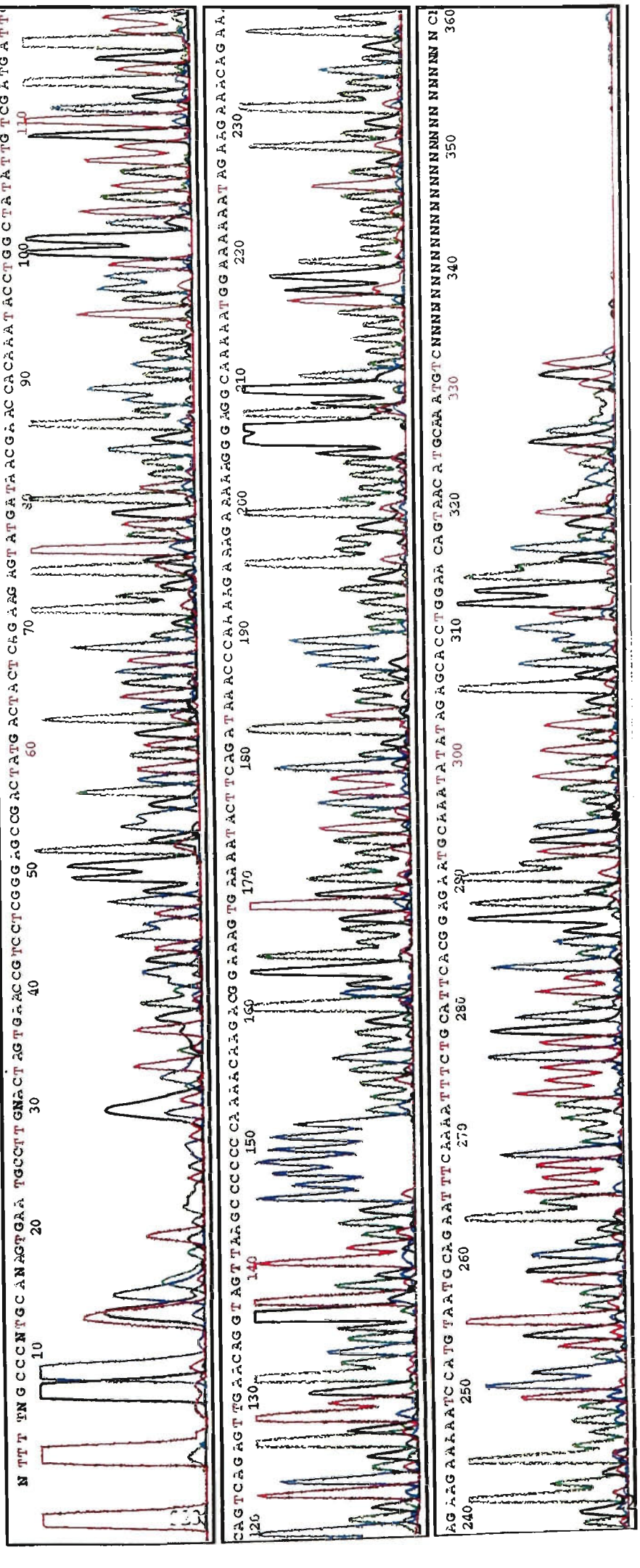
```
seq4_      KANKVLARIF KETELRKLKV LGSGVFGTVH KGVWIPEGES IKIPVCIKVI EDKSGRQSFQ  754
seq5_      APNQAQLRIL KETELKRVKV LGSGAFGTVY KGIWVPEGET VKIPVAIKIL NETTGPKANV  763
seq1_      .......... .......... .......... .......... .......... ..........  43
seq2_      .......... .......... .......... .......... .......... ..........  27
seq3_      MPNQAQMRIL KETELRKVKV LGSGAFGTVY KGIWIPDGEN VKIPVAIKVL RENTSPKANK  765
Consensus  --N----RI- KETEL---KV LGSG-FGTV- KG-W-P-GE- -KIPV-IK-- ----------  780

seq4_      AVTDHMLAIG SLDHAHIVRL LGLCPGSSLQ LVTQYLPLGS LLDHVRQHRG ALGPQLLLNW  814
seq5_      EFMDEALIMA SMDHPHLVRL LGVCLSPTIQ LVTQLMPHGC LLEYVHEHKD NIGSQLLLNW  823
seq1_      .......... .......... .......... .......... .......... ..........  43
seq2_      .......... .......... .......... .......... .......... ..........  27
seq3_      EILDEAYVMA GVGSPYVSRL LGICLTSTVQ LVTQLMPYGC LLDHVRENRG RLGSQDLLNW  825
Consensus  ---D------ --------RL LG-C-----Q LVTQ--P-G- LL--V----- --G-Q-LLNW  840

seq4_      GVQIAKGMYY LEEHGMVHRN LAARNVLLKS PSQVQVADFG VADLLPPDDK QLLYSEAKTP  874
seq5_      CVQIAKGMMY LEERRLVHRD LAARNVLVKS PNHVKITDFG LARLLEGDEK EYNADGGKMP  883
seq1_      .......... .......... .......... .......... .......... ..........  43
seq2_      .......... .......... .......... .......... .......... ..........  27
seq3_      CMQIAKGMSY LEDVRLVHRD LAARNVLVKS PNHVKITDFG LARLLDIDET EYHADGGKVP  885
Consensus  --QIAKGM-Y LE----VHR- LAARNVL-KS P--V---DFG -A-LL--D-- -------K-P  900

seq4_      IKWMALESIH FGKYTHQSDV WSYGVTVWEL MTFGAEPYAG LRLAEVPDLL EKGERLAQPQ  934
seq5_      IKWMALECIH YRKFTHQSDV WSYGVTIWEL MTFGGKPYDG IPTREIPDLL EKGERLPQPP  943
seq1_      .......... .......... .......... .......... .......... ..........  43
seq2_      .......... .......... .......... .......... .......... ..........  27
seq3_      IKWMALESIL RRRFTHQSDV WSYGVTVWEL MTFGAKPYDG IPAREIPDLL EKGERLPQPP  945
Consensus  IKWMALE-I- ----THQSDV WSYGVT-WEL MTFG--PY-G ----E-PDLL EKGERL-QP-  960

seq4_      ICTIDVYMVM VKCWMIDENI RPTFKELANE FTRMARDPPR YLVIKRESGP GIAPGPEPHG  994
seq5_      ICTIDVYMVM VKCWMIDADS RPKFKELAAE FSRMARDPQR YLVIQGDDR. MKLPSPNDSK  1002
seq1_      .......... .......... .......... .......... .......... ..........  43
seq2_      .......... .......... .......... .......... .......... ..........  27
seq3_      ICTIDVYMIM VKCWMIDSEC RPRFRELVSE FSRMARDPQR FVVIQNEDL. .GPASPLDST  1003
Consensus  ICTIDVYM-M VKCWMID--- RP-F-EL--E F-RMARDP-R --VI------ -----P----  1020

seq4_      LTNKKLEEVE LEPELDLDLD LEAEEDNLAT TTLGSALSLP VGTLNRPRGS QSLLSPSSGY  1054
seq5_      FFQNLLDEED LEDMMDAEEY LVPQAFNIPP PIYTSRARID S...NR...S EIGHSPPPAY  1056
seq1_      .......... .......... .......... .......... .......... ..........  43
seq2_      .......... .......... .......... .......... .......... ..........  27
seq3_      FYRSLLEDDD MGDLVDAEEY LVPQQGFFCP .......... .......... ..DPAPGAGG  1041
Consensus  -----L---- -----D---- L--------- ---------- ---------- -----P----  1080
```


seq4_ MPMNQGN... ..LGGSCQES AVSGSSERCP RPVSLHPMFR GCLASESSEG HVTGSEAELQ 1109
seq5_ TPMS.GNQFV YRDGGFAAEQ GVS.....VP YRAPTSTIPE APVAQ..... ...GATAEIF 1102
seq1_ 43
seq2_ 27
seq3_ MVHHRHRSSS TRSGGGDLTL GLE..... .PSEEEAER SPLAPSE... ...GAGSDVF 1086
Consensus ---------- ---GG----- ---------- --------F- ---A------ ---G------ 1140
seq4_ EKVSMCRSRS RSRSPRPRGD AAYHSGFHAL LTPTTP..LS PPG.LEEEDV NGTVMFDTHL 1166
seq5_ DDSCCNGTLR KPVAPHVQED AS..TGFYAA DPTFAPERS PRGELDEE.. .GYMTF...M 1154
seq1_ 43
seq2_ 27
seq3_ DGDLGMGAAK GLQSLPTHDP AP..LTFYAE DPTAPLP... ....SET DGIVAF...L 1131
Consensus ---------- ---------- A----AF-A- ---A------ ---------- -GI--F---- 1200
seq4_ KGTFSSREGT LSSVGLSSVL GTEEEDEDEE YEYMNRRRRH SPPHPFRPSS LEELGYEYMD 1226
seq5_ RDKF..KQEY LNPVEENPFV SRRK.NGD.. LQALDNPEYH NASNGF.PKA EDEYVNEPLY 1208
seq1_ 43
seq2_ 27
seq3_ TCSF..QPEY VNQPDVRPQP PSPREGPLPA ARPAGATLER AKTLSF...G KNGVVKDVFA 1186
Consensus ---F------ ---------- ---------- ---------- -----F---- ---------- 1260
seq4_ VGSDLSASLG STQSCPLHPV PIMPTAGTTP DEDYEIMNRQ RDGGGPGGDY AAMGACFASE 1286
seq5_ LN.TFANTLG KAEYLKNNIL SMPEKAKKAF D.NPDIWNHS LP...PR... .STLQHFDYL 1259
seq1_ 43
seq2_ 27
seq3_ FGGAVENPEY LTPQGGAAPQ PHPPPAFSPA FDNLYIWDQD PP........ .ERGAPFSTF 1237
nsensus ---------- ---------- -----A---- -----I---- ---------- ------F--- 1320
seq4_ QGYEEMRAFQ GPAHQAFHVH YARLKTLRSL EATDSAFDNP DYWHSRLFPK ANAQRT 1342
seq5_ QEYSTKYFYK QNARIRFIVA ENPEYLSEFS LKPGTVLPPP PYRHRNTVV. 1308
seq1_ 43
seq2_ 27
seq3_ KGTPTAENPE YLALDVFV.. 1255
nsensus ---------- --A---F--- ---------- ---------- ---------- ------1376

Appendix 3 AR cDNA sequence



Appendix 3a Nucleotide sequence of RT-PCR generated AR cDNA



Appendix 4 BTC cDNA sequence

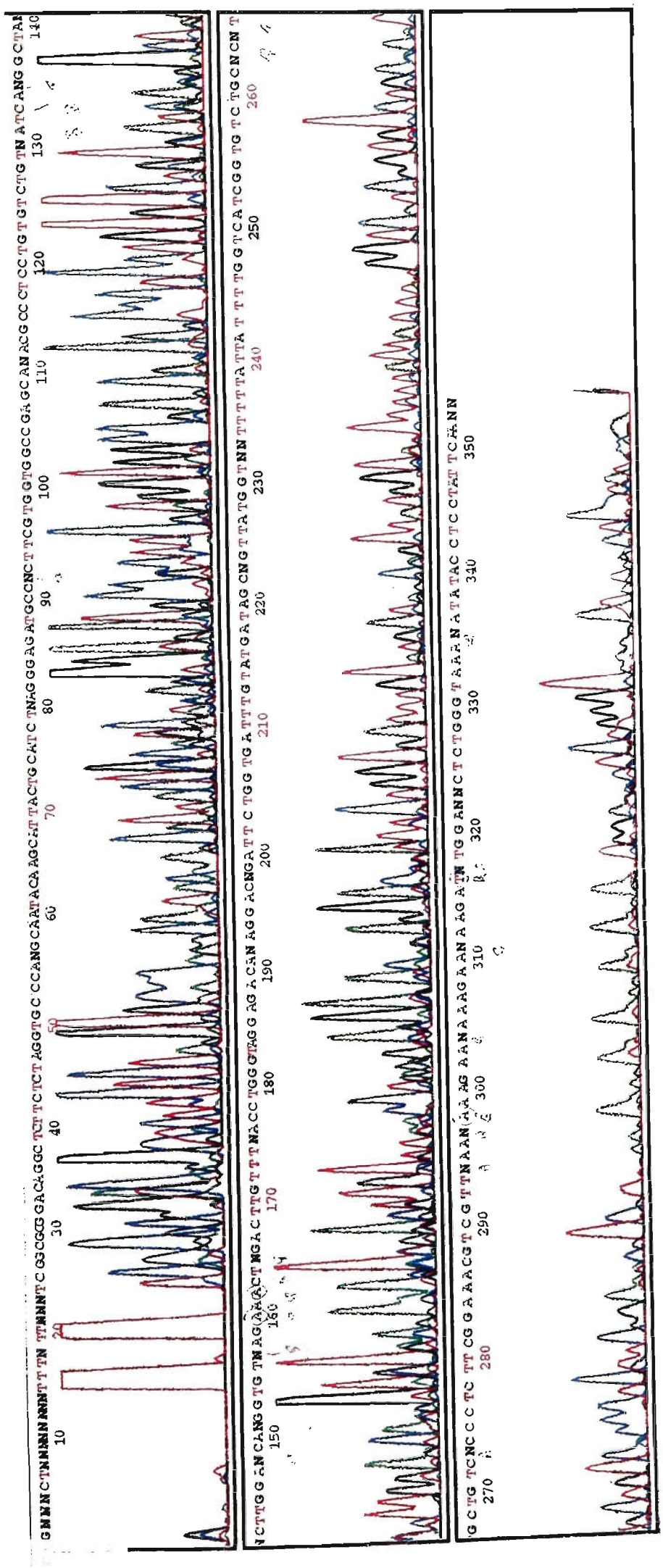
```
LOCUS       S55606       1271 bp    mRNA            PRI       29-APR-1993
DEFINITION  betacellulin [human, mRNA, 1271 nt].
ACCESSION   S55606
KEYWORDS    .
SOURCE      human.
  ORGANISM  Homo sapiens
            Unclassified.
REFERENCE   1  (bases 1 to 1271)
  AUTHORS   Sasada,R., Ono,Y., Taniyama,Y., Shing,Y., Folkman,J. and
            Igarashi,K.
  TITLE     Cloning and expression of cDNA encoding human betacellulin, a new
            member of the EGF family
  JOURNAL   Biochem. Biophys. Res. Commun. 190 (3), 1173-1179 (1993)
  MEDLINE   93176165
  REMARK    GenBank staff at the National Library of Medicine created this
            entry [NCBI gibbsq 125748] from the original journal article.
            This sequence comes from Fig. 1.
COMMENT
            NCBI gi: 265785
FEATURES             Location/Qualifiers
     source          1..1271
                     /organism="Homo sapiens"
                     /note="human"
     CDS             295..831
                     /note="Description: betacellulin, BTC;  Method: conceptual
                     translation supplied by author. This sequence comes from
                     Fig. 1.  BTC;  NCBI gi: 265786"
                     /codon_start=1
                     /product="betacellulin"
                     /translation="MDRAARCSGASSLPLLLALALGLVILHCVVADGNSTRSPETNGL
                     LCGDPEENCAATTTQSKRKGHFSRCPKQYKHYCIKGRCRFVVAEQTPSCVCDEGYIGA
                     RCERVDLFYLRGDRGQILVICLIAVMVVFIILVIGVCTCCHPLRKRRKRKKKEEEMET
                     LGKDITPINEDIEETNIA"
BASE COUNT      346 a    267 c    327 g    331 t
ORIGIN
        1 cagcgtggag gctccaagga ccaagtcctg cgcctctttg gcggggtgtg tgcaggagga
       61 gggggataa ataggaggct ccctcctccc ggcgacattc acggagccgg ccggcctccc
      121 gccctgggtg tttccctgcc ttgtagccag ggtgccagcc tgggaagtag tttcgtttcc
      181 ttctgcctcc gggattagtt tccaggcacc ctctcaggcg cccgaggccc gggaaggggg
      241 cgaagaagga gggagacttg tctaggggct gcccggcccg gcagagcggg gttgatggac
      301 cgggccgccc ggtgcagcgg cgccagctcc ctgccactgc tcctggccct tgccctgggt
      361 ctagtgatcc ttcactgtgt ggtggcagat gggaattcca ccagaagtcc tgaaactaat
      421 ggcctcctct gtggagaccc tgaggaaaac tgtgcagcta ccaccacaca atcaaagcgg
      481 aaaggccact tctctaggtg ccccaagcaa tacaagcatt actgcatcaa agggagatgc
      541 cgcttcgtgg tggccgagca gacgccctcc tgtgtctgtg atgaaggcta cattggagca
      601 aggtgtgaga gagttgactt gttttaccta agaggagaca gaggacagat tctggtgatt
      661 tgtttgatag cagttatggt agtttttatt atttggtca tcggtgtctg cacatgctgt
      721 caccctcttc ggaaacgtcg taaaagaaag aagaaagaag aagaaatgga aactctgggt
      781 aaagatataa ctcctatcaa tgaagatatt gaagagacaa atattgctta aaaggctatg
      841 aagttacctc caggttggtg gcaagctgca aagtgccttg ctcatttgaa aatggacaga
      901 atgtgtctca ggaaaaacag ctagtagaca tgaattttaa ataatgtatt tacttttat
      961 ttgcaacttt agtttgtgtt attatttttt aataagaaca ttaattatat gtatattgtc
     1021 tagtaattgg gaaaaaagca actggttagg tagcaacaac agaagggaaa tttcaataac
     1081 ctttcactta agtattgtca ccaggattac tagtcaaaca aaaaagaaaa gtagaaagga
     1141 ggttaggtct taggaattga attaataata aagctaccat ttatcaagca tttaccatgt
     1201 gctaataagt ttgaaatata ttatttcctt tattcctttc agcaatccat gagatagcta
     1261 ttataatcct c
//
```

