THE EFFECTS OF TRANSFORMING GROWTH FACTOR α ON THE EXPRESSION OF RAT PLACENTAL LACTOGENS

BY

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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This thesis is dedicated to my dear parents

Mr. Zhaoli Sun and Mrs. Fenglan Li

who are on the other side of the Pacific ocean

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If I have seen further, it is by standing on the shoulders of Giants.
Sir Isaac Newton

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ABSTRACT

The rat placental lactogens are members of a large family of prolactin-related proteins which are expressed in a developmentally specific manner by the placenta during pregnancy. The rat choriocarcinoma cell line, Rcho, expresses rat placental lactogen I (rPL-I), rat placental lactogen II (rPL-II) and rat prolactin like protein A (rPLP-A) in a similar pattern to that seen in the rat placenta. The rPL-I mRNA appears within two days after plating, when giant cells first differentiate in culture, while significant levels of rPL-II are not expressed until about 14 days after plating. The rPLP-A mRNA is expressed after day 4 in cultures. This cell line appears to be a good model system for studying the giant cells of the placenta.

In placenta, there is a switch in expression between rPL-I and rPL-II by the trophoblast giant cells at midpregnancy. The factors that influence this shift are not well understood. There is some evidence that EGF/TGFα may have a role in placental development. EGF receptors have been identified on mouse and human placenta, and TGFα mRNA has been detected in early rat decidua and human placenta. It has recently been reported that EGF stimulates the release of mPL-I but inhibits mPL-II release from primary placental cultures. Given that the Rcho cells appear to be a good model system to study giant cells in the placenta, we have investigated the effects of several placenta/decidua related growth factors on rPL-I and rPL-II mRNA levels in these cells. Exclusively, TGFα (10ng/ml) produced a 3-fold increase in the level of rPL-I mRNA but a 2-fold decrease in the level of rPL-II mRNA ($p<0.05$). TGFα had no significant effect on rPLP-A mRNA levels. These data suggest that TGFα may play a role in the switch

of rPL-I and rPL-II expression in the rat placenta at midpregnancy.

In the mouse it has been reported that within -274 bp to +1 of the mPL-I 5' region two AP-I sites and two GATA-binding sites are required for maximal placental specific expression (Shida *et al.* 1993, Ng *et al.* 1994). The 5' region of mPL-II gene from -2.7 Kb to -569 bp contains sequences which specify mPL-II placental specific expression(Shida *et al.* 1992). Little is known about the 5' regions of the equivalent rat placental lactogen genes. We have studied the reporter gene activity of different lengths of 5'-flanking DNA of rPL-I, rPL-II and rPLP-A in transient transfection assays. Luciferase reporter gene constructs containing -1.4 Kb and -300 bp of rPL-I, -4.5 Kb and -3.3 Kb of rPL-II, and -4.6 Kb and -975 bp of rPLP-A 5'-flanking regions produced significant luciferase expression in Rcho cells, but not in rat pituitary GC cells, suggesting that they may contain DNA elements sufficient for placental specific expression of PLs. A comparison of available DNA sequences for 5'-flanking regions of the rat and mouse placental lactogens I and II shows that they are closely related. Furthermore, we observed that TGFα increased the luciferase activity of both rPL-I constructs by approximately 2 fold ($p<0.001$) and both rPL-II constructs by approximately 5 fold ($p<0.05$). Taken together with the effects of TGFα on rPL-I and rPL-II mRNA levels, these data suggest that the effect of TGFα on rPL-I, at least in part, is at the level of gene transcription, but the effect of TGFα on rPL-II appears to be more complex.

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LIST OF ABBREVIATIONS

Units of measure

cm	centimetre
min	minute
h	hour
^{0}C	degrees centigrade
µl	microlitre
ml	millilitre
ng	nanogram
µg	microgram
mg	milligram
µM	micromolar
mM	millimolar
cpm	counts per minute
rpm	revolutions per minute
v/v	volume per volume
%	percent
bp	base pairs
Kb	kilobases

Reagents and Media

BSA	bovine serum albumin
CsCI	cesium chloride
ddH_2O	distilled deionized water
DMEM	Dulbecco's modified Eagle's medium
EDTA	ethylene-diamine-tetraacetic-acid
LB	Luria-Bertani medium
PBS	phosphate buffered saline
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
TE	Tris-EDTA

Proteins

PRL	prolactin
PL	placental lactogen
EGF	epidermal growth factor
TGFα	transforming growth factor α
CSF-1	colony stimulating factor 1
IGF-II	insulin-like growth factor
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
FBS	fetal bovine serum
CAT	chloramphenicol acetyltransferase
Luc	luciferase

Miscellaneous

h	human
m	mouse
r	rat
G	guanosine
A	adenosine
T	thymidine
C	cytidine
DNA	deoxyribonucleic acid
cDNA	complementary DNA
RNA	ribonucleic acid
mRNA	messenger RNA
G3PD	Glyceraldehyde-3-phosphate dehydrogenase
CMV	cytomegalovirus
TK	herpes simplex I thymidine kinase
SF	serum free
OD	optical density
UV	ultra violet

INTRODUCTION

The placenta plays a vital role in the embryonic development of mammals. It attaches the embryo to the uterus, forms vascular connections necessary for nutrient transport, and redirects maternal endocrine, immune and metabolic functions to the embryo's advantage. The placenta undergoes dramatic structural reorganization during pregnancy in order to functionally match the development of the embryonic/fetal and maternal requirements. The mammalian embryo cannot develop without the placenta (Ohlsson 1989, Cross et al. 1994b).

1. PLACENTA

1.1 Development

In mammals, the egg, after fertilization in the oviduct, undergoes a series of symmetrical cell divisions to create a mass of totipotent cells, the morula (8 cells), still enclosed within the *zona pellucida.* The first differentiation event occurs after compaction of the morula (approximately day 3.5 after fertilization in the mouse) with formation of the blastocyst. Cells on the outer layer of the blastocyst, trophectoderm, become distinguishable from those undifferentiated cells in the inner core, the inner cell mass (ICM). Trophectoderm cells are the progenitors of trophoblast cells of the placenta, while the inner cell mass will go on to differentiate into embryonic and other extraembryonic tissues. At implantation, mural trophectoderm cells located furthest from the ICM, initially adhere to antimesometrial uterine epithelial cells and then invade the uterine stroma initiating a transformation of the stomal cells termed "decidulization". After

implantation in the mouse, the polar trophectoderm proliferates to form the ectoplacental cone and later the spongiotrophoblast layer. Trophoblast stem cells in the ectoplacental cone are also precursors of the chorionic ectoderm. The outermost trophoblasts of the ectoplacental cone differentiate into secondary trophoblast giant cells. Trophoblast giant cells lie on the outside of the placenta, forming the interface with maternal cells in the decidua (Cross et al.1994b).

The placenta establishes functional connections that are critical for embryonic survival. For example, trophoblast interactions with the maternal immune system. A paradox about pregnancy is that the placenta, a semi-allograft of fetal tissue, avoids maternal immune rejection. Fetal trophoblasts, which lie in direct contact with maternal immune cells, use several mechanisms to subvert normal maternal immune response. These include secretion of factors that may suppress local immune responsiveness, selective expression 0f immune antigens critical to alloreactivity, and impaired responses to immune-activating cytokines present in the placental bed. Another important example is the establishment of a hybrid vasculature in which the fetal trophoblasts, acting like endothelial cells, are in direct contact with maternal blood, where they transport nutrients and gases. Implantation and development of the placenta occur in a stepwise manner. The major roadblock of development in the uterus is that the capacity of placenta does not meet the cardiovascular demands of the embryo during its progressive growth (Cross *et al* 1994b).

The rodent possesses two placental structures - the choriovitelline placenta and the chorioallantoic placenta. The choriovitelline placenta develops first and is comprised

of mural trophoblast cells adherent to a prominent basement membrane, Reichert's membrane, is associated with decidual cells. The choriovitelline placenta eventually degenerates and is efficiently replaced by the chorioallantoic placenta (Soares *et al.* 1991). The diagram of the midpregnant rat conceptus is shown in Fig.1 (Davies & Glasser 1968).

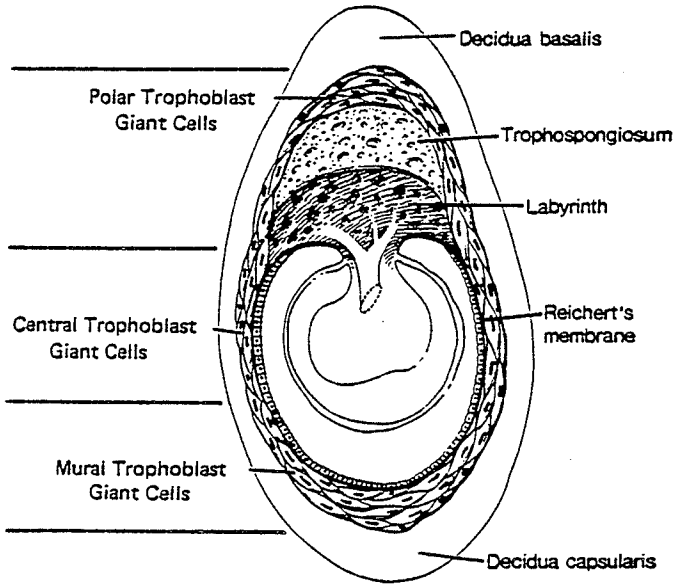


Fig.1 The diagram of midpregnancy rat conceptus (Davies & Glasser 1968).

1.2 Morphology

The mature rat choriollantoic placenta consists of two well-defined zones: the junctional or basal zone and the labyrinth (Fig.2, Soares *et al.* 1991). The junctional zone contains trophoblast giant cells, positioned at the maternal interface, and spongiotrophoblast (cytotrophoblast) and glycogen cells. The labyrinth zone contains trophoblast giant cells, syncytial trophoblast cells, fetal mesenchyme and vasculature. The

junctional zone is supplied only by maternal blood supply while the labyrinth zone is supplied by both maternal and fetal blood supplies. The labyrinth zone which is located at the fetal interface is the location of maternal-fetal nutrient exchange (Soares *et al.* 1991).