Be-ups-in

770 - 799

Be ins

Appendix 4a Nucleotide sequence of RT-PCR generated BTC cDNA

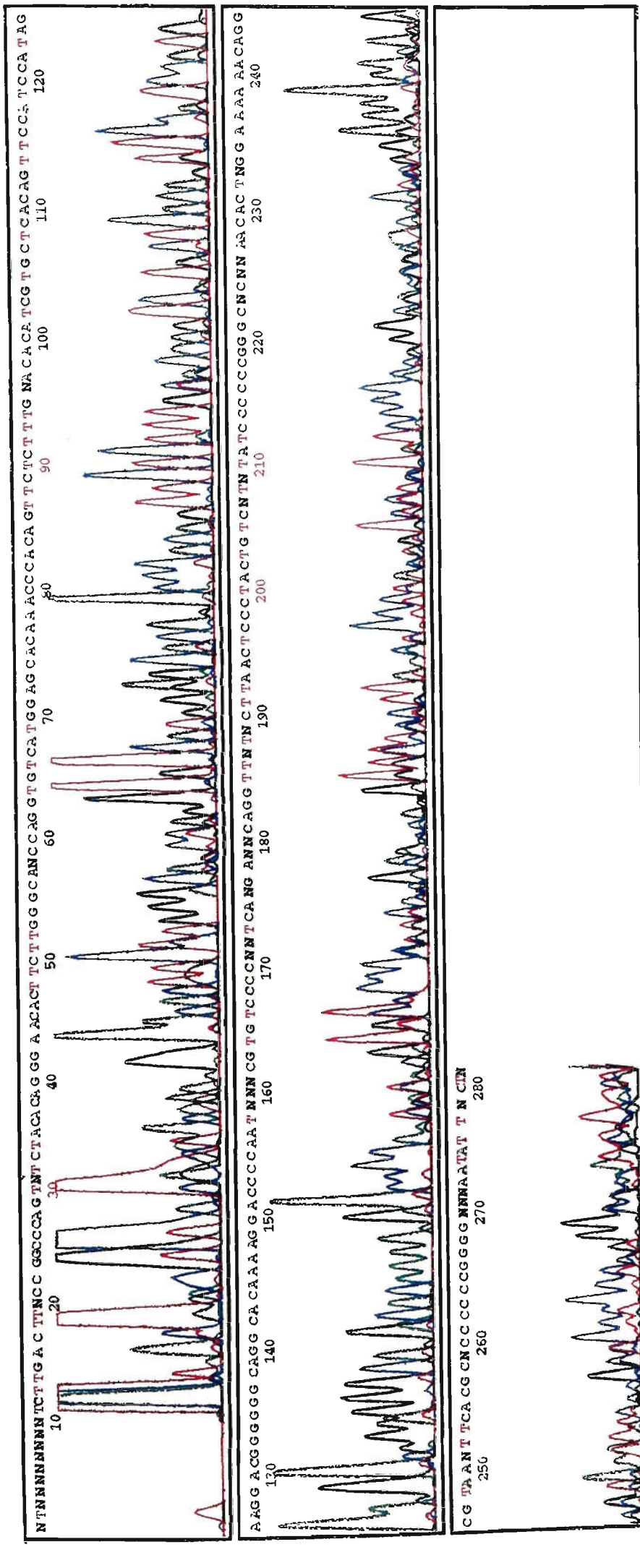


Appendix 8 CR cDNA sequence

SE COUNT 562 a 447 c 430 g 594 t
IGIN

```
   1 ggagaatccc cggaaaggct gagtctccag ctcaaggtca aaacgtccaa ggccgaaagc
  61 cctccagttt cccctggacg ccttgctcct gcttctgcta cgaccttctg gggaaaacga
 121 atttctcatt ttcttcttaa attgccattt tcgctttagg agatgaatgt tttcctttgg
 181 ctgttttggc aatgactctg aattaaagcg atgctaacgc ctcttttccc cctaattgtt
 241 aaaagctatg gactgcagga agatggcccg cttctcttac agtgtgattt ggatcatggc
 301 catttctaaa gtctttgaac tgggattagt tgccgggctg ggccatcagg aatttgctcg
 361 tccatctcgg ggatacctgg ccttcagaga tgacagcatt tggccccagg aggagcctgc
 421 aattcggcct cggtcttccc agcgtgtgcc gcccatgggg atacagcaca gtaaggagct
 481 aaacagaacc tgctgtctga atgggggaac ctgcatgctg gggtcctttt gtgcctgccc
 541 tccctccttc tacggacgga actgtgagca cgatgtgcgc aaagagaact gtgggtctgt
 601 gccccatgac acctggctgc ccaagaagtg ttccctgtgt aaatgctggc acggtcagct
 661 ccgctgcttt cctcaggcat ttctacccgg ctgtgatggc cttgtgatgg atgagcacct
 721 cgtggcttcc aggactccag aactaccacc gtctgcacgt actaccactt ttatgctagt
 781 tggcatctgc ctttctatac aaagctacta ttaatcgaca ttgacctatt tccagaaata
 841 caattttaga tatcatgcaa atttcatgac cagtaaaggc tgctgctaca atgtcctaac
 901 tgaaagatga tcatttgtag ttgccttaaa ataatgaata caatttccaa aatggtctct
 961 aacatttcct tacagaacta cttcttactt ctttgccctg ccctctccca aaaaactact
1021 tctttttca aaagaaagtc agccatatct ccattgtgcc taagtccagt gtttcttttt
1081 ttttttttt ttgagacgga gtctcactct gtcacccagg ctggactgca atgacgcgat
1141 cttggttcac tgcaacctcc gcatccgggg ttcaagccat tctcctgcct aagcctccca
1201 agtaactggg attacaggca tgtgtcacca tgcccagcta attttttgt attttagtag
1261 agatgggggt ttcaccatat tggccagtct ggtctcgaac tctgaccttg tgatccatcg
1321 atcagcctct cgagtgctga gattacacac gtgagcaact gtgcaaggcc tggtgtttct
1381 tgatacatgt aattctacca aggtcttctt aatatgttct tttaaatgat tgaattatat
1441 gttcagatta ttggagacta attctaatgt ggaccttaga atacagtttt gagtagagtt
1501 gatcaaaatc aattaaaata gtctctttaa aaggaaagaa aacatcttta aggggaggaa
1561 ccagagtgct gaaggaatgg aagtccatct gcgtgtgtgc agggagactg ggtaggaaag
1621 aggaagcaaa tagaagagag aggttgaaaa acaaaatggg ttacttgatt ggtgattagg
1681 tggtggtaga gaagcaagta aaaaggctaa atggaagggc aagtttccat catctataga
1741 aagctatata agacaagaac tccccttttt ttcccaaagg cattataaaa agaatgaagc
1801 ctccttagaa aaaaaattat acctcaatgt ccccaacaag attgcttaat aaattgtgtt
1861 tcctccaagc tattcaattc ttttaactgt tgtagaagac aaaatgttca caatatattt
1921 agttgtaaac caagtgatca aactacatat tgtaaagccc atttttaaaa tacattgtat
1981 atatgtgtat gcacagtaaa aatggaaact atattgacct aaaaaaaaaa aaa
```

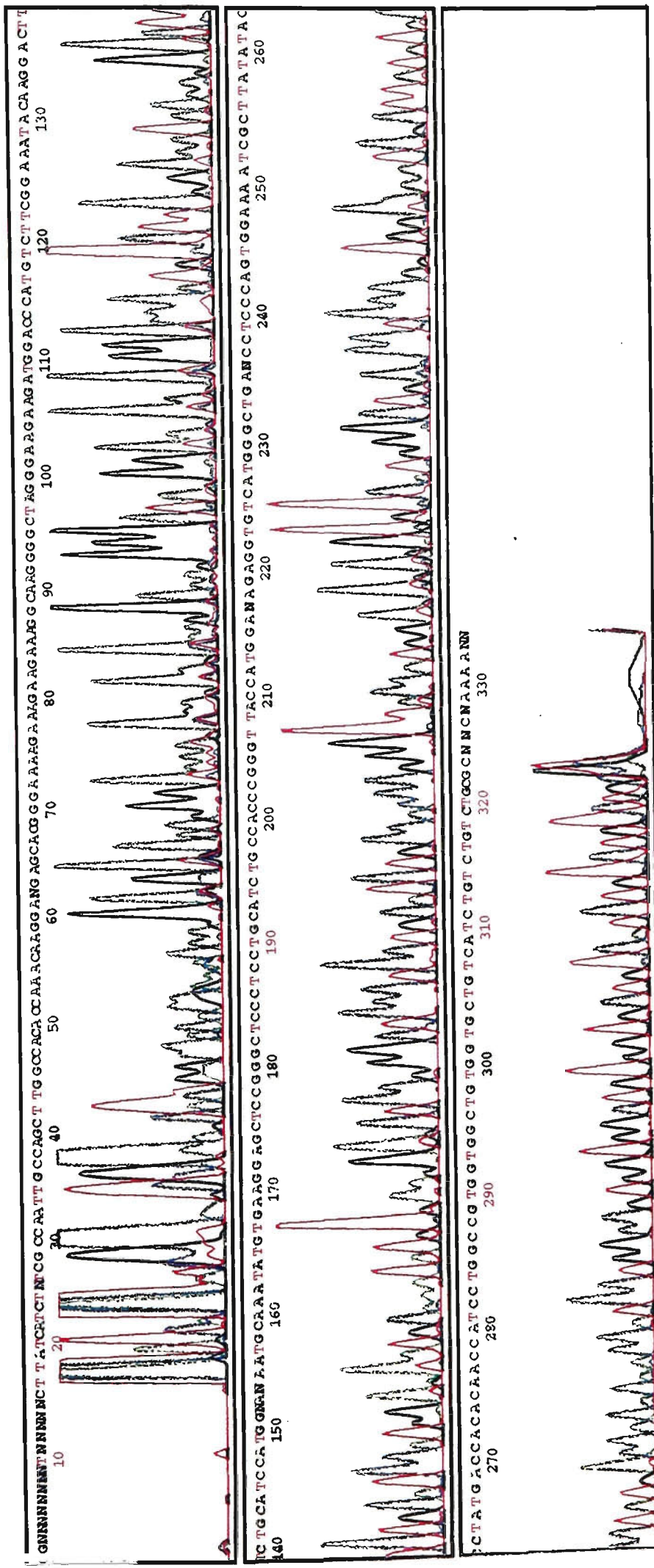
Appendix 5a Nucleotide sequence of RT-PCR generated CR cDNA



```
LOCUS       HUMHBEGF     2360 bp    mRNA            PRI       07-MAR-1991
DEFINITION  Human heparin-binding EGF-like growth factor mRNA, complete cds.
ACCESSION   M60278
KEYWORDS    heparin-binding EGF-like growth factor.
SOURCE      Human histiocytic lymphoma derived cell, cDNA to mRNA.
  ORGANISM  Homo sapiens
            Eukaryota; Animalia; Chordata; Vertebrata; Mammalia; Theria;
            Eutheria; Primates; Haplorhini; Catarrhini; Hominidae.
REFERENCE   1  (bases 1 to 2360)
  AUTHORS   Higashiyama,S., Abraham,J.A., Miller,J.L., Fiddes,J.C. and
            Klagsbrun,M.
  TITLE     A heparin-binding growth factor secreted by macrophage-like cells
            that is related to EGF
  JOURNAL   Science 251, 936-939 (1991)
  MEDLINE   91157008
COMMENT
            NCBI gi: 183866
FEATURES             Location/Qualifiers
     source          1..2360
                     /organism="Homo sapiens"
                     /cell_type="histiocytic lymphoma derived"
                     /sequenced_mol="cDNA to mRNA"
     sig_peptide     262..318
                     /note="putative"
                     /codon_start=1
     CDS             262..888
                     /note="putative;  NCBI gi: 183867"
                     /codon_start=1
                     /product="heparin-binding EGF-like growth factor"
                     /translation="MKLLPSVVLKLFLAAVLSALVTGESLERLRRGLAAGTSNPDPPT
                     VSTDQLLPLGGGRDRKVRDLQEADLDLLRVTLSSKPQALATPNKEEHGKRKKKGKGLG
                     KKRDPCLRKYKDFCIHGECKYVKELRAPSCICHPGYHGERCHGLSLPVENRLYTYDHT
                     TILAVVAVVLSSVCLLVIVGLLMFRYHRRGGYDVENEEKVKLGMTNSH"
     mat_peptide     481..702
                     /evidence=experimental
                     /codon_start=1
                     /product="heparin-binding EGF-like growth factor"
BASE COUNT      599 a    579 c    605 g    577 t
ORIGIN
        1 gctacgcggg ccacgctgct ggctggcctg acctaggcgc gcggggtcgg gcggccgcgc
       61 gggcgggctg agtgagcaag acaagacact caagaagagc gagctgcgcc tgggtcccgg
      121 ccaggcttgc acgcagaggc gggcggcaga cggtgcccgg cggaatctcc tgagctccgc
      181 cgcccagctc tggtgccagc gcccagtggc cgccgcttcg aaagtgactg gtgcctcgcc
      241 gcctcctctc ggtgcgggac catgaagctg ctgccgtcgg tggtgctgaa gctctttctg
      301 gctgcagttc tctcggcact ggtgactggc gagagcctgg agcggcttcg gagagggcta
      361 gctgctggaa ccagcaaccc ggaccctccc actgtatcca cggaccagct gctaccccta
      421 ggaggcggcc gggaccggaa agtccgtgac ttgcaagagg cagatctgga ccttttgaga
      481 gtcactttat cctccaagcc acaagcactg gccacaccaa acaaggagga gcacgggaaa
      541 agaaagaaga aaggcaaggg gctagggaag aagagggacc catgtcttcg gaaatacaag
      601 gacttctgca tccatggaga atgcaaatat gtgaaggagc tccgggctcc ctcctgcatc
      661 tgccacccgg gttaccatgg agagaggtgt catgggctga gcctcccagt ggaaaatcgc
      721 ttatatacct atgaccacac aaccatcctg gccgtggtgg ctgtggtgct gtcatctgtc
      781 tgtctgctgg tcatcgtggg gcttctcatg tttaggtacc ataggagagg aggttatgat
      841 gtggaaaatg aagagaaagt gaagttgggc atgactaatt cccactgaga gagacttgtg
      901 ctcaaggaat cggctgggga ctgctacctc tgagaagaca caaggtgatt tcagactgca
      961 gaggggaaag acttccatct agtcacaaag actccttcgt ccccagttgc cgtctaggat
     1021 tgggcctccc ataattgctt tgccaaaata ccagagcctt caagtgccaa acagagtatg
     1081 tccgatggta tctgggtaag aagaaagcaa aagcaaggga ccttcatgcc cttctgattc
     1141 ccctccacca aaccccactt cccctcataa gtttgtttaa acacttatct tctggattag
     1201 aatgccggtt aaattccata tgctccagga tctttgactg aaaaaaaaaa agaagaagaa
     1261 gaaggagagc aagaaggaaa gatttgtgaa ctggaagaaa gcaacaaaga ttgagaagcc
     1321 atgtactcaa gtaccaccaa gggatctgcc attgggaccc tccagtgctg gatttgatga
     1381 gttaactgtg aaataccaca agcctgagaa ctgaattttg ggacttctac ccagatggaa
     1441 aaataacaac tatttttgtt gttgttgttt gtaaatgcct cttaaattat atatttattt
     1501 tattctatgt atgttaattt atttagtttt taacaatcta acaataatat ttcaagtgcc
     1561 tagactgtta ctttggcaat ttcctggccc tccactcctc atccccacaa tctggcttag
     1621 tgccacccac ctttgccaca aagctaggat ggttctgtga cccatctgta gtaatttatt
     1681 gtctgtctac atttctgcag atcttccgtg gtcagagtgc cactgcggga gctctgtatg
     1741 gtcaggatgt aggggttaac ttggtcagag ccactctatg agttggactt cagtcttgcc
     1801 taggcgattt tgtctaccat ttgtgttttg aaagcccaag gtgctgatgt caaagtgtaa
     1861 cagatatcag tgtctccccg tgtcctctcc ctgccaagtc tcagaagagg ttgggcttcc
     1921 atgcctgtag ctttcctggt ccctcacccc catggcccca ggccacagcg tgggaactca
     1981 ctttcccttg tgtcaagaca tttctctaac tcctgccatt cttctggtgc tactccatgc
     2041 aggggtcagt gcagcagagg acagtctgga gaaggtatta gcaaagcaaa aggctgagaa
     2101 ggaacaggga acattggagc tgactgttct tggtaactga ttacctgcca attgctaccg
```

```
2161 agaaggttgg aggtggggaa ggctttgtat aatcccaccc acctcaccaa aacgatgaag
2221 gtatgctgtc atggtccttt ctggaagttt ctggtgccat ttctgaactg ttacaacttg
2281 tatttccaaa cctggttcat atttatactt tgcaatccaa ataaagataa cccttattcc
2341 ataaaaaaaa aaaaaaaaaa
//
```

Appendix 6a Nucleotide sequence of RT-PCR generated HB-EGF cDNA

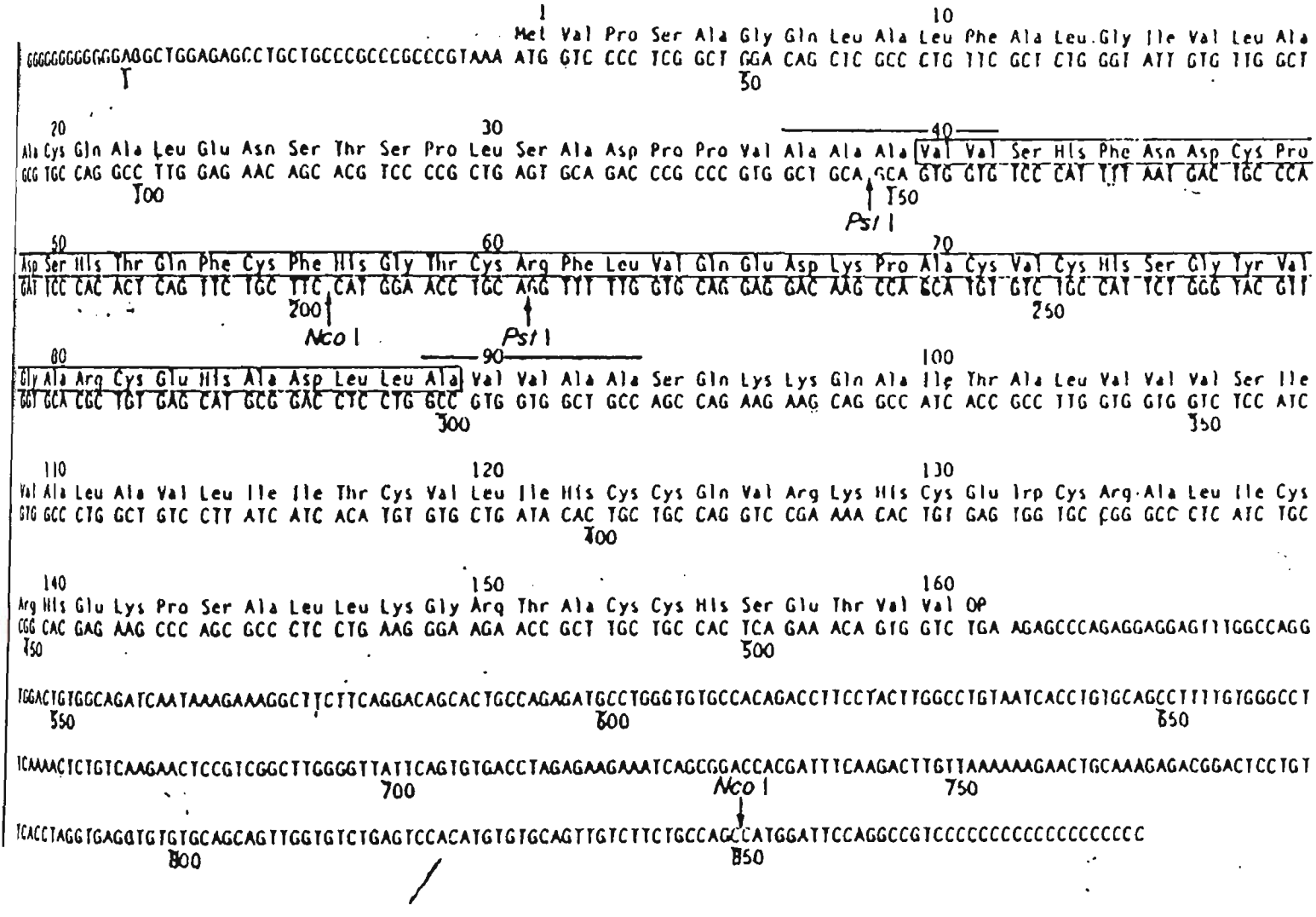


```
LOCUS       HUMHERGA     2224 bp    mRNA            PRI       31-DEC-1994
DEFINITION  Human heregulin-alpha gene, complete cds.
ACCESSION   M94165
KEYWORDS    heregulin-alpha.
SOURCE      Homo sapiens cDNA to mRNA.
  ORGANISM  Homo sapiens
            Eukaryota; Animalia; Chordata; Vertebrata; Mammalia; Theria;
            Eutheria; Primates; Haplorhini; Catarrhini; Hominidae.
REFERENCE   1  (bases 1 to 2224)
  AUTHORS   Holmes,W.E., Sliwkowski,M.X., Akita,R.W., Henzel,W.J., Lee,J.J.,
            Park,J.W., Yansura,D., Abadi,N., Raab,H., Lewis,G.D., Shepard,H.M.,
            Kuang,W.-J., Wood,W.I., Goeddel,D.V. and Vandlen,R.L.
  TITLE     Identification of heregulin, a specific activator of p185erbB2
  JOURNAL   Science 256 (5060), 1205-1210 (1992)
  MEDLINE   92271253
COMMENT
            NCBI gi: 183992
FEATURES             Location/Qualifiers
     source          1..2224
                     /organism="Homo sapiens"
                     /cell_line="MDA-MB-231"
                     /sequenced_mol="cDNA to mRNA"
     CDS             133..2055
                     /gene="heregulin"
                     /note="NCBI gi: 183993"
                     /codon_start=1
                     /product="heregulin-alpha"
                     /translation="MSERKEGRGKGKGKKKERGSGKKPESAAGSQSPALPPRLKEMKS
                     QESAAGSKLVLRCETSSEYSSLRFKWFKNGNELNRKNKPQNIKIQKKPGKSELRINKA
                     SLADSGEYMCKVISKLGNDSASANITIVESNEIITGMPASTEGAYVSSESPIRISVST
                     EGANTSSSTSTSTTGTSHLVKCAEKEKTFCVNGGECFMVKDLSNPSRYLCKCQPGFTG
                     ARCTENVPMKVQNQEKAEELYQKRVLTITGICIALLVVGIMCVVAYCKTKKQRKKLHD
                     RLRQSLRSERNNMMNIANGPHHPNPPPENVQLVNQYVSKNVISSEHIVEREAETSFST
                     SHYTSTAHHSTTVTQTPSHSWSNGHTESILSESHSVIVMSSVENSRHSSPTGGPRGRL
                     NGTGGPRECNSFLRHARETPDSYRDSPHSERYVSAMTTPARMSPVDFHTPSSPKSPPS
                     EMSPPVSSMTVSMPSMAVSPFMEEERPLLLVTPPRLREKKFDHHPQQFSSFHHNPAHD
                     SNSLPASPLRIVEDEEYETTQEYEPAQEPVKKLANSRRAKRTKPNGHIANRLEVDSNT
                     SSQSSNSESETEDERVGEDTPFLGIQNPLAASLEATPAFRLADSRTNPAGRFSTQEEI
                     QARLSSVIANQDPIAV"
BASE COUNT      685 a    594 c    505 g    440 t
ORIGIN
        1 gcgcgagcgc ctcagcgcgg ccgctcgctc tccccctcga gggacaaact tttcccaaac
       61 ccgatccgag cccttggacc aaactcgcct gcgccgagag ccgtccgcgt agagcgctcc
      121 gtctccggcg agatgtccga gcgcaaagaa ggcagaggca aagggaaggg caagaagaag
      181 gagcgaggct ccggcaagaa gccggagtcc gcggcgggca gccagagccc agccttgcct
      241 ccccgattga aagagatgaa aagccaggaa tcggctgcag gttccaaact agtccttcgg
      301 tgtgaaacca gttctgaata ctcctctctc agattcaagt ggttcaagaa tgggaatgaa
      361 ttgaatcgaa aaaacaaacc acaaaatatc aagatacaaa aaaagccagg gaagtcagaa
      421 cttcgcatta acaaagcatc actggctgat tctggagagt atatgtgcaa agtgatcagc
      481 aaattaggaa atgacagtgc ctctgccaat atcaccatcg tggaatcaaa cgagatcatc
      541 actggtatgc cagcctcaac tgaaggagca tatgtgtctt cagagtctcc cattagaata
      601 tcagtatcca cagaaggagc aaatacttct tcatctacat ctacatccac cactgggaca
      661 agccatcttg taaaatgtgc ggagaaggag aaaactttct gtgtgaatgg aggggagtgc
      721 ttcatggtga aagacctttc aaacccctcg agatacttgt gcaagtgcca acctggattc
      781 actggagcaa gatgtactga gaatgtgccc atgaaagtcc aaaaccaaga aaaggcggag
      841 gagctgtacc agaagagagt gctgaccata accggcatct gcatcgccct ccttgtggtc
      901 ggcatcatgt gtgtggtggc ctactgcaaa accaagaaac agcggaaaaa gctgcatgac
      961 cgtcttcggc agagccttcg gtctgaacga aacaatatga tgaacattgc caatgggcct
     1021 caccatccta acccaccccc cgagaatgtc cagctggtga atcaatacgt atctaaaaac
     1081 gtcatctcca gtgagcatat tgttgagaga gaagcagaga catccttttc caccagtcac
     1141 tatacttcca cagcccatca ctccactact gtcacccaga ctcctagcca cagctggagc
     1201 aacggacaca ctgaaagcat cctttccgaa agccactctg taatcgtgat gtcatccgta
     1261 gaaaacagta ggcacagcag cccaactggg ggcccaagag gacgtcttaa tggcacagga
     1321 ggccctcgtg aatgtaacag cttcctcagg catgccagag aaacccctga ttcctaccga
     1381 gactctcctc atagtgaaag gtatgtgtca gccatgacca ccccggctcg tatgtcacct
     1441 gtagatttcc acacgccaag ctcccccaaa tcgccccctt cggaaatgtc tccacccgtg
     1501 tccagcatga cggtgtccat gccttccatg gcggtcagcc ccttcatgga agaagagaga
     1561 cctctacttc tcgtgacacc accaaggctg cgggagaaga agtttgacca tcaccctcag
     1621 cagttcagct ccttccacca caacccgcg catgacagta acagcctccc tgctagcccc
     1681 ttgaggatag tggaggatga ggagtatgaa acgacccaag agtacgagcc agcccaagag
     1741 cctgttaaga aactcgccaa tagccggcgg gccaaaagaa ccaagcccaa tggccacatt
     1801 gctaacagat tggaagtgga cagcaacaca agctcccaga gcagtaactc agagagtgaa
     1861 acagaagatg aaagagtagg tgaagatacg cctttcctgg gcatacagaa ccccctggca
     1921 gccagtcttg aggcaacacc tgccttccgc ctggctgaca gcaggactaa cccagcaggc
     1981 cgcttctcga cacaggaaga aatccaggcc aggctgtcta gtgtaattgc taaccaagac
```