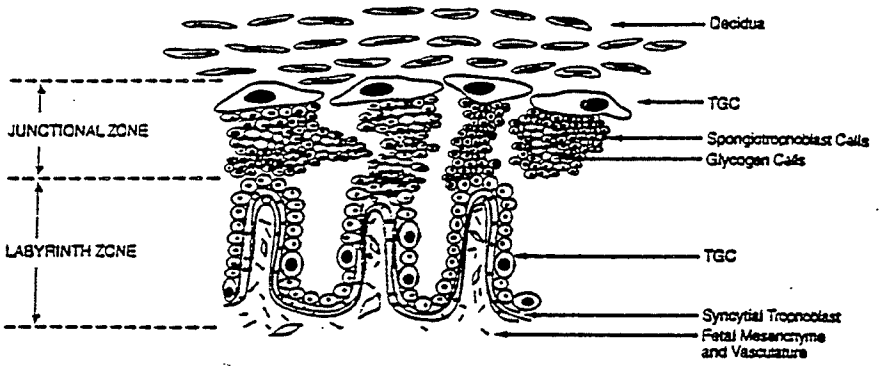


Fig. 2 Schematic diagram - the organization of trophoblast cells in a portion of the mature rat chorioallantoic placenta. (TGC = trophoblast giant cell) (Soares *et al.* 1991)

1.3 Genetic control of trophoblast differentiation

The trophoblast cell lineage is the first to differentiate during mammalian embryogenesis (Rossant *et al.* 1986, Cross *et al.* 1994b). Little is known about the transcriptional regulation of trophoblast differentiation. Several transcription factors are expressed in trophoblasts, including the zinc finger-containing factor Rex-1 (Rogers *et al* 1991), the homeodomain-containing factor Pem (Wilkinson *et al* 1990, Lin *et al* 1994), and a member of the GATA family, GATA-3 (Ng *et al* 1993). The tumour repressor retinoblastoma gene product (Rb) has also been shown to control proliferation of

trophoblast cells in the human (Roncalli *et al.* 1994). However, these factors are also expressed in other cell types, and their roles in trophoblast commitment and differentiation have not been tested.

One group of transcription factors has a helix-loop-helix (HLH) structure. An HLH motif consists of a short α helix connected by a loop to a second, long α helix. The flexibility of the loop allows one helix to fold back and pack against the other. These two helix structures bind to a second HLH protein. The second HLH protein can be the same (resulting in a homodimer) or different (resulting in a heterodimer). The basic amino acids extending from the dimerization interface of the α helix, make specific contacts with DNA. Members of the basic helix-loop-helix (bHLH) family of transcription factors are important cell lineage determinants through evolution in many cell types (Olson 1990 & 1992). Recently, two bHLH factors have been identified that give the first insights into the control of trophoblast differentiation. Mash-2, initially identified in a preneuronal cell line (Johnson *et a*l 1990), has been shown to be expressed in large amounts in trophoblasts (Guillemot *et al* 1994). There is evidence that Mash-$2^{-/-}$ embryos die from placental failure at 12 days post-coitum and the mutation of the Mash-2 gene may result in an increase in the number of giant cells, resulting in a diminished spongiotrophoblast layer (Guillemot *et al* 1994). A screen for bHLH factors expressed in blastocysts resulted in the identification of another bHLH factor, Hxt, that is expressed in trophoblasts in mice and sheep (Cross *et al* 1994a). Hxt induces the commitment of cells to differentiate into trophoblasts, as shown by injection of Hxt into uncommitted blastomeres of two cell mouse embryos (Cross *et al* 1994a). In contrast to Mash-2 , Hxt

expression persists and even increases in mouse trophoblasts as they form giant cells. Overexpression of Hxt in rat trophoblast stem cells reduces their proliferation and promotes differentiation (Cross *et al* 1994a), consistent with the hypothesis that Hxt regulates trophoblast giant cell formation.

In rodents, the balance between proliferation of trophoblasts and differentiation into nonproliferative trophoblast giant cells is regulated by these trophoblast-specific bHLH transcription factors, as well as by the native HLH factors the Id proteins (Id). These proteins dimerize with members of the basic helix-loop-helix protein family, but due to the absence of the basic region, the resulting heterodimers cannot bind DNA. Therefore Id type proteins negatively regulate DNA binding of the bHLH proteins (Barone *et al.* 1994). The expression of Id-1 (Evans & O'Brien 1993, Cross *et al* 1994a) and Id-2 (Cross *et al.* 1994a, Janatpour *et al* 1994) is high in proliferative cells and is down-regulated during differentiation. Ectopic expression of Id-1 reduces the ability of rat trophoblasts to differentiate *in vitro* (Cross *et al* 1994a), an effect similar to the activity of this gene in regulating the differentiation of the other cell lineages (Barone *et al* 1994).

Failures in implantation and placental development are clinically important. About one-third of normal human pregnancies end in spontaneous abortion. In humans, for example, abnormalities in the vascular connections result in preeclampsia, a disease of pregnancy with significant morbidity and mortality to both mother and fetus (Cross *et al.* 1994b). Another severe placental disease is molar pregnancy. It has been reported that 6-28% cases of molar pregnancy have persistent gestational trophoblastic proliferation, which is responsible for the production of HCG, luteinizing hormone, prolactin, and

testosterone (Brittain & Bayliss 1995). Currently, the approaches for diagnosis and treatment of diseases of pregnancy are limited mainly because of our inability to understand the mechanisms of placental development. Understanding the control of placental development is fundamental to our understanding of mammalian development.

2. RAT PLACENTAL PROLACTIN FAMILY OF HORMONES

The human placental lactogen was first characterized and shown to be structurally and immunologically related to human growth hormone (GH) (Josimovich & McLaren 1962). In the rat, 6 different members of the PRL gene family are expressed in the placenta: rat placental lactogen I (rPL-I), rPL-I variant (rPL-Iv), rat placental lactogen II (rPL-II), rat PRL-like protein A (rPLP-A), rPLP-B, and rPLP-C (Duckworth *et al.* 1993). The placental lactogens of this family (rPL-I and rPL-II) were identified by their biological activities, which were similar to pituitary PRL (stimulation of mammary gland growth/function and corpus luteum maintenance). Robertson *et al.* (1981) determined that rPL-I has a molecular weight of 40,000 and rPL-II of 20,000. Interestingly, an antibody to rPL-II did not crossreact with rPL-I suggesting that these proteins were not closely related, although both could bind to a rat prolactin (PRL) receptor and sequence information however has shown that they are related. Additional members of the family were identified during the purification of known members and /or during the course of cloning cDNAs encoding known members. All 6 members of the placental PRL family have been characterized, and their cDNA cloned (rPL-I by Robertson *et al.* 1990, rPL-Iv by Robertson *et al.* 1991, rPL-II by Duckworth *et al.*1986a, rPLP-A by Duckworth *et al.*

1986b, rPLP-B by Duckworth *et al.* 1988, rPLP-C by Deb *et al.*1991c). Rat PLP-B is not only present in the placenta but also expressed in antimesometrial decidual tissue if the pregnancy and pseudopregnancy rat (Croze *et al.* 1990). Another protein expressed in the decidua is decidual prolactin related protein (dPRP) (Gu *et al.* 1994). Two relatives - PRL and growth hormone are present in the pituitary (Soares *et al.* 1991). Each of those proteins is expressed in a temporal and cellular specific manner during the rat placental development (Soares *et al.* 1991).

2.1 Temporal, tissue-, and cell-specific expression

The placental PRL family is characterized by distinct cell and temporal patterns of expression (Table.1, Duckworth *et al.*1993). The expression of rPL-I is initiated shortly after implantation in mural trophoblast giant cells of the implanting blastocyst and later extends to the giant cells of the chorioallantoic placentas. Within a 48 h period between day 11 and 13 of gestation (In rat the day 1 gestation was defined as the day sperm was observed in the vagina) rPL-II expression is initiated and rPL-I expression is terminated (Soares *et al.* 1985, Faria *et al.* 1990a). rPL-II is initially expressed exclusively by trophoblast giant cells of the junctional zone and then, toward the end of gestation, trophoblast giant cells of the labyrinth zone also acquire the capability of expressing rPL-II (Campbell *et al.* 1989, Duckworth *et al.* 1990). During the overlapping period of rPL-I and rPL-II expression, the same trophoblast giant cells are responsible for the production of both hormones (Duckworth *et al.* 1993). Mechanisms responsible for the initiation of rPL-II production and the termination of rPL-I production termed the "placental lactogen midpregnancy shift/switch" are unknown. Rat prolactin like protein-A

(rPLP-A), rat prolactin like protein-C (rPLP-C) and rat placental lactogen-I variant (rPL-Iv) are restricted to the junctional zone of the chorioallantoic placenta. Expression of these three PRL-related hormones is initiated just after midgestation, confined to spongiotrophoblast cells and some trophoblast giant cells of the junctional zone, and increases as gestation advances (Campbell *et al.* 1989, Duckworth *et al.* 1990, Deb *et al.* a,b,c 1991). The expression of rPLP-B in placenta is restricted to the spongiotrophoblast cells. Temporally, rPLP-B exhibits an expression pattern similar to that of rPLP-A, rPLP-C, and rPL-Iv expression in placenta. rPLP-B expression represents a specific marker of placental spongiotrophoblast cells (Duckworth *et al.* 1990 &1993).

Table 1 Patterns of expression of rat placental PRL-like proteins (Duckworth et al. 1993).

Name of protein	Time of appearance	Placental cell type expressing protein
rPL-I	Day 7–13	Giant cells
rPL-Iv	Day 15–term	Cytotrophoblasts and giant cells
rPL-II	Day 11–term	Giant cells
rPLP-A	Day 14–term	Cytotrophoblasts and giant cells
rPLP-B	Day 8–14	Antimesometrial decidua
	Day 12–term	Cytotrophoblasts
rPLP-C	Day 15–term	Cytotrophoblasts and giant cells

* Day 1 gestation was defined as the day sperm was observed in the vagina.

2.2 Other species

A placental PRL family of proteins, including PLs and other PRL-related proteins have been identified in several species, including the mouse (Linzer & Nathans 1985, Linzer *et al.* 1985, Jackson *et al.* 1986, Colosi, *et al.* 1987/1988), cow (Schuler *et al.*

1987/1988, Kessler *et al.* 1989), sheep (Colosi *et al.* 1989), and hamster (Southard *et al.* 1989). In rat and mouse, the two placental lactogens are closely related (Jackson *et al.* 1986, Robertson *et al.* 1990), but the other PRL-like placental proteins, although structurally related, are not homologous.