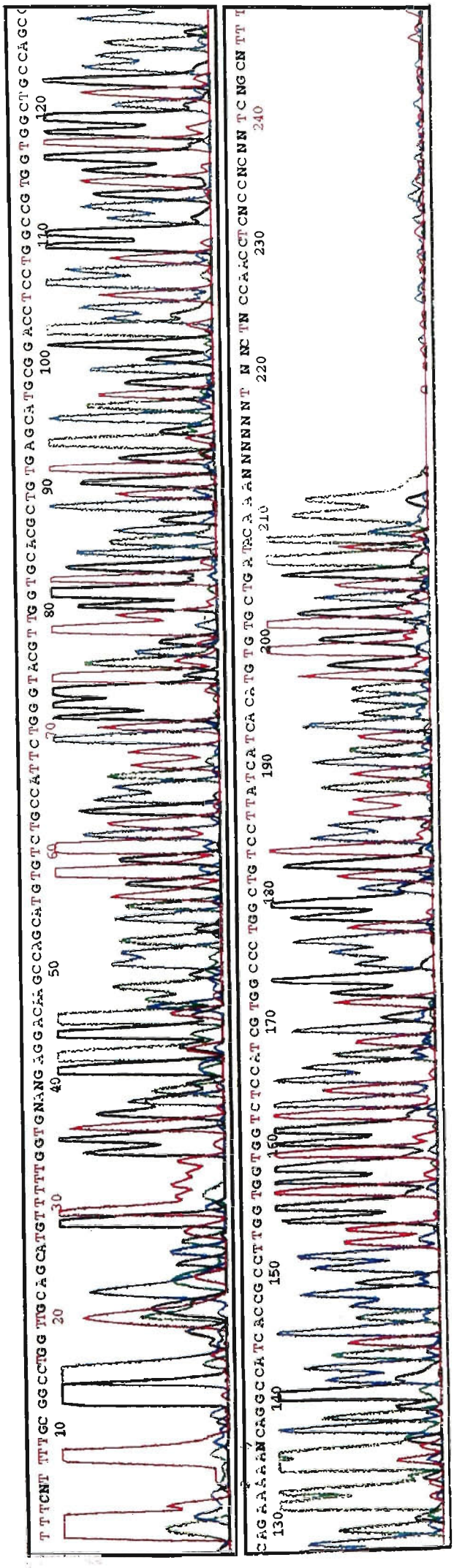
```
2041 cctattgctg tataaaacct aaataaacac atagattcac ctgtaaaact ttattttata
2101 taataaagta ttccacctta aattaaacaa tttattttat tttagcagtt ctgcaaatag
2161 aaaacaggaa aaaaactttt ataaattaaa tatatgtatg taaaaatgaa aaaaaaaaaa
2221 aaaa
//
```

Appendix 7a Nucleotide sequence of RT-PCR generated HRG cDNA

Appendix 8 TGFα cDNA sequence



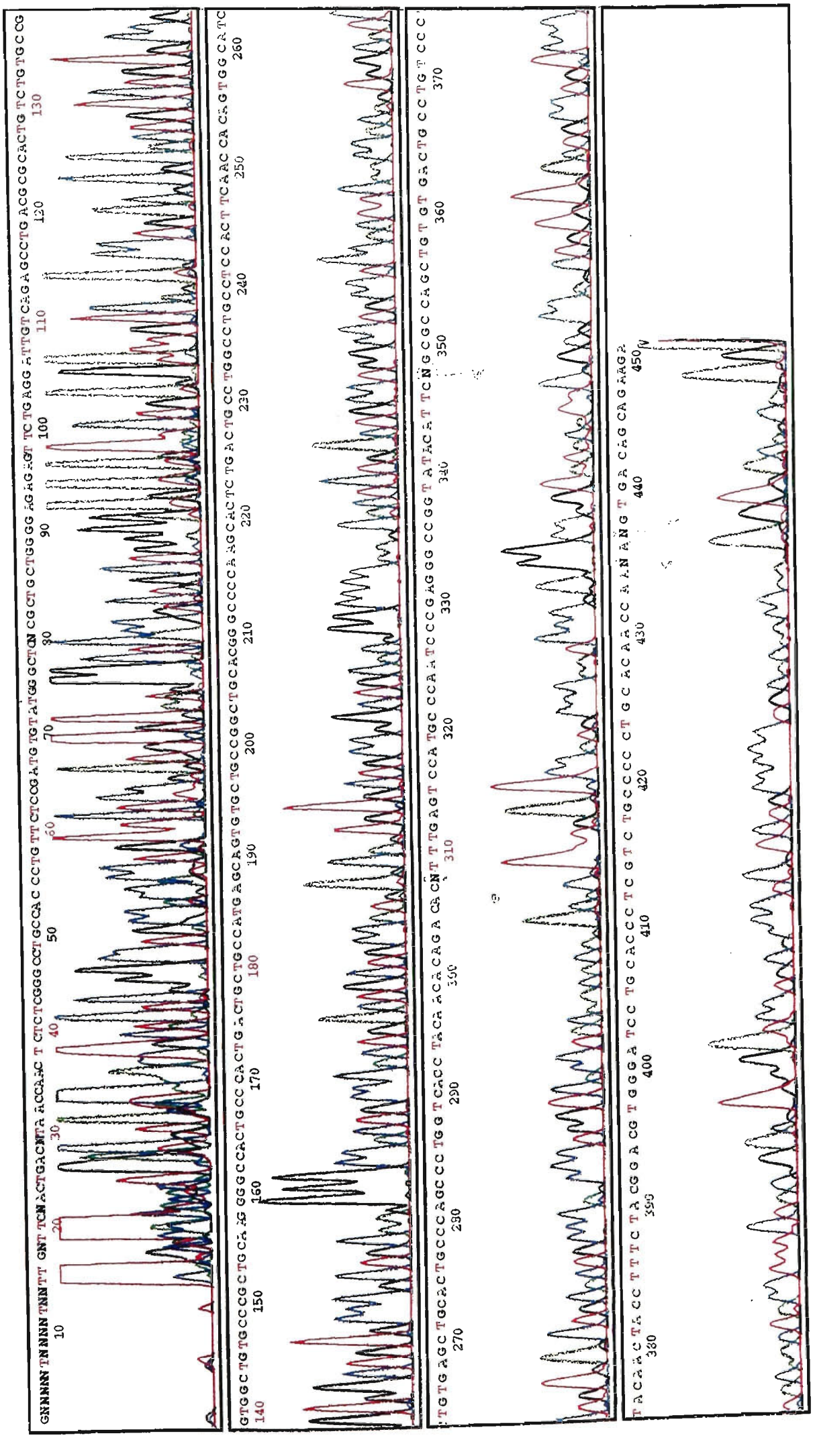
Appendix 8a Nucleotide sequence of RT-PCR generated TGFα cDNA



M11730
Human tyrosine kinase-type receptor (HER2) mRNA, complete cds
Seq: M11730 Length: 4530 Fri Dec 13 10:48:36 1996 Check: 4936

```
   1 AATTCTCGAG CTCGTCGACC GGTCGACGAG CTCGAGGGTC GACGAGCTCG
  51 AGGGCGCGCG CCCGGCCCCC ACCCCTCGCA GCACCCCGCG CCCCGCGCCC
 101 TCCCAGCCGG GTCCAGCCGG AGCCATGGGG CCGGAGCCGC AGTGAGCACC
 151 ATGGAGCTGG CGGCCTTGTG CCGCTGGGGG CTCCTCCTCG CCCTCTTGCC
 201 CCCCGGAGCC GCGAGCACCC AAGTGTGCAC CGGCACAGAC ATGAAGCTGC
 251 GGCTCCCTGC CAGTCCCGAG ACCCACCTGG ACATGCTCCG CCACCTCTAC
 301 CAGGGCTGCC AGGTGGTGCA GGGAAACCTG GAACTCACCT ACCTGCCCAC
 351 CAATGCCAGC CTGTCCTTCC TGCAGGATAT CCAGGAGGTG CAGGGCTACG
 401 TGCTCATCGC TCACAACCAA GTGAGGCAGG TCCCACTGCA GAGGCTGCGG
 451 ATTGTGCGAG GCACCCAGCT CTTTGAGGAC AACTATGCCC TGGCCGTGCT
 501 AGACAATGGA GACCCGCTGA ACAATACCAC CCCTGTCACA GGGGCCTCCC
 551 CAGGAGGCCT GCGGGAGCTG CAGCTTCGAA GCCTCACAGA GATCTTGAAA
 601 GGAGGGGTCT TGATCCAGCG GAACCCCCAG CTCTGCTACC AGGACACGAT
 651 TTTGTGGAAG GACATCTTCC ACAAGAACAA CCAGCTGGCT CTCACACTGA
 701 TAGACACCAA CCGCTCTCGG GCCTGCCACC CCTGTTCTCC GATGTGTAAG
 751 GGCTCCCGCT GCTGGGGAGA GAGTTCTGAG GATTGTCAGA GCCTGACGCG
 801 CACTGTCTGT GCCGGTGGCT GTGCCCGCTG CAAGGGGCCA CTGCCCACTG
 851 ACTGCTGCCA TGAGCAGTGT GCTGCCGGCT GCACGGGCCC CAAGCACTCT
 901 GACTGCCTGG CCTGCCTCCA CTTCAACCAC AGTGGCATCT GTGAGCTGCA
 951 CTGCCCAGCC CTGGTCACCT ACAACACAGA CACGTTTGAG TCCATGCCCA
1001 ATCCCGAGGG CCGGTATACA TTCGGCGCCA GCTGTGTGAC TGCCTGTCCC
1051 TACAACTACC TTTCTACGGA CGTGGGATCC TGCACCCTCG TCTGCCCCCT
1101 GCACAACCAA GAGGTGACAG CAGAGGATGG AACACAGCGG TGTGAGAAGT
1151 GCAGCAAGCC CTGTGCCCGA GTGTGCTATG GTCTGGGCAT GGAGCACTTG
1201 CGAGAGGTGA GGGCAGTTAC CAGTGCCAAT ATCCAGGAGT TTGCTGGCTG
1251 CAAGAAGATC TTTGGGAGCC TGGCATTTCT GCCGGAGAGC TTTGATGGGG
1301 ACCCAGCCTC CAACACTGCC CCGCTCCAGC CAGAGCAGCT CCAAGTGTTT
1351 GAGACTCTGG AAGAGATCAC AGGTTACCTA TACATCTCAG CATGGCCGGA
1401 CAGCCTGCCT GACCTCAGCG TCTTCCAGAA CCTGCAAGTA ATCCGGGGAC
1451 GAATTCTGCA CAATGGCGCC TACTCGCTGA CCCTGCAAGG GCTGGGCATC
1501 AGCTGGCTGG GGCTGCGCTC ACTGAGGGAA CTGGGCAGTG GACTGGCCCT
1551 CATCCACCAT AACACCCACC TCTGCTTCGT GCACACGGTG CCCTGGGACC
1601 AGCTCTTTCG GAACCCGCAC CAAGCTCTGC TCCACACTGC CAACCGGCCA
1651 GAGGACGAGT GTGTGGGCGA GGGCCTGGCC TGCCACCAGC TGTGCGCCCG
1701 AGGGCACTGC TGGGGTCCAG GGCCCACCCA GTGTGTCAAC TGCAGCCAGT
1751 TCCTTCGGGG CCAGGAGTGC GTGGAGGAAT GCCGAGTACT GCAGGGGCTC
1801 CCCAGGGAGT ATGTGAATGC CAGGCACTGT TTGCCGTGCC ACCCTGAGTG
1851 TCAGCCCCAG AATGGCTCAG TGACCTGTTT TGGACCGGAG GCTGACCAGT
1901 GTGTGGCCTG TGCCCACTAT AAGGACCCTC CCTTCTGCGT GGCCCGCTGC
1951 CCCAGCGGTG TGAAACCTGA CCTCTCCTAC ATGCCCATCT GGAAGTTTCC
2001 AGATGAGGAG GGCGCATGCC AGCCTTGCCC CATCAACTGC ACCCACTCCT
2051 GTGTGGACCT GGATGACAAG GGCTGCCCCG CCGAGCAGAG AGCCAGCCCT
2101 CTGACGTCCA TCGTCTCTGC GGTGGTTGGC ATTCTGCTGG TCGTGGTCTT
2151 GGGGGTGGTC TTTGGGATCC TCATCAAGCG ACGGCAGCAG AAGATCCGGA
2201 AGTACACGAT GCGGAGACTG CTGCAGGAAA CGGAGCTGGT GGAGCCGCTG
2251 ACACCTAGCG GAGCGATGCC CAACCAGGCG CAGATGCGGA TCCTGAAAGA
2301 GACGGAGCTG AGGAAGGTGA AGGTGCTTGG ATCTGGCGCT TTTGGCACAG
2351 TCTACAAGGG CATCTGGATC CCTGATGGGG AGAATGTGAA AATTCCAGTG
2401 GCCATCAAAG TGTTGAGGGA AAACACATCC CCCAAAGCCA ACAAAGAAAT
2451 CTTAGACGAA GCATACGTGA TGGCTGGTGT GGGCTCCCCA TATGTCTCCC
2501 GCCTTCTGGG CATCTGCCTG ACATCCACGG TGCAGCTGGT GACACAGCTT
2551 ATGCCCTATG GCTGCCTCTT AGACCATGTC CGGGAAAACC GCGGACGCCT
2601 GGGCTCCCAG GACCTGCTGA ACTGGTGTAT GCAGATTGCC AAGGGGATGA
2651 GCTACCTGGA GGATGTGCGG CTCGTACACA GGGACTTGGC CGCTCGGAAC
2701 GTGCTGGTCA AGAGTCCCAA CCATGTCAAA ATTACAGACT TCGGGCTGGC
2751 TCGGCTGCTG GACATTGACG AGACAGAGTA CCATGCAGAT GGGGGCAAGG
2801 TGCCCATCAA GTGGATGGCG CTGGAGTCCA TTCTCCGCCG GCGGTTCACC
2851 CACCAGAGTG ATGTGTGGAG TTATGGTGTG ACTGTGTGGG AGCTGATGAC
2901 TTTTGGGGCC AAACCTTACG ATGGGATCCC AGCCCGGGAG ATCCCTGACC
2951 TGCTGGAAAA GGGGGAGCGG CTGCCCCAGC CCCCCATCTG CACCATTGAT
3001 GTCTACATGA TCATGGTCAA ATGTTGGATG ATTGACTCTG AATGTCGGCC
3051 AAGATTCCGG GAGTTGGTGT CTGAATTCTC CCGCATGGCC AGGGACCCCC
3101 AGCGCTTTGT GGTCATCCAG AATGAGGACT TGGGCCCAGC CAGTCCCTTG
3151 GACAGCACCT TCTACCGCTC ACTGCTGGAG GACGATGACA TGGGGGACCT
3201 GGTGGATGCT GAGGAGTATC TGGTACCCCA GCAGGGCTTC TTCTGTCCAG
3251 ACCCTGCCCC GGGCGCTGGG GGCATGGTCC ACCACAGGCA CCGCAGCTCA
3301 TCTACCAGGA GTGGCGGTGG GGACCTGACA CTAGGGCTGG AGCCCTCTGA
3351 AGAGGAGGCC CCCAGGTCTC CACTGGCACC CTCCGAAGGG GCTGGCTCCG
3401 ATGTATTTGA TGGTGACCTG GGAATGGGGG CAGCCAAGGG GCTGCAAAGC
```

Appendix 9a Nucleotide sequence of RT-PCR generated c-erb-B2 cDNA



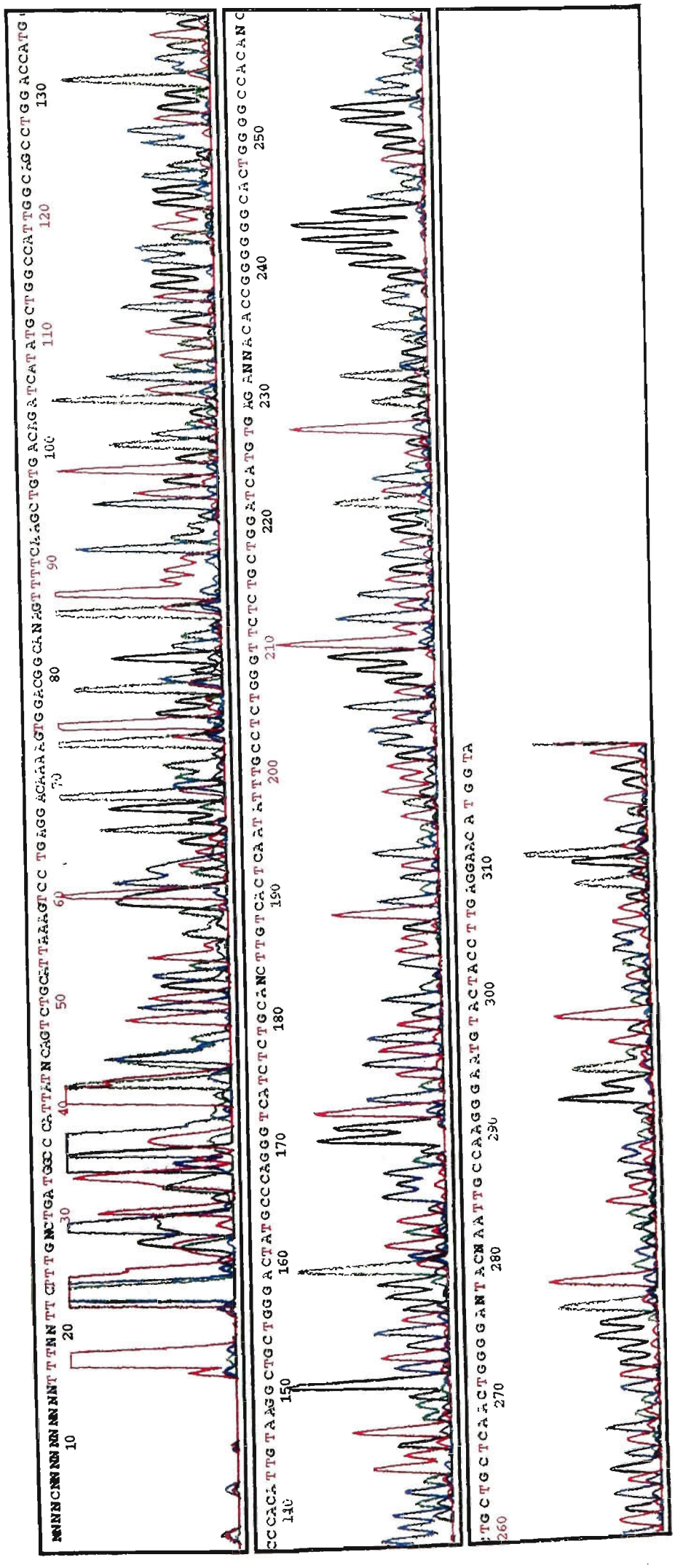
Appendix 10 c-erb-B3 cDNA sequence

M34309
Human epidermal growth factor receptor (HER3) mRNA, complete cds
Seq: M34309 Length: 4975 Fri Dec 13 10:48:42 1996 Check: 4646 ..

```
   1  CTCTCACACA CACACACCCC TCCCCTGCCA TCCCTCCCCG GACTCCGGCT
  51  CCGGCTCCGA TTGCAATTTG CAACCTCCGC TGCCGTCGCC GCAGCAGCCA
 101  CCAATTCGCC AGCGGTTCAG GTGGCTCTTG CCTCGATGTC CTAGCCTAGG
 151  GGCCCCCGGG CCGGACTTGG CTGGGCTCCC TTCACCCTCT GCGGAGTCAT
 201  GAGGGCGAAC GACGCTCTGC AGGTGCTGGG CTTGCTTTTC AGCCTGGCCC
 251  GGGGCTCCGA GGTGGGCAAC TCTCAGGCAG TGTGTCCTGG GACTCTGAAT
 301  GGCCTGAGTG TGACCGGCGA TGCTGAGAAC CAATACCAGA CACTGTACAA
 351  GCTCTACGAG AGGTGTGAGG TGGTGATGGG GAACCTTGAG ATTGTGCTCA
 401  CGGGACACAA TGCCGACCTC TCCTTCCTGC AGTGGATTCG AGAAGTGACA
 451  GGCTATGTCC TCGTGGCCAT GAATGAATTC TCTACTCTAC CATTGCCCAA
 501  CCTCCGCGTG GTGCGAGGGA CCCAGGTCTA CGATGGGAAG TTTGCCATCT
 551  TCGTCATGTT GAACTATAAC ACCAACTCCA GCCACGCTCT GCGCCAGCTC
 601  CGCTTGACTC AGCTCACCGA GATTCTGTCA GGGGGTGTTT ATATTGAGAA
 651  GAACGATAAG CTTTGTCACA TGGACACAAT TGACTGGAGG GACATCGTGA
 701  GGGACCGAGA TGCTGAGATA GTGGTGAAGG ACAATGGCAG AAGCTGTCCC
 751  CCCTGTCATG AGGTTTGCAA GGGGCGATGC TGGGGTCCTG GATCAGAAGA
 801  CTGCCAGACA TTGACCAAGA CCATCTGTGC TCCTCAGTGT AATGGTCACT
 851  GCTTTGGGCC CAACCCCAAC CAGTGCTGCC ATGATGAGTG TGCCGGGGGC
 901  TGCTCAGGCC CTCAGGACAC AGACTGCTTT GCCTGCCGGC ACTTCAATGA
 951  CAGTGGAGCC TGTGTACCTC GCTGTCCACA GCCTCTTGTC TACAACAAGC
1001  TAACTTTCCA GCTGGAACCC AATCCCCACA CCAAGTATCA GTATGGAGGA
1051  GTTTGTGTAG CCAGCTGTCC CCATAACTTT GTGGTGGATC AAACATCCTG
1101  TGTCAGGGCC TGTCCTCCTG ACAAGATGGA AGTAGATAAA AATGGGCTCA
1151  AGATGTGTGA GCCTTGTGGG GGACTATGTC CCAAAGCCTG TGAGGGAACA
1201  GGCTCTGGGA GCCGCTTCCA GACTGTGGAC TCGAGCAACA TTGATGGATT
1251  TGTGAACTGC ACCAAGATCC TGGGCAACCT GGACTTTCTG ATCACCGGCC
1301  TCAATGGAGA CCCCTGGCAC AAGATCCCTG CCCTGGACCC AGAGAAGCTC
1351  AATGTCTTCC GGACAGTACG GGAGATCACA GGTTACCTGA ACATCCAGTC
1401  CTGGCCGCCC CACATGCACA ACTTCAGTGT TTTTTCCAAT TTGACAACCA
1451  TTGGAGGCAG AAGCCTCTAC AACCGGGGCT TCTCATTGTT GATCATGAAG
1501  AACTTGAATG TCACATCTCT GGGCTTCCGA TCCCTGAAGG AAATTAGTGC
1551  TGGGCGTATC TATATAAGTG CCAATAGGCA GCTCTGCTAC CACCACTCTT
1601  TGAACTGGAC CAAGGTGCTT CGGGGGCCTA CGGAAGAGCG ACTAGACATC
1651  AAGCATAATC GGCCGCGCAG AGACTGCGTG GCAGAGGGCA AAGTGTGTGA
1701  CCCACTGTGC TCCTCTGGGG GATGCTGGGG CCCAGGCCCT GGTCAGTGCT
1751  TGTCCTGTCG AAATTATAGC CGAGGAGGTG TCTGTGTGAC CCACTGCAAC
1801  TTTCTGAATG GGGAGCCTCG AGAATTTGCC CATGAGGCCG AATGCTTCTC
1851  CTGCCACCCG GAATGCCAAC CCATGGGGGG CACTGCCACA TGCAATGGCT
1901  CGGGCTCTGA TACTTGTGCT CAATGTGCCC ATTTTCGAGA TGGGCCCCAC
1951  TGTGTGAGCA GCTGCCCCCA TGGAGTCCTA GGTGCCAAGG GCCCAATCTA
2001  CAAGTACCCA GATGTTCAGA ATGAATGTCG GCCCTGCCAT GAGAACTGCA
2051  CCCAGGGGTG TAAAGGACCA GAGCTTCAAG ACTGTTTAGG ACAAACACTG
2101  GTGCTGATCG GCAAAACCCA TCTGACAATG GCTTTGACAG TGATAGCAGG
2151  ATTGGTAGTG ATTTTCATGA TGCTGGGCGG CACTTTTCTC TACTGGCGTG
2201  GGCGCCGGAT TCAGAATAAA AGGGCTATGA GGCGATACTT GGAACGGGGT
2251  GAGAGCATAG AGCCTCTGGA CCCCAGTGAG AAGGCTAACA AAGTCTTGGC
2301  CAGAATCTTC AAAGAGACAG AGCTAAGGAA GCTTAAAGTG CTTGGCTCGG
2351  GTGTCTTTGG AACTGTGCAC AAAGGAGTGT GGATCCCTGA GGGTGAATCA
2401  ATCAAGATTC CAGTCTGCAT TAAAGTCATT GAGGACAAGA GTGGACGGCA
2451  GAGTTTTCAA GCTGTGACAG ATCATATGCT GGCCATTGGC AGCCTGGACC
2501  ATGCCCACAT TGTAAGGCTG CTGGGACTAT GCCCAGGGTC ATCTCTGCAG
2551  CTTGTCACTC AATATTTGCC TCTGGGTTCT CTGCTGGATC ATGTGAGACA
2601  ACACCGGGGG GCACTGGGGC CACAGCTGCT GCTCAACTGG GGAGTACAAA
2651  TTGCCAAGGG AATGTACTAC CTTGAGGAAC ATGGTATGGT GCATAGAAAC
2701  CTGGCTGCCC GAAACGTGCT ACTCAAGTCA CCCAGTCAGG TTCAGGTGGC
2751  AGATTTTGGT GTGGCTGACC TGCTGCCTCC TGATGATAAG CAGCTGCTAT
2801  ACAGTGAGGC CAAGACTCCA ATTAAGTGGA TGGCCCTTGA GAGTATCCAC
2851  TTTGGGAAAT ACACACACCA GAGTGATGTC TGGAGCTATG GTGTGACAGT
2901  TTGGGAGTTG ATGACCTTCG GGGCAGAGCC CTATGCAGGG CTACGATTGG
2951  CTGAAGTACC AGACCTGCTA GAGAAGGGGG AGCGGTTGGC ACAGCCCCAG
3001  ATCTGCACAA TTGATGTCTA CATGGTGATG GTCAAGTGTT GGATGATTGA
3051  TGAGAACATT CGCCCAACCT TTAAAGAACT AGCCAATGAG TTCACCAGGA
3101  TGGCCCGAGA CCCACCACGG TATCTGGTCA TAAAGAGAGA GAGTGGGCCT
3151  GGAATAGCCC CTGGGCCAGA GCCCCATGGT CTGACAAACA AGAAGCTAGA
3201  GGAAGTAGAG CTGGAGCCAG AACTAGACCT AGACCTAGAC TTGGAAGCAG
3251  AGGAGGACAA CCTGGCAACC ACCACACTGG GCTCCGCCCT CAGCCTACCA
3301  GTTGGAACAC TTAATCGGCC ACGTGGGAGC CAGAGCCTTT TAAGTCCATC
3351  ATCTGGATAC ATGCCCATGA ACCAGGGTAA TCTTGGGGGG TCTTGCCAGG
3401  AGTCTGCAGT TTCTGGGAGC AGTGAACGGT GCCCCCGTCC AGTCTCTCTA
```

```
3451  CTCCCCACAC ATGACCCCAG CCCTCTACAG CGGTACAGTG AGGACCCCAC
3501  AGTACCCCTG CCCTCTGAGA CTGATGGCTA CGTTGCCCCC CTGACCTGCA
3551  GCCCCCAGCC TGAATATGTG AACCAGCCAG ATGTTCGGCC CCAGCCCCCT
3601  TCGCCCCGAG AGGGCCCTCT GCCTGCTGCC CGACCTGCTG GTGCCACTCT
3651  GGAAAGGGCC AAGACTCTCT CCCCAGGGAA GAATGGGGTC GTCAAAGACG
3701  TTTTTGCCTT TGGGGGTGCC GTGGAGAACC CCGAGTACTT GACACCCCAG
3751  GGAGGAGCTG CCCCTCAGCC CCACCCTCCT CCTGCCTTCA GCCCAGCCTT
3801  CGACAACCTC TATTACTGGG ACCAGGACCC ACCAGAGCGG GGGGCTCCAC
3851  CCAGCACCTT CAAAGGGACA CCTACGGCAG AGAACCCAGA GTACCTGGGT
3901  CTGGACGTGC CAGTGTGAAC CAGAAGGCCA AGTCCGCAGA AGCCCTGATG
3951  TGTCCTCAGG GAGCAGGGAA GGCCTGACTT CTGCTGGCAT CAAGAGGTGG
4001  GAGGGCCCTC CGACCACTTC CAGGGGAACC TGCCATGCCA GGAACCTGTC
4051  CTAAGGAACC TTCCTTCCTG CTTGAGTTCC CAGATGGCTG GAAGGGGTCC
4101  AGCCTCGTTG GAAGAGGAAC AGCACTGGGG AGTCTTTGTG GATTCTGAGG
4151  CCCTGCCCAA TGAGACTCTA GGGTCCAGTG GATGCCACAG CCCAGCTTGG
4201  CCCTTTCCTT CCAGATCCTG GGTACTGAAA GCCTTAGGGA AGCTGGCCTG
4251  AGAGGGGAAG CGGCCCTAAG GGAGTGTCTA AGAACAAAAG CGACCCATTC
4301  AGAGACTGTC CCTGAAACCT AGTACTGCCC CCCATGAGGA AGGAACAGCA
4351  ATGGTGTCAG TATCCAGGCT TTGTACAGAG TGCTTTTCTG TTTAGTTTTT
4401  ACTTTTTTTG TTTTGTTTTT TTAAAGACGA AATAAAGACC CAGGGGAGAA
4451  TGGGTGTTGT ATGGGGAGGC AAGTGTGGGG GGTCCTTCTC CACACCCACT
4501  TTGTCCATTT GCAAATATAT TTTGGAAAAC
```