2.3 Regulation

The highly specific cellular and temporal expression of these PRL-related placental proteins raises the question of what factors regulate their patterns of expression. Little is known about the regulation of these genes at the molecular level. Although the genes of the placental PRL family may vary from species to species, the regulatory mechanisms controlling their placental specific expression may be conserved.

Shida *et al.* (1993) have reported that the mouse PL-I gene promoter sequences extending 274bp up-stream from the start site of transcription contain all of the elements necessary for maximal expression of mouse PL-I in Rcho cells. From stably transfected cells they have observed that the expression from this promoter increases as the percentage of differentiated cells in culture increases. It has also been shown that two AP-1 binding and GATA-binding sites are required for the maximal expression of mPL-I from this 274 bp 5'-flanking region DNA (Shida *et al.*1993, Ng. *et al.*1994). GATA-2 and GATA-3 are expressed in placental trophoblast cells, with peak levels of their mRNAs and mPL-I mRNA each accumulating at mid gestation. The importance of these transcription factors has been demonstrated by their ability to induce transcription from a -274 bp mPL-I promoter when transfected into non-trophoblast (fibroblast) cells (Ng *et al.* 1994).

In the transgenic mouse model, it has been also reported that one or more elements required for placental trophoblast giant cell expression are localized between -2700 and -569 of the 5'-flanking region of the mPL-II gene in transgenic mice (Shida et al. 1992).

In the rat, the only report by Vuille et al. (1993) showed that a -975 bp of 5'-flanking region of rPLP-A is sufficient to specify placental expression a *cat* reporter gene in Rcho cells. The results suggested that the presence of additional further enhancing elements between -4.6Kb and -975 bp in the rPLP-A 5'-flanking region.

2.4 Function

The prolactin family of hormones plays a key role in the establishment and maintenance of pregnancy in the rat. Early in pregnancy, pituitary PRL directly stimulates the growth and development of the mammary glands, a necessary prerequisite to lactation, and stimulates the milk protein synthesis (Thordarson & Talamantes 1988, Forsyth 1988). In the ovary, PRL is a necessary participant in the extension of the functional lifespan of the corpus luteum of the estrous cycle (Morishige *et al.* 1974). Beginning at day 2 after mating, pituitary PRL is secreted as diurnal and nocturnal surges. These surges decline at midpregnancy (Freeman & Neill 1972, Smith *et al.* 1975). It has been showed that the hypophysectomy of a pregnant rat before day 12 of gestation results in abortion; after midterm the outcome of pregnancy is unaffected (Astwood & Greep 1938), suggesting that pituitary hormones are essential in the earlier stages, but that the placental proteins are able to take over their roles after that time. It has been speculated that the rat placental PRL proteins might replace the functions of the rat pituitary PRL, and that they may also have a role in regulating the decline of these prolactin surges. The

data show that rPL-I itself might inhibit PRL release via the hypothalamus (Yogev & Terkel 1978, 1980; Voogt 1980, Tomogane *et al.* 1993).

Mouse PL-II has been reported to be present in the fetal serum from day 14 (Ogren & Talamantes 1988) and binding sites for mPL-II have been identified in the fetal mouse liver from day 17 (Harigaya *et al.*1988). Recently functional rPL-II binding sites have been revealed in the fetal adrenal cortex, renal tubules, small intestinal villi, pancreatic islets and hepatic parenchyma (Royster *et al.* 1995). The widespread expression of PRL receptor in the fetal rat suggests novel roles for the lactogenic hormones in fetal metabolism and growth, neural development, fluid and electrolyte balance, and development of the immune system (Royster *et al.* 1995). Very recently, it has been reported that the birth rate of PRL receptor negative transgenic mice is much declined (Ormandy *et al.* 1995). This critical effect is unlikely to be the result from loss of PRL binding alone. It has been reported that prolactin is not expressed in the rat fetus until close to birth (Gash *et al.* 1982, Aubert *et al.* 1985, Tonh *et al.* 1989), suggesting that the placental lactogens which bind to PRL receptors may have vital roles in fetal development.

Although rPLP-A, rPLP-B, rPLP-C, and rPL-Iv are structurally analogous to pituitary PRL, their biological actions are yet to be determined. Recently it has been reported that recombinant rPL-Iv is able to compete with ovine PRL for rat ovarian and liver PRL receptors, which might give us some clue of the physiological function of rPL-Iv (Cohick *et al.* 1995).

3. RAT CHORIOCARCINOMA CELLS

The rat choriocarcinoma cell line, Rcho, is derived from a transplantable rat choriocarcinoma tumour, and is composed of pure trophoblast cells. Cultures are composed of small cells which multiply and differentiate into giant cells. Rcho cells are hormonally active as demonstrated by the presence of lactogens and progesterone (Verstuyf *et al.* 1990). The tumour itself, when transplanted under the kidney capsule of female rats, has been shown to produce rPL-I, but not rPL-II, rPLP-A, and rPLP-B (Faria *et al.* 1990b). In culture, the Rcho cell line has been shown to produce rPL-I, rPL-II, rPLP-A, and rPLP-C all of which are expressed in placental giant cells, but not rPLP-B which is spongiotrophoblast specific (Faria *et al.* 1991, Duckworth *et al.* 1993, Hamlin *et al.* 1994). *In situ* hybridization studies show that rPL-I, rPL-II, and rPLP-A are localized exclusively to giant cells in both rat placenta and Rcho cultures (Duckworth et al. 1990 & 1993). These observations suggest that the Rcho cells do not have the potential to develop into spongiotrophoblast, but are already committed to a giant cell identity (Faria *et al.* 1991, Duckworth *et al.* 1993, Hamlin *et al.* 1994). The Rcho cell line provides a unique *in vitro* model for studying placental lactogen expression during trophoblast differentiation.

4. PLACENTA/DECIDUA RELATED GROWTH FACTORS

The growth factors are like the classical hormones in the microenviroument. They affect the target cells either by autocrine or paracrine mechanisms (Fritz 1988). The initial event of a cellular growth factor's action is its binding to a specific usually high

affinity receptor which spans the outer membrane. The binding of a growth factor by its receptor increases soluble, intracellular second messengers which transmit a signal to the nucleus. These second messengers may be phosphoprotein, inositol phosphates, diacylglycerol, cyclic nucleotides, monovalent or divalent ions. Within minutes after the formation of the growth factor-receptor complex, changes in gene expression can often be detected. Growth factors may function to control proliferation, differentiation, and morphogenesis during mammalian development (Mercola & Stiles 1988). The following discussion refers to some of the growth factors, for which there is evidence such as the presence of protein and/or receptors in placenta, which suggests that they might have a role in fetal/placental development.

4.1 Epidermal growth factor / Transforming growth factor α

Epidermal growth factor (EGF) and transforming growth factor α (TGFα) are monomeric polypeptides (6KDa) of similar structure released by proteolysis from membrane-bound precursors (Derynck 1988). TGFα can bind to and activate the EGF receptor in an analogous manner to EGF (Pike *et al.* 1982). Therefore some of the functions attributed to EGF may be as a result of TGFα activity. EGF receptor (c-erb-B1) has been demonstrated to be a important protein kinase which can be autophosphorylated at tyrosine residues (Ushiro & Cohen 1980), as well as being phosphorylated at serine and threonine residues by protein kinase C (hunter & Cooper 1981). EGF receptors are found in a wide variety of cell types (Fisher & Lakshmanan 1990).

One of the earliest events following EGF binding to its receptor is an increase in the phosphorylation of a number of key intracellular regulatory proteins (Cohen *et al.*

1982), including general phospholipases (Margolis *et al.* 1989), protein kinases and phosphatases (Yang et al. 1989). By complex signal transduction cascades, signals are transmitted and resulted in gene expression (Haley 1990).

In the mouse, EGF receptors are expressed in a wide range of fetal tissues including trophoblast cells of placenta (Adamson 1990). During pregnancy in the mouse, the number of EGF receptors is lower at day 14 than either day 10 or day 17 (Smith & Talamantes 1986). These data could reflect a developmental process in placenta in which there is a change in the types of cells that express the EGF receptor or in their relative numbers. In the human, EGF receptors are found predominantly present in the syncytiotrophoblast cells of the placenta which are the source of placental lactogen. Immunohistochemical analysis of cellular EGF receptor levels in the syncytiotrophoblasts revealed remarkably higher levels in early placenta compared to those in mid-term and term placentas (Maruo *et al.* 1987).

TGFα is a strong candidate for an important role in preimplantation and implantation embryo development. TGFα is expressed in a temporal and cell-type-specific manner in the mouse uterus during the preimplantation period (Tamada *et al.* 1991). In the rat TGFα mRNA is found in maternal decidua with the highest concentration in the region adjacent to the embryo between day 8 and day 16 (the plug day defined as day 1 of gestation), and low or nondetectable levels in the uterus placenta and other maternal tissues (Han *et al.* 1987). In the human, TGFα mRNA and protein are detected in early, mid, and late gestation placenta (Bissonnette et al. 1986).

EGF has been shown to have profound effects on the developing fetus (Thorburn *et al.* 1981). EGF may play a multifunctional role during development, and it may stimulate proliferation in stem cells and differentiation, or expression of a differentiated phenotype in mature cells (Mercola & Stiles 1988). EGF is also found in gestational decidua and placenta in humans (Hofmann *et al.* 1991). There is also evidence that EGF induces implantation and decidualization in the rat (Tamada *et al.* 1994). In human, it has been shown that EGF stimulates cell proliferation and inhibits prolactin secretion of human decidual cells in culture (Saji *et al.* 1990). EGF and EGF-receptor protein and mRNAs are present in cytotrophoblast cells of primary human placental cultures. EGF, via its receptors on the syncytiotrophoblasts, stimulates the release of hCG and hPL in normal early placenta (Maruo *et al.* 1987, Amemiya *et al.* 1994). Light and electronmicroscopic studies show that EGF induces differentiation of cytotrophoblast to form syncytiotrophoblast (Morrish *et al.* 1987), which suggests that EGF may play an important role in the functional differentiation of human trophoblast cells by autocrine or paracrine mechanisms. Taken together, these findings suggest that EGF/TGFα may have role in placenta development.

EGF regulates hCG and hPL (Maruo *et al.* 1987, Amemiya *et al.* 1994). Do EGF/TGFα regulate placental lactogens? Yamaguchi et al.(1992) report that EGF stimulates mouse PL-I secretion, but inhibits mouse PL-II secretion in primary placental cultures. In Rcho cells it has been observed that EGF has a growth promoting effect, which suggests that EGF may affect Rcho cell proliferation (Verstuyf et al. 1993). EGF at doses from 0.001 to 10 μg/ml did not affect rPL-II secretion in primary rat placental

cultures after 2-3 days of incubation (Kishi et al. 1993). The effect of EGF/TGFα on rat placental lactogens at the molecular level is totally unknown.

4.2 Colony Stimulating Factor 1

Colony stimulating factor 1 (CSF-1) is a glycoprotein growth factor first identified as essential for the proliferation and differentiation of mononuclear phagocytic cells (Stanley et al. 1983). The CSF-1 receptor is a transmembrane glycoprotein tyrosine kinase identified as the product of the *c-fms* proto-oncogene (Sherr et al. 1985).

In the mouse, CSF-1 concentrations in urine exhibit a dramatic elevation throughout pregnancy such that, at term, it is approximately 1000-fold higher than that of non-pregnant mouse (Bartocci *et al.* 1986). Uterine synthesis of CSF-I has been demonstrated by the presence of an alternatively spliced 2.3 Kb CSF-1 mRNA in the mouse (Pollard *et al.* 1987). CSF-1 is present in the mouse uterus (Muller *et al.* 1983). During the pregnancy, CSF-1 has been found at a low but constant concentration in the mouse placenta (Bartocci *et al.* 1986) and high levels in murine amniotic fluid where it reaches a peak about day 14 of gestation (Azoulay *et al.* 1987).

C-fms mRNA has been identified in the mouse placenta (Muller *et al.* 1983) and in human trophoblasts (Hoshina *et al.* 1985). Significant expression of *c-fms* mRNA is found to be confined to the mouse placenta (Muller *et al* 1983, Arceci *et al* 1989) and the mouse uterus before placentation (Arceci *et al* 1989). The amount of c-fms transcripts in the placenta increases approximately 15-fold during development reaching a plateau at day 14 to day 15 of gestation in the mouse (Arceci et al. 1989). Detailed *in situ* hybridization studies in the mouse have shown that a high level of CSF-1 receptor mRNA

is detected in the decidua starting at day 6, persists in the decidua basalis during formation of the placenta but declines once the mature placenta is formed. Throughout pregnancy, the expression of *c-fms* is found in decreasing amounts in giant trophoblasts, spongiotrophoblasts and cells of the labyrinth layer (Arceci et al. 1989, Regenstreif & Rossant 1989).

The temporal relationship of uterine CSF-1 synthesis with both decidual and trophoblast cell expression of the CSF-1 receptor and placental growth strongly suggest a role for CSF-1 in placental growth and development. Data showing CSF-1 stimulating the proliferation of primary murine placental cells and the established placental cell lines support this hypothesis (Athanassakis et al. 1987).

4.3 Insulin-like growth factors

The insulin-like growth factors (IGF-I and IGF-II) are growth-promoting peptides that are structurally related to insulin (Blundell et al. 1983). They interact primarily with the IGF-I receptor, a transmembrane tyrosine kinase structurally related to the insulin receptor (Ullrich et al. 1986). IGF-II has a lower binding affinity for the insulin receptor than for the IGF-I receptor, but has its greatest affinity for a distinct receptor - the type II (IGF-II) receptor (Sara & Hall 1884, Rechler 1985, Gammeltoft 1989).

Gene targeting studies show that both IGFs play critical roles during fetal development (DeChiara *et al.* 1990 & 1991, Liu et al. 1993). Germ-line transmission of the inactive IGF-II gene from male chimeras yield heterozygous progeny that are smaller than their embryonic stem cell-derived wild type littermates (about 60% of normal wight)

(DeChiara *et al.* 1990). It has been also demonstrated, that transmission of this mutation, through the male germ line results in heterozygous progeny that are growth deficient, but through the female germ line results in heterozygous progeny that are phenotypically normal (DeChiara *et al.* 1991). These results indicate that IGF-II is indispensable for normal embryonic growth and the IGF-II gene is subject to tissue specific parental printing. Furthermore, in the litter of 16-day-old embryos, the average weight ratio of heterozygotes to wild type embryos is the same as that of their respective placentas (DeChiara *et al.* 1991).

In human, the *in situ* hybridization studies show that IGF-II gene expression is particularly active in the cytotrophoblasts (Fant *et al.* 1986, Ohlsson et al. 1989). In rat, IGF-II receptor mRNA is detected in the developing labyrinth, and is expressed from the chorioallantoic placenta established persistently throughout the remainder of gestation (Senior *et al.* 1990). These data indicate that IGF-II may function as a placental growth factor.

4.4 Granulocyte-macrophage colony stimulating factor (GM-CSF)

GM-CSF is produced by numerous cell types including endothelial cells, fibroblasts, and T-lymphocytes (Azoulay *et al.* 1987). GM-CSF has also been shown to stimulate proliferation of murine placental cell lines (Athanassakis *et al.*1987). GM-CSF and other lymphokines, produced as a consequence of T cell activation by the allogenic fetus, have a role in promoting placental growth (Wegmann 1984 & 1988). It has been determined that T cell growth factors such as GM-CSF are produced locally at the

conceptus sites and at specific time during pregnancy (Burgess *et al.* 1977, Azoulay *et al.* 1987).

Several other growth factors, including basic broblast growth factor (bFGF), transforming growth factor β (TGFβ), platelet-derived growth factor (PDGF) and interleukins, may also play some role in growth and development of the uteroplacental unit (Brigstock *et al.* 1991). For example, FGFs are highly angiogenic and their presence in the uterus suggests that they might play important roles in the rapid angiogenesis that occurs in the neovascularization of the uterus or placenta during pregnancy. A role for FGFs in regulation of placental growth is suggested by the presence of high- and low-affinity bFGF receptors on mouse placental cell membranes (Brigstock *et al.* 1991). Furthermore, Cattini *et al.* (1991) have reported that bFGF is present in the nucleus as well as the cytoplasm of dividing placental cells, which suggests that bFGF may has an intracrine role in trophoblast proliferation.

Overall, the presence of variety of growth factor and their receptors on the uteroplacental unit suggest that they regulate various aspects of placenta function through complex autocrine and/or paracrine pathways (Brigstock 1991).

5. REGULATION OF EUKARYOTIC GENE EXPRESSION

Differential gene expression not only underlies the molecular mechanisms of eukaryotic development but also forms the basis of cellular response to extracellular stimuli (e.g. growth factors). From the standpoint of a protein molecule, its activity could be adjusted by controlling its abundance (transcriptional, post-transcriptional, translational

regulation) or by augmenting its activity after its production (post-translational regulation) (Grunstein 1990, Jackson 1991). This brief review section is focused only on the regulation of mRNA levels by gene transcription and mRNA stability mechanisms.

5.1 Transcription

The past several years have witnessed considerable effort to elucidate the mechanisms that control the transcription of genes by RNA polymerase II (Pol-II). Specifically, the effort has been devoted to two areas: the identification of specific *cis*-acting DNA elements involved in the regulation of transcription and isolation, characterization, and the cloning of *trans*-acting protein factors that interact with these DNA sequences. *Trans*-acting factors or transcription factors can be grouped into general and regulatory types (transcriptional activators or repressor), the former being involved in the basal transcription process and the latter concerned with achieving an induced or repressed state of transcription.

5.1.1 Basal transcription by RNA polymerase II

Promoters are located immediately upstream of the transcription start site and are required for accurate and efficient initiation of transcription (Lewin 1987, Dynan 1989). Sequence analysis of many eukaryotic Pol-II promoters reveals a common pattern of organization. Many promoters of higher eukaryotic genes contains an AT-rich "TATA box" (TATAA) region at position 25 to 30 bp upstream of the transcriptional start site which serves to specify the position of transcription initiation. Further upstream at around 75 bp is the "CAT box" (CCAAT) which functions to enhance the frequency of transcription (Lewin 1987). Many Pol II-transcribed genes contain a TATA box promoter.

TATA box-containing promoters usually belong to the class of genes known as non-housekeeping genes. Housekeeping genes, however, often lack both the TATA and CAT boxes. In these cases, positioning of the transcription start site is determined by other discrete elements such as a "GC' box (GGGCG). In view of the large assortment of promoter regulatory elements for Pol-II genes, one can only say that each gene has its own combination and arrangement of *cis*-acting elements that act in concert to confer a unique pattern of expression. In recent years, gene transcription factors which assist Pol-II in the process of transcription have been isolated and purified to a high degree. General transcription factors can be classified as transcription initiation factors and elongation factors, each required in separate steps of transcription. The most commonly used nomenclature for general transcription initiation factors is TFIIA, TFIIB, TFIID, and TFIIE (Molecular Biology of The Cell, 1994). Along with Pol-II, these transcription initiation factors are thought to assemble into stable preinitiation complexes in the promoter region prior to actual initiation of transcription. In the presence of ATP and all the other required nucleotides, this rapid-start complex can initiate RNA synthesis (Saltzman & Weinmann 1989, Sawadogo & Sentenac 1990).

5.1.2 Regulated transcription

Eukaryotic genes are regulated differentially in response to a complex set of environmental and developmental cues. In a multicellular organism, they would constitute stimuli such as hormones, growth factors and environmental stresses. Regulation at the transcriptional level is now known to be the most widely used primary control point to achieve differential gene expression. As in the case of basal transcription, this process

is mediated by sequence-specific transcriptional regulatory proteins which could either activate or repress transcription by augmenting the activity of the basal transcription apparatus. The *cis*-acting DNA elements include enhancers and repressors. Enhancers are traditionally defined as *cis*-acting regulatory elements that modulate the activity of promoters irrespective of their orientation, position and distance from the start of transcription. Enhancers are as large as 50 - 150 bp and they can be situated and function at distances up to thousands of basepairs upstream or downstream from the start site. They are usually not considered as a basic requirement for the basal level transcription although some genes do need enhancers to stimulate transcription to detectable levels. The enhancer elements contain short consensus sequences for DNA-binding proteins, many of which are tissue- or cell-specific. Binding of activator proteins to enhancer elements in turn augments the activity of promoters by increasing the frequency of transcriptional initiation. The repressors have similar features as enhancers, but normally suppress the transcription of genes (Lewin 1987, Ptashne 1988, Dynan 1989). In some cases, adjacent *cis* elements may cooperate to exert a positive synergistic effect, in others, overlapping or superimposed binding sites for different factors can result in different factors competing for the same site leading to negative, inhibitory effects. Furthermore, the same *cis* element may confer different physiological responses in different cell types, depending on the abundance of the factor (s) and their level (s) of activity in different cell types (Mitchell & Tjian 1989).