Appendix 10a Nucleotide sequence of RT-PCR generated c-erb-B3 cDNA

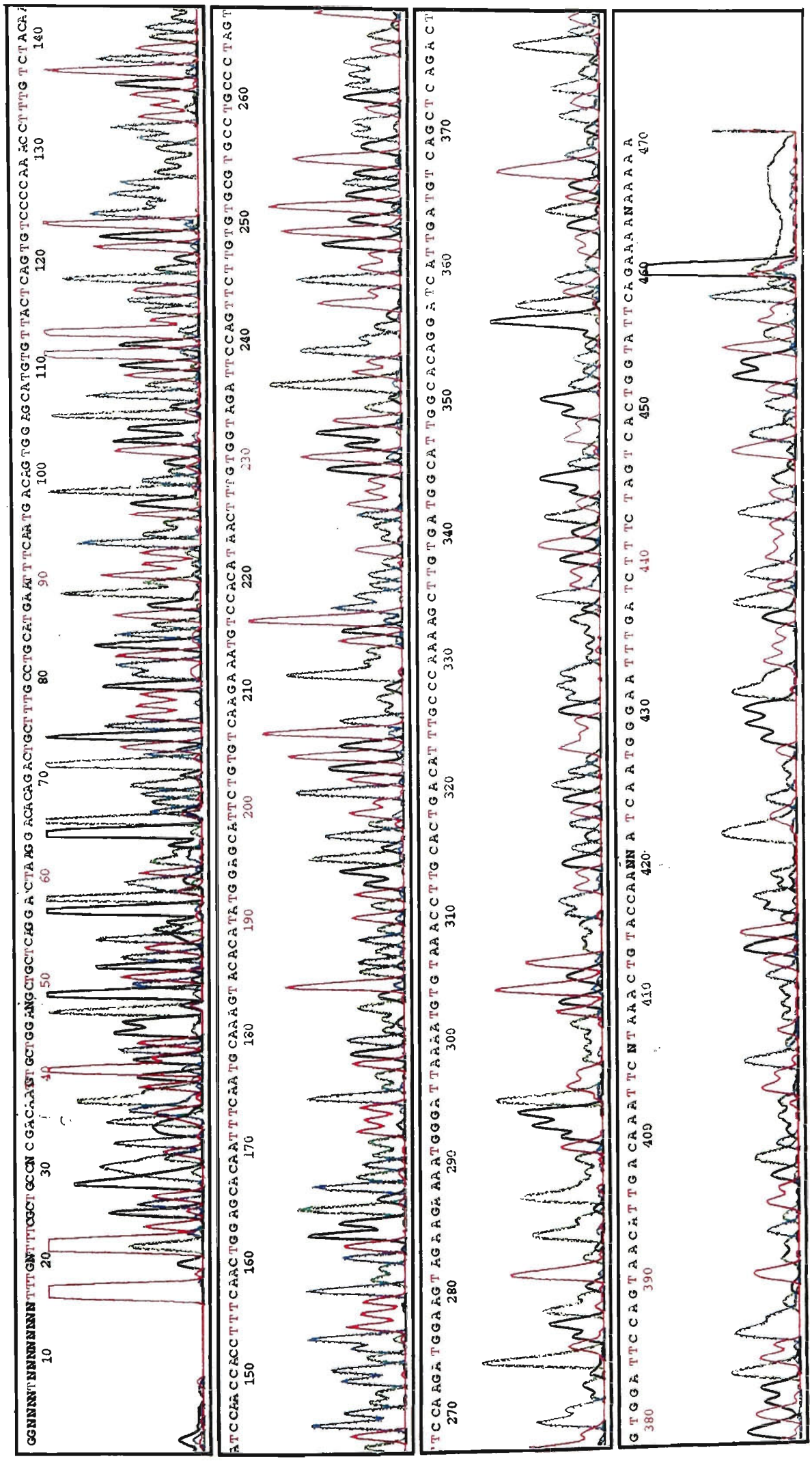


```
LOCUS       HUMRETYKIN   5484 bp    mRNA            PRI       09-JAN-1995
DEFINITION  Homo sapiens receptor tyrosine kinase (ERBB4) gene, complete cds.
ACCESSION   L07868
KEYWORDS    EGF receptor-related; Her4 tyrosine kinase;
            receptor protein-tyrosine kinase.
SOURCE      Homo sapiens (tissue library: lambda ZAPII) heart cDNA to mRNA.
  ORGANISM  Homo sapiens
            Eukaryota; Animalia; Chordata; Vertebrata; Mammalia; Theria;
            Eutheria; Primates; Haplorhini; Catarrhini; Hominidae.
REFERENCE   1  (bases 1 to 5484)
  AUTHORS   Plowman,G.D., Culouscou,J.M., Whitney,G.S., Green,J.M.,
            Carlton,G.W., Foy,L., Neubauer,M.G. and Shoyab,M.
  TITLE     Ligand-specific activation of HER4/p180erbB4, a fourth member of
            the epidermal growth factor receptor family
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 90 (5), 1746-1750 (1993)
  MEDLINE   93189574
COMMENT
            NCBI gi: 337359
FEATURES             Location/Qualifiers
     source          1..5484
                     /organism="Homo sapiens"
                     /cell_line="MDA-MB-453"
                     /sequenced_mol="cDNA to mRNA"
                     /tissue_type="heart"
                     /tissue_lib="lambda ZAPII"
     sig_peptide     34..108
                     /gene="ERBB4"
                     /codon_start=1
     CDS             34..3960
                     /gene="ERBB4"
                     /standard_name="Her4 tyrosine kinase-EGF receptor related"
                     /note="putative;  NCBI gi: 337360"
                     /codon_start=1
                     /product="receptor tyrosine kinase"
                     /translation="MKPATGLWVWVSLLVAAGTVQPSDSQSVCAGTENKLSSLSDLEQ
                     QYRALRKYYENCEVVMGNLEITSIEHNRDLSFLRSVREVTGYVLVALNQFRYLPLENL
                     RIIRGTKLYEDRYALAIFLNYRKDGNFGLQELGLKNLTEILNGGVYVDQNKFLCYADT
                     IHWQDIVRNPWPSNLTLVSTNGSSGCGRCHKSCTGRCWGPTENHCQTLTRTVCAEQCD
                     GRCYGPYVSDCCHRECAGGCSGPKDTDCFACMNFNDSGACVTQCPQTFVYNPTTFQLE
                     HNFNAKYTYGAFCVKKCPHNFVVDSSSCVRACPSSKMEVEENGIKMCKPCTDICPKAC
                     DGIGTGSLMSAQTVDSSNIDKFINCTKINGNLIFLVTGIHGDPYNAIEAIDPEKLNVF
                     RTVREITGFLNIQSWPPNMTDFSVFSNLVTIGGRVLYSGLSLLILKQQGITSLQFQSL
                     KEISAGNIYITDNSNLCYYHTINWTTLFSTINQRIVIRDNRKAENCTAEGMVCNHLCS
                     SDGCWGPGPDQCLSCRRFSRGRICIESCNLYDGEFREFENGSICVECDPQCEKMEDGL
                     LTCHGPGPDNCTKCSHFKDGPNCVEKCPDGLQGANSFIFKYADPDRECHPCHPNCTQG
                     CNGPTSHDCIYYPWTGHSTLPQHARTPLIAAGVIGGLFILVIVGLTFAVYVRRKSIKK
                     KRALRRFLETELVEPLTPSGTAPNQAQLRILKETELKRVKVLGSGAFGTVYKGIWVPE
                     GETVKIPVAIKILNETTGPKANVEFMDEALIMASMDHPHLVRLLGVCLSPTIQLVTQL
                     MPHGCLLEYVHEHKDNIGSQLLLNWCVQIAKGMMYLEERRLVHRDLAARNVLVKSPNH
                     VKITDFGLARLLEGDEKEYNADGGKMPIKWMALECIHYRKFTHQSDVWSYGVTIWELM
                     TFGGKPYDGIPTREIPDLLEKGERLPQPPICTIDVYMVMVKCWMIDADSRPKFKELAA
                     EFSRMARDPQRYLVIQGDDRMKLPSPNDSKFFQNLLDEEDLEDMMDAEEYLVPQAFNI
                     PPPIYTSRARIDSNRSEIGHSPPPAYTPMSGNQFVYRDGGFAAEQGVSVPYRAPTSTI
                     PEAPVAQGATAEIFDDSCCNGTLRKPVAPHVQEDSSTQRYSADPTVFAPERSPRGELD
                     EEGYMTPMRDKPKQEYLNPVEENPFVSRRKNGDLQALDNPEYHNASNGPPKAEDEYVN
                     EPLYLNTFANTLGKAEYLKNNILSMPEKAKKAFDNPDYWNHSLPPRSTLQHPDYLQEY
                     STKYFYKQNGRIRPIVAENPEYLSEFSLKPGTVLPPPPYRHRNTVV"
     mat_peptide     109..3957
                     /gene="ERBB4"
                     /note="putative"
                     /codon_start=1
                     /product="receptor tyrosine kinase"
     misc_difference replace(3169,"")
                     /gene="ERBB4"
                     /note="sequence diverges in several cDNA clones"
     misc_difference replace(3228,"g")
                     /gene="ERBB4"
                     /note="1 clone"
     polyA_site      5484
BASE COUNT     1588 a   1173 c   1230 g   1493 t
ORIGIN
        1 aattgtcagc acgggatctg agacttccaa aaaatgaagc cggcgacagg actttgggtc
       61 tgggtgagcc ttctcgtggc ggcggggacc gtccagccca gcgattctca gtcagtgtgt
      121 gcaggaacgg agaataaact gagctctctc tctgacctgg aacagcagta ccgagccttg
      181 cgcaagtact atgaaaactg tgaggttgtc atgggcaacc tggagataac cagcattgag
```

```
 241 cacaaccggg acctctcctt cctgcggtct gttcgagaag tcacaggcta cgtgttagtg
 301 gctcttaatc agtttcgtta cctgcctctg gagaatttac gcattattcg tgggacaaaa
 361 ctttatgagg atcgatatgc cttggcaata tttttaaact acagaaaaga tggaaacttt
 421 ggacttcaag aacttggatt aaagaacttg acagaaatcc taaatggtgg agtctatgta
 481 gaccagaaca aattcctttg ttatgcagac accattcatt ggcaagatat tgttcggaac
 541 ccatggcctt ccaacttgac tcttgtgtca acaaatggta gttcaggatg tggacgttgc
 601 cataagtcct gtactggccg ttgctgggga cccacagaaa atcattgcca gactttgaca
 661 aggacggtgt gtgcagaaca atgtgacggc agatgctacg gaccttacgt cagtgactgc
 721 tgccatcgag aatgtgctgg aggctgctca ggacctaagg acacagactg ctttgcctgc
 781 atgaatttca atgacagtgg agcatgtgtt actcagtgtc cccaaacctt tgtctacaat
 841 ccaaccacct ttcaactgga gcacaatttc aatgcaaagt acacatatgg agcattctgt
 901 gtcaagaaat gtccacataa ctttgtggta gattccagtt cttgtgtgcg tgcctgccct
 961 agttccaaga tggaagtaga agaaaatggg attaaaatgt gtaaaccttg cactgacatt
1021 tgcccaaaag cttgtgatgg cattggcaca ggatcattga tgtcagctca gactgtggat
1081 tccagtaaca ttgacaaatt cataaactgt accaagatca atgggaattt gatctttcta
1141 gtcactggta ttcatgggga cccttacaat gcaattgaag ccatagaccc agagaaactg
1201 aacgtctttc ggacagtcag agagataaca ggtttcctga acatacagtc atggccacca
1261 aacatgactg acttcagtgt tttttctaac ctggtgacca ttggtggaag agtactctat
1321 agtggcctgt ccttgcttat cctcaagcaa cagggcatca cctctctaca gttccagtcc
1381 ctgaaggaaa tcagcgcagg aaacatctat attactgaca acagcaacct gtgttattat
1441 cataccatta actggacaac actcttcagc acaatcaacc agagaatagt aatccgggac
1501 aacagaaaag ctgaaaattg tactgctgaa ggaatggtgt gcaaccatct gtgttccagt
1561 gatggctgtt ggggacctgg gccagaccaa tgtctgtcgt gtcgccgctt cagtagagga
1621 aggatctgca tagagtcttg taacctctat gatggtgaat ttcgggagtt tgagaatggc
1681 tccatctgtg tggagtgtga cccccagtgt gagaagatgg aagatggcct cctcacatgc
1741 catggaccgg gtcctgacaa ctgtacaaag tgctctcatt ttaaagatgg cccaaactgt
1801 gtggaaaaat gtccagatgg cttacagggg gcaaacagtt tcattttcaa gtatgctgat
1861 ccagatcggg agtgccaccc atgccatcca aactgcaccc aagggtgtaa cggtcccact
1921 agtcatgact gcatttacta cccatggacg ggccattcca ctttaccaca acatgctaga
1981 actcccctga ttgcagctgg agtaattggt gggctcttca ttctggtcat tgtgggtctg
2041 acatttgctg tttatgttag aaggaagagc atcaaaaaga aaagagcctt gagaagattc
2101 ttggaaacag agttggtgga accattaact cccagtggca cagcacccaa tcaagctcaa
2161 cttcgtattt tgaaagaaac tgagctgaag agggtaaaag tccttggctc aggtgctttt
2221 ggaacggttt ataaaggtat ttgggtacct gaaggagaaa ctgtgaagat tcctgtggct
2281 attaagattc ttaatgagac aactggtccc aaggcaaatg tggagttcat ggatgaagct
2341 ctgatcatgg caagtatgga tcatccacac ctagtccggt tgctgggtgt gtgtctgagc
2401 ccaaccatcc agctggttac tcaacttatg ccccatggct gcctgttgga gtatgtccac
2461 gagcacaagg ataacattgg atcacaactg ctgcttaact ggtgtgtcca gatagctaag
2521 ggaatgatgt acctggaaga aagacgactc gttcatcggg atttggcagc ccgtaatgtc
2581 ttagtgaaat ctccaaacca tgtgaaaatc acagattttg ggctagccag actcttggaa
2641 ggagatgaaa aagagtacaa tgctgatgga ggaaagatgc caattaaatg gatggctctg
2701 gagtgtatac attacaggaa attcacccat cagagtgacg tttggagcta tggagttact
2761 atatgggaac tgatgacctt tggaggaaaa ccctatgatg gaattccaac gcgagaaatc
2821 cctgatttat tagagaaagg agaacgtttg cctcagcctc ccatctgcac tattgacgtt
2881 tacatggtca tggtcaaatg ttggatgatt gatgctgaca gtagacctaa atttaaggaa
2941 ctggctgctg agttttcaag gatggctcga gaccctcaaa gatacctagt tattcagggt
3001 gatgatcgta tgaagcttcc cagtccaaat gacagcaagt tctttcagaa tctcttggat
3061 gaagaggatt tggaagatat gatggatgct gaggagtact tggtccctca ggctttcaac
3121 atcccacctc ccatctatac ttccagagca agaattgact cgaataggag tgaaattgga
3181 cacagccctc ctcctgccta cacccccatg tcaggaaacc agtttgtata ccgagatgga
3241 ggttttgctg ctgaacaagg agtgtctgtg ccctacagag ccccaactag cacaattcca
3301 gaagctcctg tggcacaggg tgctactgct gagatttttg atgactcctg ctgtaatggc
3361 accctacgca agccagtggc accccatgtc caagaggaca gtagcaccca gaggtacagt
3421 gctgacccca ccgtgtttgc cccagaacgg agcccacgag gagagctgga tgaggaaggt
3481 tacatgactc ctatgcgaga caaacccaaa caagaatacc tgaatccagt ggaggagaac
3541 ccttttgttt ctcggagaaa aaatggagac cttcaagcat tggataatcc cgaatatcac
3601 aatgcatcca atggtccacc caaggccgag gatgagtatg tgaatgagcc actgtacctc
3661 aacacctttg ccaacacctt gggaaaagct gagtacctga agaacaacat actgtcaatg
3721 ccagagaagg ccaagaaagc gtttgacaac cctgactact ggaaccacag cctgccacct
3781 cggagcaccc ttcagcaccc agactacctg caggagtaca gcacaaaata tttttataaa
3841 cagaatgggc ggatccggcc tattgtggca gagaatcctg aatacctctc tgagttctcc
3901 ctgaagccag gcactgtgct gccgcctcca ccttacagac accggaatac tgtggtgtaa
3961 gctcagttgt ggtttttag gtggagagac acacctgctc caatttcccc acccccctct
4021 ctttctctgg tggtcttcct tctaccccaa ggccagtagt tttgacactt cccagtggaa
4081 gatacagaga tgcaatgata gttatgtgct tacctaactt gaacattaga gggaaagact
4141 gaaagagaaa gataggagga accacaatgt ttcttcattt ctctgcatgg gttggtcagg
4201 agaatgaaac agctagagaa ggaccagaaa atgtaaggca atgctgccta ctatcaaact
4261 agctgtcact ttttttcttt ttctttttct ttctttgttt ctttcttcct cttctttttt
4321 tttttttttt taaagcagat ggttgaaaca cccatgctat ctgttcctat ctgcaggaac
4381 tgatgtgtgc atatttagca tccctggaaa tcataataaa gtttccatta gaacaaaaga
4441 ataacatttt ctataacata tgatagtgtc tgaaattgag aatccagttt ctttccccag
4501 cagtttctgt cctagcaagt aagaatggcc aactcaactt tcataattta aaaatctcca
4561 ttaaagttat aactagtaat tatgttttca acacttttg gttttttca ttttgttttg
4621 ctctgaccga ttcctttata tttgctcccc tatttttggc tttaatttct aattgcaaag
4681 atgtttacat caaagcttct tcacagaatt taagcaagaa atattttaat atagtgaaat
4741 ggccactact ttaagtatac aatctttaaa ataagaaagg gaggctaata tttttcatgc
4801 tatcaaatta tcttcaccct catcctttac atttttcaac attttttttt ctccataaat
```

```
4861 gacactactt gataggccgt tggttgtctg aagagtagaa gggaaactaa gagacagttc
4921 tctgtggttc aggaaaacta ctgatacttt caggggtggc ccaatgaggg aatccattga
4981 actggaagaa acacactgga ttgggtatgt ctacctggca gatactcaga aatgtagttt
5041 gcacttaagc tgtaatttta tttgttcttt ttctgaactc cattttggat tttgaatcaa
5101 gcaatatgga agcaaccagc aaattaacta atttaagtac atttttaaaa aaagagctaa
5161 gataaagact gtggaaatgc caaaccaagc aaattaggaa ccttgcaacg gtatccaggg
5221 actatgatga gaggccagca cattatcttc atatgtcacc tttgctacgc aaggaaattt
5281 gttcagttcg tatacttcgt aagaaggaat gcgagtaagg attggcttga attccatgga
5341 atttctagta tgagactatt tatatgaagt agaaggtaac tctttgcaca taaattggta
5401 taataaaaag aaaaacacaa acattcaaag cttagggata ggtccttggg tcaaaagttg
5461 taaataaatg tgaaacatct tctc
```
//