Analysis of recently cloned sequence-specific transcription factors has revealed that they consist of discrete structural domains with sequence-specific DNA binding and

transcriptional activation functions (Mitchell & Tjian 1989, Johnson & McKnight 1989, Latchman 1990). Four common protein motifs have been identified to be important for sequence-specific DNA binding: the helix-turn-helix (homeodomain proteins are a specific class of HTH) (Pabo & Saner 1984) , the leucine zipper (Bush & Sassone-Corsi 1990, McKnight 1990), zinc fingers (Berg 1990 a,b), and the basic helix-loop-helix (Murre *et al.* 1989). The leucine zipper and the helix-loop-helix motifs do not act directly as DNA-binding domains, but they rather act as a dimerization interface, serving to bring into correct positioning the adjacent highly basic amino acid regions which form the actual DNA contact surfaces. The members of basic helix-loop-helix (bHLH) family of transcription factors are able to form dimers that directly bind on DNA (Ferre-D'Amare *et al.* 1993)

The products of the protooncogenes, *c-fos* and *c-jun*, are nuclear transcription factor gene families containing leucine zipper domains (Ransone & Verma 1990, Distel & Spiegelman 1990, Vogt & Bos 1990). They are two components of a transcription factor historically known as activator protein-1 (AP-1) which is now known to consist of a family of related but distinct protein factors which all share the common property of binding to the DNA sequence TGACTCA, termed the TPA responsive element (TRE) or AP-1 binding site (Lee *et al.* 1987). *In vitro* assays indicated that the Fos protein by itself is incapable of binding to AP-1 sites whereas the Jun protein alone can bind as a homodimer. Dimerization is a prerequisite for Jun-DNA binding. The inability of Fos to achieve DNA binding is due to its inability to form homodimers. However, Fos can dimerize via the leucine zipper with a Jun molecule and this heterodimeric complex is

found to be more stable than a Jun-Jun homodimer (Ransone & Verma 1990, Distel & Spiegelman 1990, Vogt & Bos 1990). Thus, the prevailing physiological concentrations and /or activity of these two factors in a cell, at a given time, will dictate the binding characteristics of Jun and Fos complexes for the AP-1 site (Curran *et al.* 1988).

GATA is another transcription factor family. All the members in this family share a conserved, cysteine-rich, metal-binding motif, which is essential for DNA binding and which contains two finger domains (Bockamp *et al.* 1994). All the GATA proteins bind DNA sequences containing a GATA core consensus element. Binding-site enrichment studies have shown overlapping but distinct sequence preferences for different GATA family members. GATA-1 is expressed in multipotent progenitors capable of giving rise to cells that do not express GATA-1, and maintained or increased during differentiation along several lineages, but down-regulated during differentiation along other pathways (Bockamp *et al.* 1994). Recently it has been demonstrated that GATA-2 and GATA-3 are transcriptional regulators of placental trophoblast cells in the mouse (Ng *et al.* 1994).

Although gene control through transcriptional activation has gained the most attention, a growing number of cases have been described in which selective repression of transcription acts as an important mechanism of transcriptional control (Renkawitz 1990).

5.2 mRNA stability

Next to transcriptional regulation, the control of steady-state mRNA levels through regulation of cytoplasmic mRNA stability is perhaps the most widely studied and appreciated aspect of gene expression (Ross 1989). Theoretically, the steady-state level

of a particular mRNA can be controlled at any step in its biogenesis, including initiation or elongation of transcription, post-transcriptional maturation events (addition of 5' cap, addition of 3' poly (A) residues, and splicing), transport from the nucleus to the cytoplasm, and cytoplasmic degradation of the mature mRNA. The stability of different mRNAs changes considerably. It is now clear that in mammalian cells many messages are short lived while others have half lives of many hours to days. For the cells to respond rapidly to changes in the extracelluar environment, the levels of mRNAs encoding proteins that mediate these changes must be able to change rapidly. These mRNAs, in turn must be capable of rapid turnover, so that different rates of transcription can be rapidly converted into changes in steady-state mRNA levels. On the other hand, it is important that the mRNAs for many structural genes be long-lived, so that constitutive, high-level transcription of these genes is not required to supply the basic components of cellular machinery (Atwater *et al.* 1990).

Among the genes most studied with regard to mRNA stability are the protooncogenes involved in the early response to growth factors, especially *c-fos* and *c-myc*. These are part of a larger group that contains a structural motif common to many unstable mRNAs, an AU-rich 3' untranslated region. A series of investigations have attempted to define the structural features of *c-fos* mRNA that specify rapid turnover. Experiments to address this issue have employed mutant *c-fos* genes (both chimeric genes and deletion mutants) under the transcriptional control of either the *c-fos* promoter, in which case, decay following transient transcriptional induction with serum is monitored, or a constitutive promoter, in which case, mRNA decay is measured following the

addition of transcription-blocking drugs like Actinomycin D. The results of experiments using both techniques support the hypothesis that an AU-rich sequence contained in the 3' untranslated region of *c-fos* mRNA can function as a dominant element to confer instability to an otherwise stable mRNA like β-globin. The sequence identified in these experiments is similar to sequences present in the 3' untranslated region of many unstable mRNA (Atwater *et al.* 1990)

There are in general two common experimental approaches to measure the decay rates of individual mRNA. One involves *in vivo* labelling using a radioactive marker followed by monitoring the disappearance with time of specific mRNAs after a chase with radiolabelled nucleotide triphosphates (pulse chase experiments). Alternatively, transcription is inhibited and the decay rates derived by determining the abundance of the mRNA of interest at various times after such inhibition.

BASED ON THE DATA WHICH SHOW THAT EGF STIMULATES mPL-1 SECRETION AND INHIBITS mPL-II SECRETION IN PRIMARY MOUSE PLACENTAL CELL CULTURES, OUR HYPOTHESIS IS THAT THE REGULATION OF RAT PLACENTAL LACTOGENS BY EGF/TGFα OCCURS AT THE LEVEL OF mRNA.

The studies in this thesis were focused on three approaches to examine the regulation of the rat placental prolactin family of hormones. The first study was to determine the expression patterns of rPL-I, rPL-II and rPLP-A in Rcho cells and assess the value of this cell line for placental specific gene regulation studies.

The factors involved in the regulation of the rat placental PRL family of genes are largely unknown. The second study was to investigate the factors that might be involved in the regulation of this gene family. Several placenta/decidua related growth factors (EGF, TGFα, CSF-1, IGF-II and GM-CSF) were tested.

The molecular determinants controlling tissue-specific gene expression in the placenta are poorly defined. The third study was to analyze the 5'-flanking regions of rPL-I and rPL-II gene and to identify the fragments sufficient to determine placental specific expression.

MATERIALS AND METHODS

MATERIALS

1. CELL LINES

The rat choriocarcinoma cell line, Rcho, was originally derived from a choriocarcinoma transplanted beneath the kidney capsule. The cell line is composed of pure trophoblast cells which are a mixture of cell types - small undifferentiated cells growing in clusters and differentiated giant cells possessing large nuclei. The morphology of the cells is very similar to normal rat cytotrophoblasts and giant cells. In culture only the small cells multiply and differentiate (Verstuyf *et al.* 1990). It has also been shown that Rcho cells express rPL-I, rPL-II, rPLP-A and rPLP-C (Faria *et al.* 1991, Duckworth *et al.* 1993, Hamlin *et al.* 1994). Our original Rcho cells were kindly provided by Drs. A.Verstuyf and M.Vandeputte (Rega Institute for Medical Research, University of Louvain, B-3000 Louvain, Belgiun)

The rat anterior pituitary tumour cell line (GC) was used to identify and isolate a nuclear protein, variously called pit1/GHF1 (Catanzaro *et al.* 1987, West *et al.* 1987, Lefevre *et al.* 1987, Ingraham *et al.* 1988) to explain pituitary specific expression of hGH-N as well as rGH after gene transfer (Cattini *et al.* 1986). The GC cells were kindly provided by Dr. P.A. Cattini (Department of Physiology, University of Manitoba, Winnipeg, Manitoba, Canada).

2. OTHER MATERIALS

Purchased materials:

GIBCO Bethesda Research Laboratories Inc., Burlington, Ontario: RPMI-1640 medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin, growth factors (mouse EGF, human TGFα, and human IGF-II), phosphate buffered saline/calcium and magnesium free (PBS/CMF), lipofectin and lipofectamine.

R & D systems Inc., Minneapolis, Minnesota: growth factors (murine GM-CSF, human CSF-1).

Sigma Chemical Company, St. Louis, Missouri: NCTC-135 medium, β-mercaptoethanol, sodium pyruvate.

ICN Biomedicals Canada Ltd., Toronto, Ontario: L-glutamine.

Amersham Corporation, Oakville, Ontario: nick translation kit, ^{3}H-Acetyl CoA.

Pharmacia Biotechnology Inc., Baie d'Urfe, Quebec: NICK™ columns, DNA markers (lambda DNA and ϕx 174 RF DNA), restriction endonucleases.

Promega corporation, Madison, Wisconsin, USA: Magic Minipreps kits, luciferase assay reagents, restriction endonucleases.

Qiagen, Chatsworth, California: maxi plasmid kits.

New England Biolabs, Mississauga, Ontario: T4 DNA ligase, restriction endonucleases.

Boehringer Mannheim, Laval, Quebec: calf intestinal phosphatase.

United States Biochemical, Cleveland, Ohio: DNA sequenase kits.

BIO RAD Laboratories, Mississauga, Ontario: Bio-Rad protein assay reagent.

Fisher Scientific, Edmonton, Alberta: Scintilene, general laboratory chemicals.

Dupont Canada New England Nuclear, Mississauga, Ontario: $dCTP^{32}$, $dATP^{35}S$.

Eastman Kodak Company, Rochester, New York: XAR xray film.

Gift materials:

The rPL-I cDNA clone and genomic fragment clone (7bES) were from Dr.M.C.Robertson; cytomegalovirus luciferase (CMVp.Luc.) and herpes simplex I thymidine kinase (pT81Luc) plasmids were from Dr.R.J.Matusik; the glyceraldehyde-3-phosphate dehydrogenase (G3PD) cDNA clone and CMV promoter chloramphenicol acetyltransferase (pcDNA3.*cat*) plasmid clone were from Dr.R.P.C.Shiu. All the people above are from Department of Physiology, University of Manitoba, Winnipeg, Manitoba, Canada

The rat EGF receptor cDNA clone was from Dr.H.S.Earp (UNC Lineberger Comprehensive Cancer Centre, University of North Carolina at Chapel Hill, Chapel Hill, NC, U.S.A.). All the other cDNA and genomic clones were from our laboratory.

Thanks to Drs. R.J.Matusik and P.A.Cattini for the use of the TROPIX™ luminometer (BIO/CAN Scientific, Mississauga, Ontario) and Dr.L.Murphy for the use of the 1450 Microbeta Counter (Wallac, Turku, Finland).

METHODS

1. CULTURE CONDITIONS

The Rcho cells were routinely grown in RPMI-1640 medium with 20% fetal bovine serum (FBS) supplemented with 50 μM β-mercaptoethanol, 1 mM sodium pyruvate, and 50 u/ml of penicillin and 50 μg/ml of streptomycin (20% FBS-RPMI 1640) for 4 days. To promote differentiation, cultures were shifted into NCTC-135 medium with 10% FBS supplemented as above (10% FBS-NCTC 135), and maintained in this medium to the designated time.

The GC cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS supplemented with 0.4 mM L-glutamine and 50 u/ml of penicillin and 50 μg/ml of streptomycin (10% FBS-DMEM).

2. ROUTINE CULTURE PROCEDURES

Rcho: Cells were split at greater than 90% confluence every 3 days. Usually Rcho cells were split 1:4 or 1:5 to avoid small cells largely differentiated. When the cells were continually growing, they were always in 20% FBS-RPMI 1640. GC: Cells were split at less than 80% confluence (greater than 80% confluence caused cells to lift). Usually GC cells were split 1:3.

Medium was changed every other day. To split the cells, the growth medium was aspirated and the plate surface was rinsed with PBS; 0.05% trypsin/0.53 mM EDTA solution (3 ml/10 cm plate) was added and incubated for 1-2 min in a 37^{0}C incubator. The regular medium was added to wash off cells and stop the effect of trypsin. The lifted

cells were collected by spinning for 3 min at 800 rpm (in eppendorf centrifuge) at room temperature. All the cell cultures were incubated in a 37^0 C incubator containing 5% CO_2 and 95% humidity. Tissue culture was routinely done in 10 cm plates.

3. STANDARD EXPERIMENTAL MANIPULATIONS OF CULTURES

In all experiments cells were cultured as described in section 2.

3.1. Expression pattern study

Cells were harvested at every other day after plating, as indicated, up to 32 days. The cells were rinsed with PBS, then stored at -70°C for subsequent RNA preparation.

3.2. Growth factor treatment study

The cultures were maintained until day 7 or day 14 after plating. The cells were rinsed with PBS and maintained in serum-free NCTC-135 medium supplemented with 50 μM β-mercaptoethanol, 1 mM sodium pyruvate, and 50 u/ml of penicillin and 50μg/ml of streptomycin (SF-NCTC 135) for 24 hours, then given growth factors dissolved in PBS/0.25% BSA with SF-NCTC 135 for 3 days. The medium was changed every day during the 3 days. Control plates were given the same volumes of PBS/0.25% BSA with SF-NCTC 135. The cells were harvested for RNA as described above.

3.3 Transient transfection study

At day 14 after plating, cells were transiently transfected using lipofectin and lopofectamine (GIBCO BRL) or calcium phosphate methods (Howley *et al.* 1983).

3.3.1 Lipofection method

Lipofectin and lipofectamine were used according to manufacturer's instructions (GIBCO BRL) in preliminary transfection experiments. CMV luciferase (CMVp.Luc) was used to optimize conditions. Gene transfer was for 6 h and gene expression was for 48h. Different amounts of CMVp.Luc and transfection reagents - lipofectin and lipofectamine were tested. The experiments were done in 35 mm plates.

3.3.2 Calcium phosphate method

The calcium phosphate method was carried out routinely in 10 cm plates. Ten μg of supercoiled rPL-I and rPL-II 5'-flanking luciferase reporter plasmids, pXP1/pXP2 (-p.Luc.) as promoterless controls or CMV luciferase (CMVp.Luc.) as a positive control were transiently transfected into Rcho cells. The transfection procedures were as described in Howley *et al* (1983). All samples were co-transfected with 1 μg CMVp.*cat* for determination of plasmid uptake. The gene transfer was for 24h, followed by gene expression for 24h. 10% FBS-NCTC 135 supplemented medium was used during all the procedures. The Rcho cells were harvested 48 h after the start of gene transfer.

The GC cells were grown to 40% or 50% confluence, then transfected with the same plasmids as described above. A 20% glycerol shock for 2 min was given at 6 h after the initiation of gene transfer. Cultures were washed twice with PBS and given 10% FBS-DMEM. The GC cells were harvested 48 h after the glycerol shock.

To test the effects of TGFα on hybrid rPL-I and rPL-II gene promoter activity, at day 14 after plating, the rPL-I and rPL-II reporter plasmids were transiently transfected into Rcho cells for 24 h as described above. Following transfection the cultures were

washed with PBS and maintained 24 h in SF-NCTC with and without TGFα (10ng/ml) for gene expression. The cultures were harvested 48 h after the start of gene transfer.

For preparation of extracts for reporter gene analyses, at the time of harvesting, the transfected cultures were rinsed with PBS/CMF (calcium and magnesium free); 4.5 ml PBS-CMF/0.1mM EDTA was added, and cells were incubated at room temperature for 1 to 2 min. The cells were resuspended using a pipette and spun down for 3 min at 3,000 rpm (PR-6000) at room temperature. The cell pellets were resuspended in 400 µl of cold 100 mM Tris pH 7.8/0.1% Triton X100 (cell lysis Tris/Triton buffer) and lysed on ice for 15 min, then spun for 15 min at 12000 rpm (in eppendrof centrifuge) at 4^0C. The supernatants were transferred to new tubes. The luciferase assay was done immediately and the remaining cell extracts were frozen at -70^0C for subsequent protein and CAT assays.

4. RNA ANALYSIS

4.1 Total RNA isolation

The total cellular RNA was isolated from frozen cell cultures by the method of Chomczynski and Sacchi (1987). The concentration of RNA was determined by measuring the relative absorbance of a diluted RNA sample at 260 nm using the formula: $1OD_{260}$ = 40 µg/ml RNA.

4.2 RNA blotting

For northern blots, 30 µg of total RNA was mixed with 1.5 µl 10X RNA running buffer (0.2M MOPS, 50mM NaAc,10mM EDTA pH 7.0), 1.5 µl 37% formaldehyde, and

5.25 µl deionized formamide to a final volume of 30 µl. The samples were heated at 65°C for 15 min; 3 µl of loading buffer was then added. The samples were separated by electrophoresis on 1.2% agarose/2.2 M formaldehyde gels with ethidium bromide. Following electrophoresis the gels were washed in sterile 20X SSC (20X SSC = 3M NaCL and 0.3 M sodium citrate) for 30 min with gentle shaking. After photographing, the gels were blotted overnight onto Nitroplus membranes as for routine Southern blotting (Current Protocols in Molecular Biology, 1989). The next day, the Nitroplus membranes were soaked briefly in 5X SSC to remove salt. The membranes were air-dried and baked for 2h at 80°C under vacuum.

To optimize the binding capacity of the slot blot 1µg, 2µg and 5 µg of total RNA were tested; the result showed that 2 µg was optimal. For the routine slot blot analysis, 2 µg of total RNA were diluted into 100 µl with ddH_2O, then added into 200 µl of 50% (v/v) 37% formaldehyde and 50% (v/v) 20X SSC. All the samples were denatured for 15 min at 65°C. The denatured RNA samples were loaded onto slots under low vacuum. After loading all the samples, the slots were rinsed with 250 µl of 10X SSC under low vacuum. The membranes were air-dried and baked for 2 h at 80°C under vacuum.

4.3 Hybridization

The blots were first prehybridized at least 2 h at 42°C in 50% (v/v) of deionized formamide, 4X SSC, 0.4X Denhardt's solution (0.4X DH = 0.08% each of BSA, Ficoll 400 and polyvinylpyrrolidone), 10mM Na_2HPO_4 pH 7.4, 0.1% SDS, 1mM EDTA pH 8.0, 250µg/ml sonicated salmon sperm DNA .

Hybridizations were performed in the same solution, containing ^{32}P-labelled cDNA probes (see below*) 16-20 h at 42^{0}C. The membranes were washed twice in 2X SSC and 0.1% SDS for 15 min at 65^{0}C. An extra wash in 0.2X SSC and 0.1% SDS for 15 min at 65^{0}C was performed if necessary.

For reuse, blots were stripped in boiling 0.1X SSC and 0.1% SDS for 3 min.

* Labelling of cDNA probes with ^{32}P-dCTP: All nick translation reactions were performed using a nick translation kit according to the manufacturer's instructions (Amersham). The amount of DNA used in each reaction ranged from 100 to 200 ng. Labelled DNA was separated from the unincorporated free nucleotide by passing through NICK^{TM} columns (Pharmacia) equilibrated with TE pH 7.5 according to the manufacturer's instruction. The specific activity of each labelled probe was more than 10^8 dpm/μg. Before addingthe probe into the hybridization solution, it was boiled for 5 min and cooled rapidly on ice to separate the two DNA strands.

4.4 Exposure

The blots were exposed to XAR Kodak film at -70^{0}C with intensifying screens for a period of several hours to 14 days.

4.5 Quantitative analysis

Quantitation was achieved by densitometric scanning of various exposures of the autoradiographs using the PhotoFinish and Scanplot programs. All the quantitation was corrected for RNA loading by comparison to a housekeeping gene, glyceraldehyde 3 phosphate dehydrogenase (G3PD). Graphs were prepared using the computer programs Freelance or SigmaPlot.

5. ISOLATION AND PURIFICATION OF PLASMIDS AND DNA FRAGMENTS

5.1 Plasmids

5.1.1 Plasmid Transformation

Competent cells (TG1λ) were prepared as described in Nishimura *et al.* (1990), and transformations were carried out according to standard protocols in Current Protocols in Molecular Biology (1989).