Appendix 11a Nucleotide sequence of RT-PCR generated c-erb-B4 cDNA

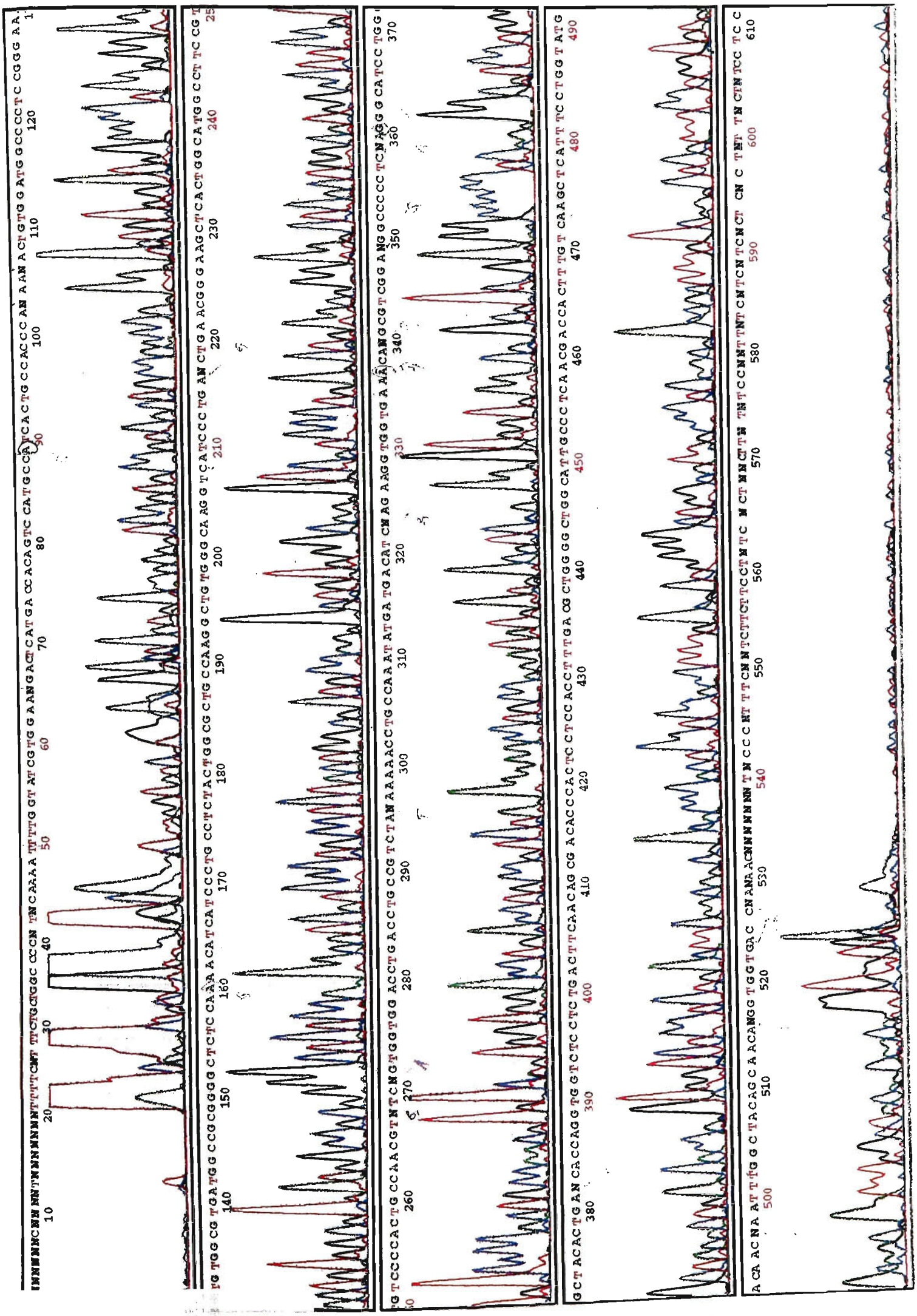


Appendix 12 GAPDH cDNA sequence

```
LOCUS       HSGAPDR      1272 bp    RNA             PRI       23-NOV-1995
DEFINITION  Human liver mRNA for glyceraldehyde-3-phosphate dehydrogenase
            (G3PD, EC 1.2.1.12).
ACCESSION   X01677 K03121 M17851 X01110
NID         g31644
KEYWORDS    dehydrogenase; glyceraldehyde-3-phosphate dehydrogenase.
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;
            Vertebrata; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 1272)
  AUTHORS   Arcari,P., Martinelli,R. and Salvatore,F.
  TITLE     The complete sequence of a full length cDNA for human liver
            glyceraldehyde-3-phosphate dehydrogenase: evidence for multiple
            mRNA species
  JOURNAL   Nucleic Acids Res. 12 (23), 9179-9189 (1984)
  MEDLINE   85087928
REFERENCE   2  (bases 1 to 1272)
  AUTHORS   Tso,J.Y., Sun,X.H., Kao,T.H., Reece,K.S. and Wu,R.
  TITLE     Isolation and characterization of rat and human
            glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity
            and molecular evolution of the gene
  JOURNAL   Nucleic Acids Res. 13 (7), 2485-2502 (1985)
  MEDLINE   85215629
REFERENCE   3  (bases 1 to 1272)
  AUTHORS   Hanauer,A. and Mandel,J.L.
  TITLE     The glyceraldehyde 3 phosphate dehydrogenase gene family: structure
            of a human cDNA and of an X chromosome linked pseudogene; amazing
            complexity of the gene family in mouse
  JOURNAL   EMBO J. 3 (11), 2627-2633 (1984)
  MEDLINE   85076585
FEATURES             Location/Qualifiers
     source          1..1272
                     /organism="Homo sapiens"
     CDS             61..1068
                     /EC_number="1.2.1.12"
                     /note="(aa 1-335)"
                     /codon_start=1
                     /product="glyceraldehyde-3-phosphate dehydrogenase"
                     /db_xref="PID:g31645"
                     /db_xref="SWISS-PROT:P04406"
                     /translation="MGKVKVGVNGFGRIGRLVTRAAFNSGKVDIVAINDPFIDLNYMV
                     YMFQYDSTHGKFHGTVKAENGKLVINGNPITIFQERDPSKIKWGDAGAEYVVESTGVF
                     TTMEKAGAHLQGGAKRVIISAPSADAPMFVMGVNHEKYDNSLKIISNASCTTNCLAPL
                     AKVIHDNFGIVEGLMTTVHAITATQKTVDGPSGKLWRDGRGALQNIIPASTGAAKAVG
                     KVIPELDGKLTGMAFRVPTANVSVVDLTCRLEKPAKYDDIKKVVKQASEGPLKGILGY
                     TEHQVVSSDFNSDTHSSTFDAGAGIALNDHFVKLISWYDNEFGYSNRVVDLMAHMASK
                     E"
     polyA_signal    1249..1254
     polyA_site      1272
BASE COUNT      301 a    381 c    330 g    260 t
ORIGIN
       1 gctcggctgg cgacgcaaaa gaagatgcgg ctgactgtcg agccacatcg ctcagacacc
      61 atggggaagg tgaaggtcgg agtcaacgga tttggtcgta ttgggcgcct ggtcaccagg
     121 gctgctttta actctggtaa agtggatatt gttgccatca atgacccctt cattgacctc
     181 aactacatgg tttacatgtt ccaatatgat tccacccatg gcaaattcca tggcaccgtc
     241 aaggctgaga acgggaagct tgtcatcaat ggaaatccca tcaccatctt ccaggagcga
```

```
     301 gatccctcca aaatcaagtg gggcgatgct ggcgctgagt acgtcgtgga gtccactggc
     361 gtcttcacca ccatggagaa ggctggggct catttgcagg ggggagccaa aagggtcatc
     421 atctctgccc cctctgctga tgcccccatg ttcgtcatgg gtgtgaacca tgagaagtat
     481 gacaacagcc tcaagatcat cagcaatgcc tcctgcacca ccaactgctt agcacccctg   A
     541 gccaaggtca tccatgacaa ctttggtatc gtggaaggac tcatgaccac agtccatgcc
     601 atcactgcca cccagaagac tgtggatggc ccctccggga aactgtggcg tgatggccgc
     661 ggggctctcc agaacatcat ccctgcctct actggcgctg ccaaggctgt gggcaaggtc
     721 atccctgagc tagacgggaa gctcactggc atggccttcc gtgtccccac tgccaacgtg
     781 tcagtggtgg acctgacctg ccgtctagaa aaacctgcca aatatgatga catcaagaag
     841 gtggtgaagc agcgtcgga gggccccctc aaaggcatcc tgggctacac tgagcaccag
     901 gtggtctcct ctgacttcaa cagcgacacc cactcctcca cctttgacgc tggggctggc
     961 attgccctca acgaccactt tgtcaagctc atttcctggt atgacaacga atttggctac   B
    1021 agcaacaggg tggtggacct catggcccac atggcctcca aggagtaaga cccctggacc
    1081 accagcccca gcaagagcac aagaggaaga gagagaccct cactgctggg gagtccctgc
    1141 cacactcaga cccccaccac actgaatctc ccctcctcac agttgccatg tagacccctt
    1201 gaagagggga ggggcctatg ggagccgcac cttgtcatgt atccatctaa taaagtatcc
    1261 ctgtgctcaa cc
//
```