5.1.2 Large scale plasmid preparation

Escherichia coli cultures were grown in LB medium in the presence of ampicillin with and without chloramphenicol amplification. Large scale preparations of plasmid DNA were carried out by alkaline/SDS lysis followed by centrifugation on a cesium chloride gradients as described in Molecular Cloning (1989) or by separation on a Qiagen maxiprep column according to the manufacturer's instructions. The concentration of the plasmid DNA solution was determined from the absorbance of a diluted samples at 260 nm using the formula: $1OD_{260}$ = 50 μg/ml DNA. The plasmid sample was stored at -20°C or -70°C.

5.2 Isolation of DNA fragments

Agarose gel electrophoresis was used to separate restriction-digested DNA fragments. The DNA band of interest was cut out of the gel, and put inside of a piece of Spectrapor dialysis membrane (#132670) with 400 μl of 4X AGB (Tris/Acetate/EDTA buffer). An electric current was applied to the membrane until the DNA was removed from the gel fragment. The DNA containing buffer was collected, extracted with

phenol/chloroform and the DNA precipitated with ethanol. This method was used for both cDNA and genomic DNA fragment isolations.

6. CONSTRUCTION OF HYBRID PLASMIDS

Restriction enzyme mapping: A variety of restriction enzymes were used according to manufacturer's instructions for mapping the rPL-II genomic clone. In blunt end and single enzyme digested ligations alkaline phosphatase (18 u/μl) was used to reduce the self religation.

Ligation: Ligations were performed as follows: 1 μl of 10X ligase buffer, 1 μl of T4 DNA ligase, molar ratio 3:1 for insert DNA vs vector DNA and H_2O to 10 μl. The ligation reaction was done at room temperature overnight, and heat denatured for 15 min at 65 ^{0}C; the final volume was brought up to 25 μl with TE pH 7.5. Five μl of the ligation mix were used for transfromation of 100 μl of competent cells.

Clone selection: The resulting colonies were tested for the presence of interested clone by small scale plasmid preparation according to alkaline lysis method described in Molecular Cloning (1989), followed by a diagnostic restriction enzyme digestion. All the new clones created in this study were purified by Magic Minipreps (Promega) and sequenced by DNA sequencing kit (USB) to check the boundaries between the 5' and 3' end of the rPL-I and rPL-II gene and the reporter gene vector.

7. REPORTER GENE ACTIVITY ANALYSIS OF rPL-I AND rPL-II HYBRID PLASMIDS

For preparation of extracts for reporter gene analyses, at the time of harvesting, the transfected cultures were rinsed with PBS/CMF (calcium and magnesium free); 4.5 ml PBS-CMF/0.1mM EDTA was added, and cells were incubated at room temperature for 1 to 2 min. The cells were resuspended using a pipette and spun down for 3 min at 3,000 rpm (PR-6000) at room temperature. The cell pellets were resuspended in 400 µl of cold 100 mM Tris pH 7.8/0.1% Triton X100 (cell lysis Tris/Triton buffer) and lysed on ice for 15 min, then spun for 15 min at 12000 rpm (in eppendrof centrifuge) at 4°C. The supernatants were removed to new tubes, the luciferase assay was performed immediately and the remaining cell extracts were frozen at -70°C for subsequent protein and CAT assays.

7.1 Luciferase assays

Twenty µl of cell extract was used for each luciferase assay. Cell lysis Tris/Triton buffer was used as the blank control. The luciferase activity was measured on a TROPIX™ luminometer with 100 µl luciferase assay reagent (Promega). Activity was measured in relative light units.

7.2 CAT assay

The CAT activity was measured by a two-phase fluor diffusion assay (Neumann *et al.* 1987). Briefly, 200 µg of cell extract were added to a 7 ml scintillation vial with sufficient cell lysis Tris/Triton buffer to give a total volume of 200 µl. The solution was heated for 15 min at 65°C to inactivate some CAT inhibitors, and cooled to room

temperature. A reaction mix (75 µl), containing 2 µl of [^{3}H] acetyl-CoA (Specific activity 0.5 µCi/assay, Amersham), 50 µl of 5 mM chloramphenicol (in H_2O), 7.5 µl of 1 M Tris/HCI pH 7.8, and 15.5 µl of H_2O, was added. The reaction mixture was carefully overlaid with 3 ml of organic-phase scintillation cocktail (Fisher-Scintilene). After a 30 min incubation at room temperature, the samples were cycle-counted for 1 min each during 3 to 5 cycles. Quantitative values for CAT activity were determined by regression analysis to give cpm/min/mg protein.

7.3 Quantitation of Proteins

The Bio-Rad protein assay reagent was used according to the manufacturer's instructions (Bio-Rad). The samples for a standard curve were prepared using 0, 2, 4, 6, 8, 10, 15, 20 µl of BSA (1 mg/ml). Five µl of a 1:5 diluted cell extract were analyzed. Water was added to total 800 µl for each of the samples, and 200 µl of Bio-Rad protein assay dye reagent was added. The mixtures were incubated at room temperature for at least 10 min. The OD was measured at 595 nm wavelength.

8. STATISTICS

All the statistics were done by an unpaired student's t-test in SigmaStart program. The definition of statistical significance was $p < 0.05$.

RESULTS

1. THE EXPRESSION PATTERNS OF rPL-I, rPL-II AND rPLP-A IN RCHO CELLS

1.1 Optimization of culture conditions

The Rcho cell line is composed of pure trophoblast cells which are a mixture of cell types - small undifferentiated cells growing in clusters and differentiated giant cells possessing large nuclei. In the culture only the small cells multiply and differentiate. The giant cells in the culture express rPL-I, rPL-II, rPLP-A and rPLP-C (Faria *et al.* 1991, Duckworth *et al.* 1993, Han *et al.* 1994). The optimal culture conditions for the growth and differentiation of small cells are not well characterized. In Shida *et al* (1993) the Rcho cells were grown to confluence in 10% RPMI 1640 medium, then shifted into 10% NCTC 135 medium to promote differentiation. In this study 20% FBS-RPMI 1640 medium and 10% FBS-NCTC 135 medium were tested. At the same day after plating, the numbers of giant cells in the cultures appeared as: 10% FBS-NCTC 135 > 10% FBS-RPMI 1640 > 20% FBS-RPMI 1640; the small undifferentiated cells appeared as: 20% FBS-RPMI 1640 > 10% FBS-RPMI 1640 > 10% FBS-NCTC 135. These observations indicated that 20% FBS-RPMI 1640 is better for cell growth and division, while 10% FBS-NCTC 135 favours differentiation. It was also observed that the high cell density facilitates giant cell formation. For the standard experiments, Rcho cells were grown in 20% FBS-RPMI 1640 for 4 days, then shifted into 10% FBS-NCTC 135 to promote differentiation.

1.2 Morphology

After plating, the small cells began to differentiate into giant cells beginning at day 2. By day 4 giant cells covered approximately half of the plate surface. By day 14, giant cells covered more than 80% of the plate surface, and the small rapidly growing cells began to pile up. After day 14, no obvious morphological changes were seen in the cultures. Although clusters of small cells were often washed off during the medium changes, but grew up rapidly again.

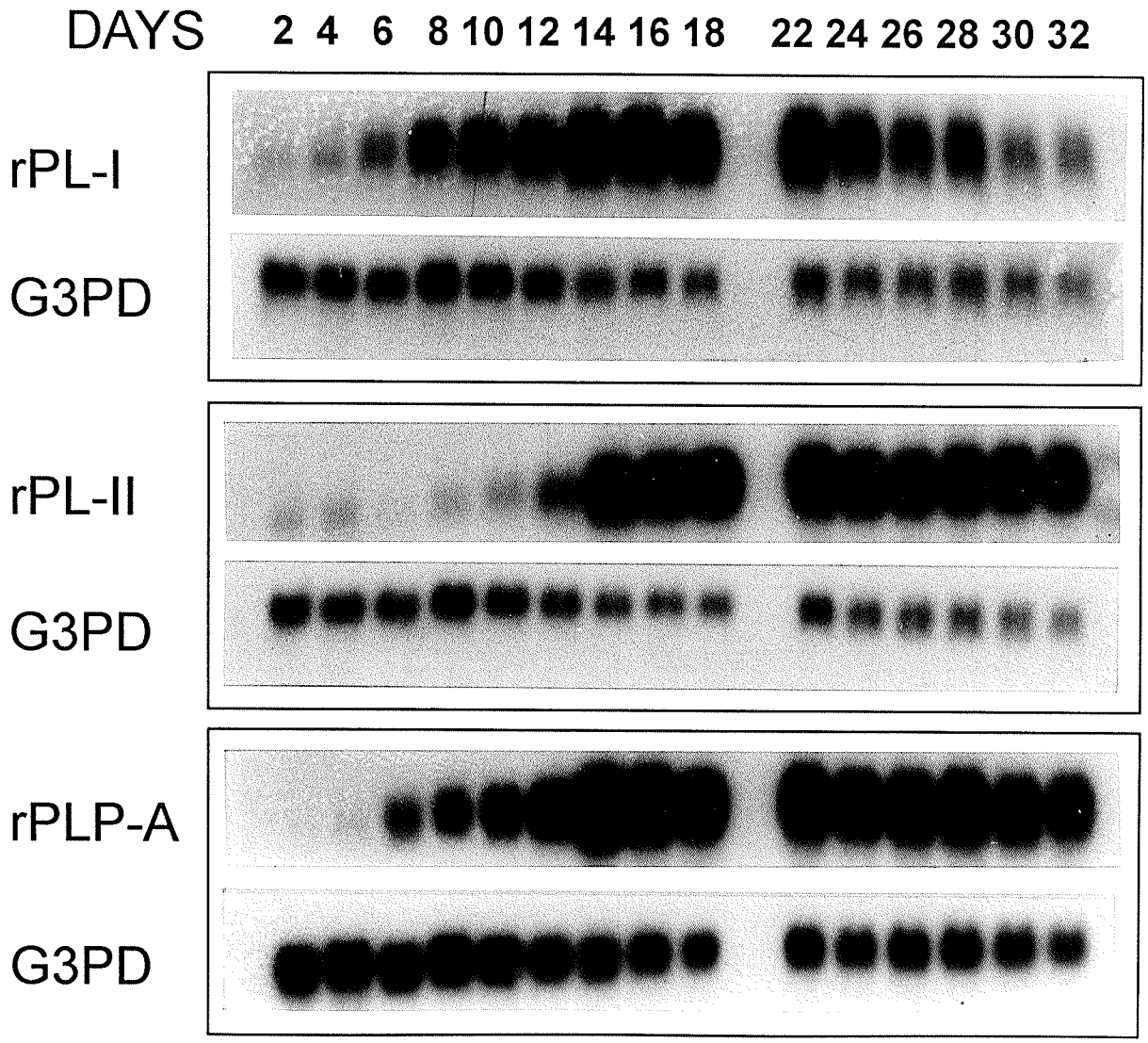
1.3 RNA analysis

Rcho cells express all rat placental PRL family members known to be expressed in the placental giant cells: rPL-I, rPL-II rPLP-A, rPLP-C (Faria *et al.* 1991, Duckworth *et al.* 1993, Hamlin *et al.* 1994), but the expression patterns during long term culture have not been described. RNA blot analysis was used to study the expression patterns of rPL-I, rPL-II and rPLP-A. Rcho cells, were grown and maintained as described, were harvested every other day from day 2 to day 32. The blots of rPL-I, rPL-II and rPLP-A all showed a single hybridizing bands of approximately 1.0 Kb (Figure 3). Using probes of similar specific activity, the exposure time for rPL-I was 2 days, rPL-II, 12 days, and rPLP-A, 3 days, suggesting that the expression of rPL-II mRNA in these cultures is much lower than that of rPL-I and rPLP-A. The rPL-I mRNA began to appear as soon as giant cells differentiated in the cultures (day 2), gradually increased and peaked at day 16 to day 24, after which levels gradually declined. Detectable amounts of rPL-II mRNA did not appear until about day 14, and unlike rPL-I, rPL-II mRNA levels were maintained at more or less peak levels until day 32 when the experiment was terminated. The mRNA