Appendix 12a Nucleotide sequence of RT-PCR generated GAPDH cDNA

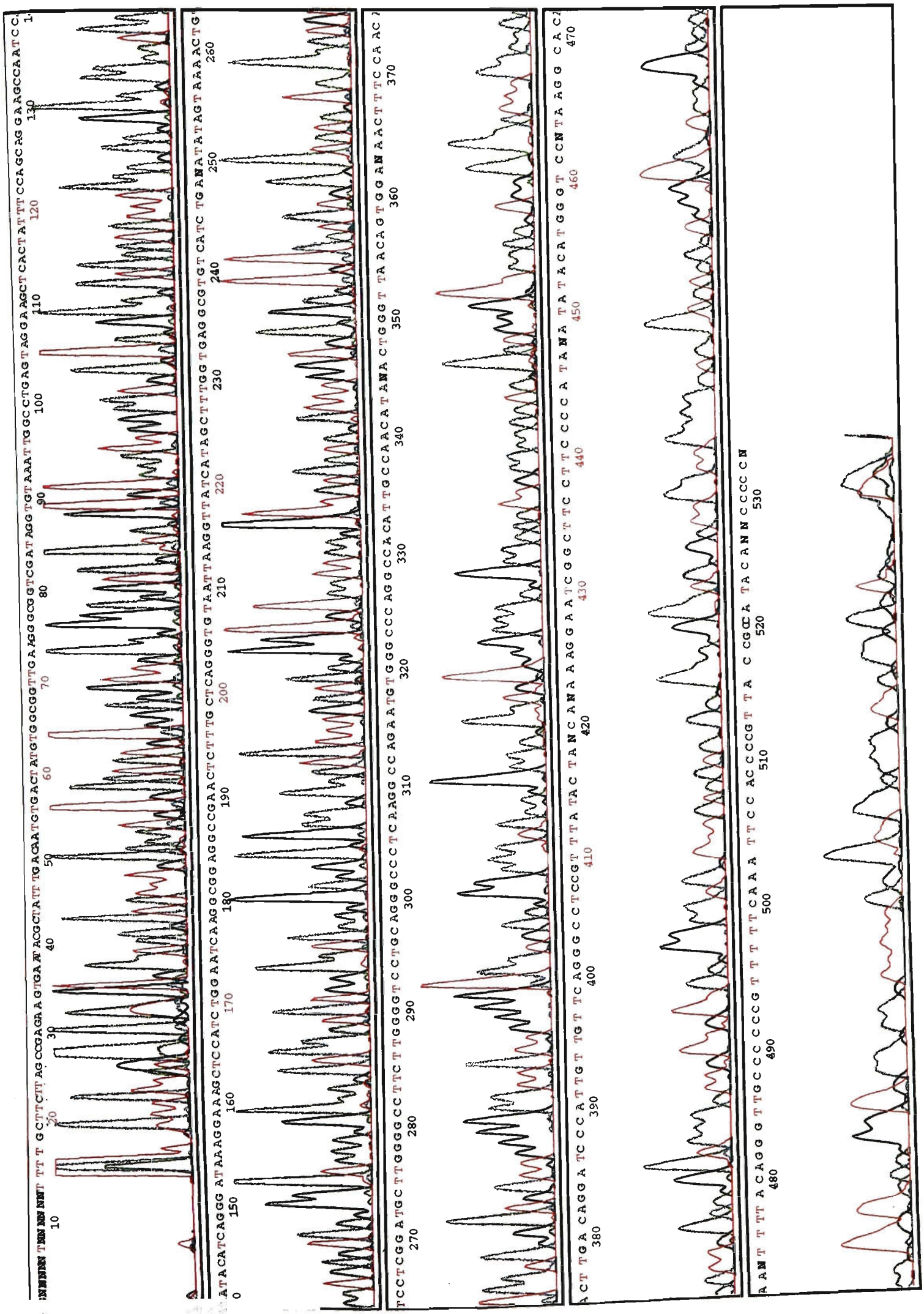


Appendix 13 3βHSD cDNA sequence

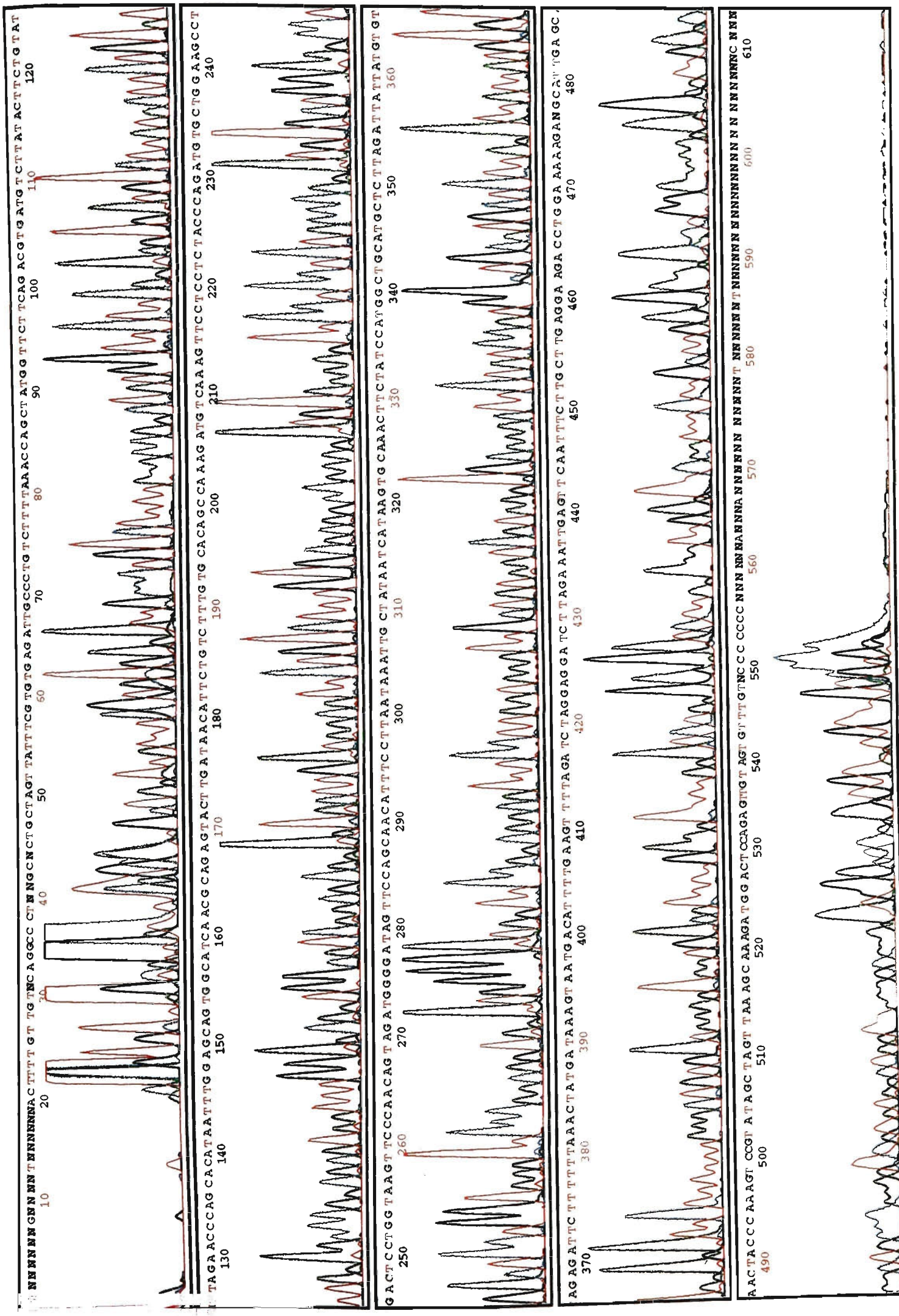
X53321
H.sapiens mRNA for 3Beta-hydroxysteroid dehydrogenase/ Delta(5)->(4)isomerase
Seq: X53321 Length: 1574 Thu Nov 7 12:32:44 1996 Check: 3147

```
   1  GCGGAGTGAT TCCTGCTACT TTGGATGGCC ATGACGGGCT GGAGCTGCCT
  51  TGTGACAGGA GCAGGAGGGT TTCTGGGACA GAGGATCATC CGCCTCTTGG
 101  TGAAGGAGAA GGAGCTGAAG GAGATCAGGG TCTTGGACAA GGCCTTCGGA
 151  CCAGAATTGA GAGAGGAATT TTCTAAACTC CAGAACAAGA CCAAGCTGAC
 201  AGTGCTGGAA GGAGACATTC TGGATGAGCC ATTCCTGAAG AGAGCCTGCC
 251  AGGACGTCTC GGTCATCATC CACACCGCCT GTATCATTGA TGTCTTCGGT
 301  GTCACTCACA GAGAGTCTAT CATGAATGTC AATGTGAAAG GTACCCAGCT
 351  CCTGTTAGAG GCCTGTGTCC AAGCTAGTGT GCCAGTCTTC ATCTACACCA
 401  GTAGCATAGA GGTAGCCGGG CCCAACTCCT ACAAGGAAAT CATCCAGAAT
 451  GGCCATGAAG AAGAGCCTCT GGAAAACACA TGGCCCGCTC CATACCCACA
 501  CAGCAAAAAG CTTGCTGAGA AGGCTGTACT GGCGGCTAAC GGGTGGAATC
 551  TGAAAAACGG CGGCACCCTG TACACTTGTG CCTTACGACC CATGTATATC
 601  TATGGGGAAG GAAGCCGATT CCTTTCTGCT AGTATAAACG AGGCCCTGAA
 651  CAACAATGGG ATCCTGTCAA GTGTTGGAAA GTTCTCCACT GTTAACCCAG
 701  TCTATGTTGG CAATGTGGCC TGGGCCCACA TTCTGGCCTT GAGGGCCCTG
 751  CAGGACCCCA AGAAGGCCCC AAGCATCCGA GGACAGTTCT ACTATATCTC
 801  AGATGACACG CCTCACCAAA GCTATGATAA CCTTAATTAC ACCCTGAGCA
 851  AAGAGTTCGG CCTCCGCCTT GATTCCAGAT GGAGCTTTCC TTTATCCCTG
 901  ATGTATTGGA TTGGCTTCCT GCTGGAAATA GTGAGCTTCC TACTCAGGCC
 951  AATTTACACC TATCGACCGC CCTTCAACCG CCACATAGTC ACATTGTCAA
1001  ATAGCGTATT CACCTTCTCT TATAAGAAGG CTCAGCGAGA TCTGGCGTAT
1051  AAGCCACTCT ACAGCTGGGA GGAAGCCAAG CAGAAAACGG TGGAGTGGGT
1101  TGGTTCCCTT GTGGACCGGC ACAAGGAGAC CCTGAAGTCC AAGACTCAGT
1151  GATTTAAGGA TGACAGAGAT GTGCATGTGG GTATTGTTAG GAGATGTCAT
1201  CAAGCTCCAC CCTCCTGGCC TCATACAGAA AGTGACAAGG GCACAAGCTC
1251  AGGTCCTGCT GCCTCCCTTT CATACAATGG CCAACTTATT GTATTCCTCA
1301  TGTCATCAAA ACCTGCGCAG TCATTGGCCC AACAAGAAGG TTTCTGTCCT
1351  AATCATATAC CAGAGGAAAG ACCATGTGGT TTGCTGTTAC CAAATCTCAG
1401  TAGCTGATTC TGAACAATTT AGGGACTCTT TTAACTTGAG GGTCGTTTTG
1451  ACTACTAGAG CTCCATTTCT ACTCTTAAAT GAGAAAGGAT TTCCTTTCTT
1501  TTTAATCTTC CATTCCTTCA CATAGTTTGA TAAAAAGATC AATAAATGTT
1551  TGAATGTTTA ATGTGAAAAA AAAA
```

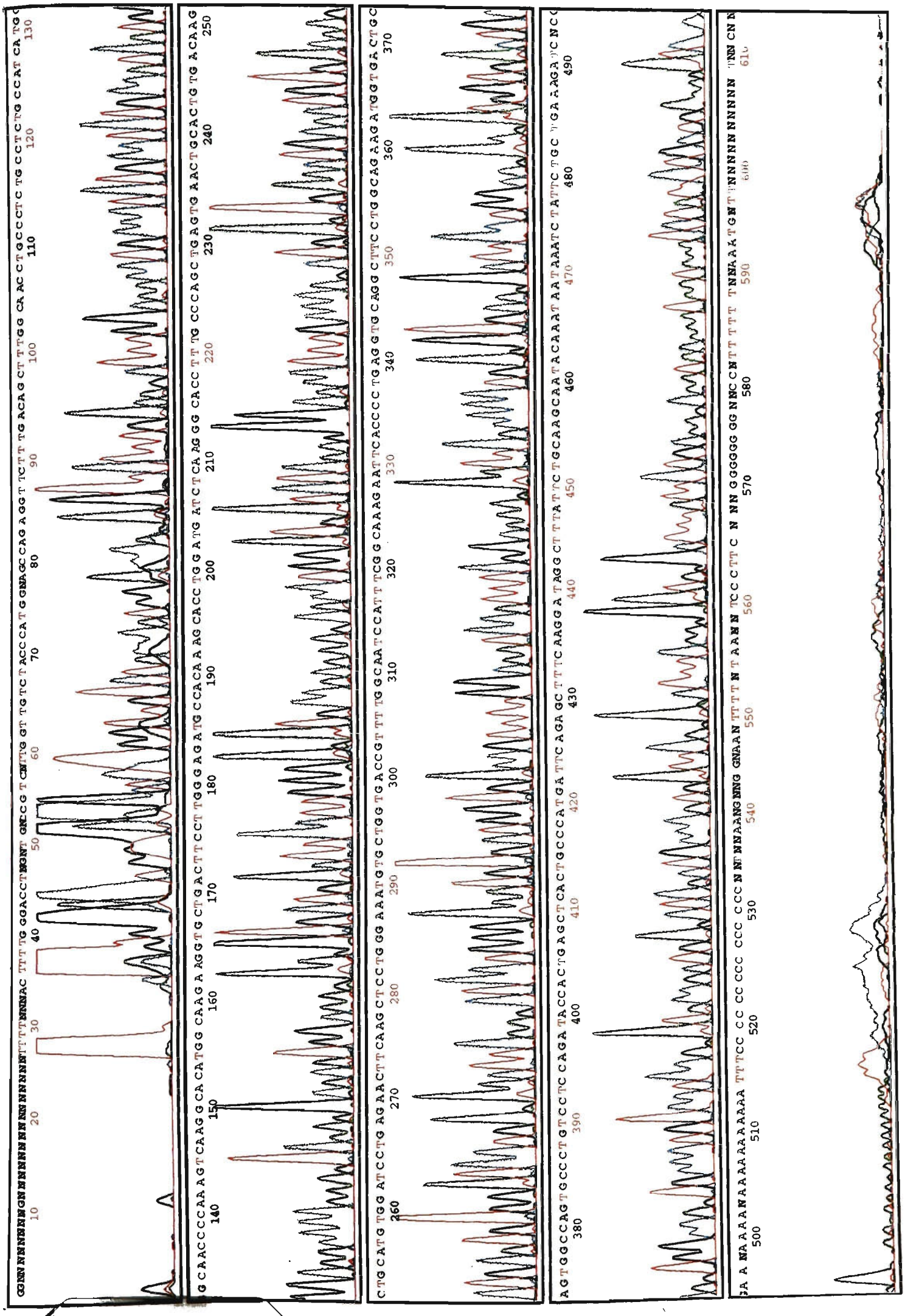
Appendix 13a Nucleotide sequence of RT-PCR generated 3βHSD cDNA



Appendix 14 Nucleotide sequence of 580bp cDNA fragment obtained through RDA



Appendix 15 Nucleotide sequence of 520bp cDNA fragment obtained through RDA



Appendix 16 Nucleotide sequence of 310bp cDNA fragment obtained through RDA