Figure 3 →

Fig.3 The expression patterns of rPL-I, rPL-II and rPLP-A mRNA in Rcho cells.

Rcho cells were grown and maintained as described. Total RNAs from individual dishes were isolated at different days after plating as indicated. 30μg per lane of total RNA were loaded on a 1.2 % agarose/2.2 M formaldehyde gel, blotted to Nitroplus membrane and hybridized to nick-translated rPL-I, rPL-II or rPLP-A cDNA probes. In the autoradiographs of RNA blots, the exposure time for rPL-I was 2 days, rPL-II was 12 days and rPLP-A was 3 days. Glyceraldehyde 3-phosphate dehydrogenase (G3PD) mRNA exposed for 4 h was used as a loading control. The relative absorbance units of rPL-I, rPL-II and rPLP-A mRNA bands vs G3PD mRNA bands were compared to determine mRNA levels of the three different prolactin-like proteins.


DAYS
2 4 6 8 10 12 14 16 18 22 24 26 28 30 32
rPL-I
G3PD
rPL-II
G3PD
rPLP-A
G3PD

levels of rPLP-A were barely detectable before day 4, increased between days 6 and 10; the high levels of expression were maintained after day 12 to the termination of the experiment. Overall, rPL-I and rPLP-A were expressed throughout the test period at high or low levels, but detectable levels of rPL-II were expressed mainly during the second half of the experimental period. The rPL-II result was somewhat similar to that seen in the rat placenta in which rPL-II was expressed from day 11 to term (Duckworth *et al.* 1993).

2. THE EFFECTS OF SOME PLACENTA/DECIDUA RELATED GROWTH FACTORS ON rPL-I, rPL-II, AND rPLP-A mRNA EXPRESSION

2.1 Preliminary experiments

Several growth factors and/or their receptors have been shown to be expressed in placenta and/or decidua as described in the Introduction. To investigate whether they could be involved in the regulation of the rat placental PRL family of genes, the effects of different concentrations (Table 2) of EGF, TGFα, CSF-1, IGF-II and GM-CSF on the levels of rPL-I, rPL-II and rPLP-A mRNAs were tested in Rcho cells.

Rcho cells were grown and maintained as described in the Methods. At day 14 after plating, cells were incubated for 24 h in SF-NCTC 135, followed by 3 days of treatment with different concentrations of the growth factors. Total RNAs were isolated and RNA blot analysis was performed. EGF and TGFα appeared to increase the mRNA levels of rPL-I and rPLP-A, and decrease the mRNA levels of rPL-II; CSF-1 appeared

Table 2 & Figure 4 →

Table 2 The concentrations of growth factors

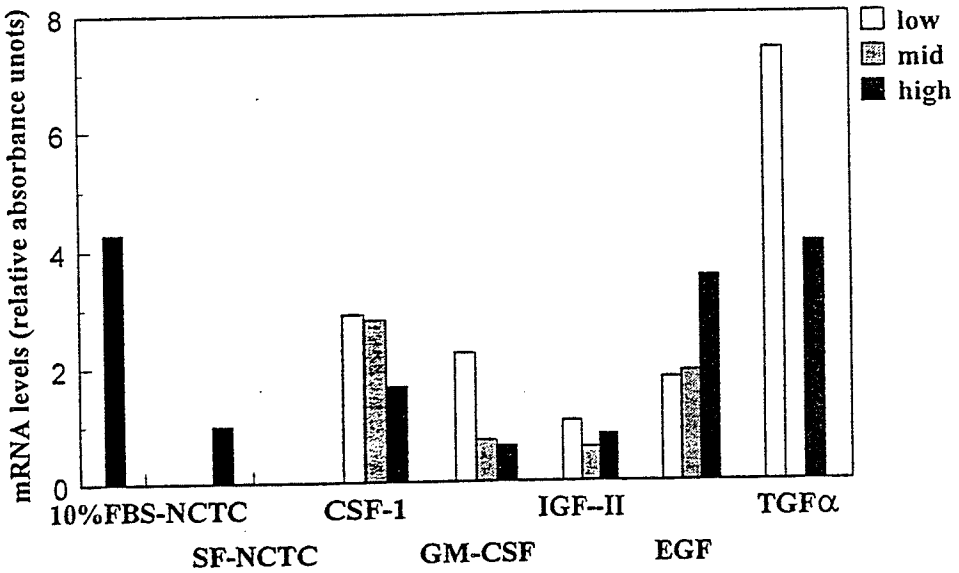
	low (ng/ml)	mid (ng/ml)	high (ng/ml)
EGF	1	5	10
TGFα	1	5	10
CSF-1	1	2	5
IGF-II	5	10	20
GM-CSF	0.1	1	2

The above five growth factors at different concentrations were used in preliminary growth factor experiment.

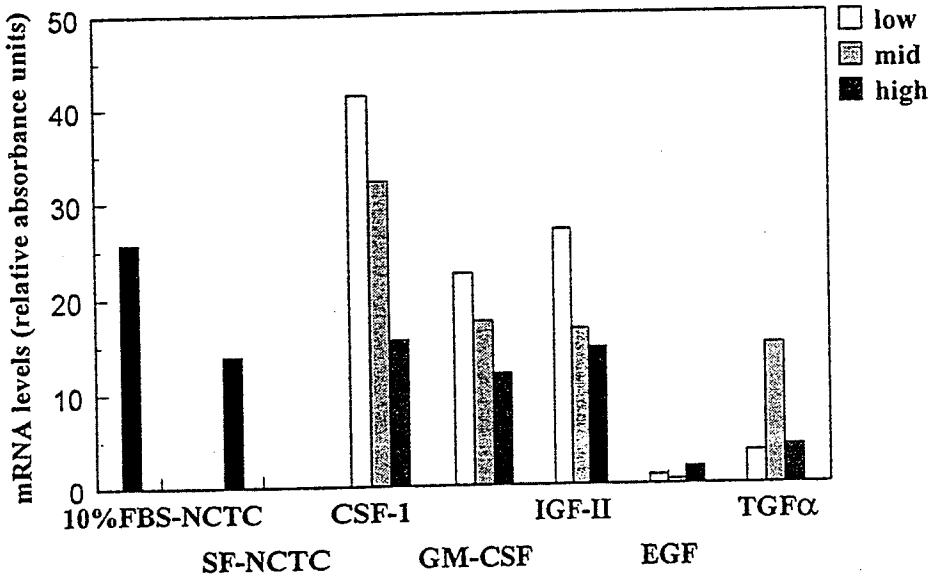
Fig.4 The effects of different growth factors on rPL-I, rPL-II and rPLP-A mRNA expression

Rcho cells were routinely maintained to day 14 after plating, and given 24 h SF-NCTC 1640, then supplemented with different concentrations of growth factors as indicated in Table 2 for 3 days. SF-NCTC and 10% FBS-NCTC 135 were given in control plates. Medium was changed every day during the growth factor incubation period. The total RNAs were isolated and northern blot analysis was performed. G3PD cDNA probe image was used as a loading control. For each growth factor in the graphs, left/middle/right column represented low/medium/high concentration as in Table 2. One sample is for each column.

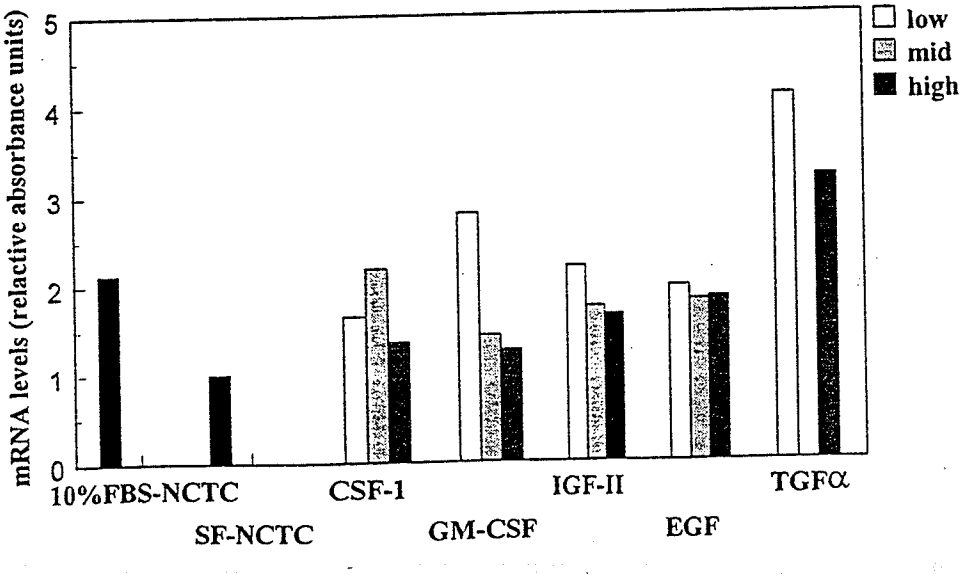
rPL-I



rPL-II



rPLP-A



to increase the mRNA levels of rPL-I and rPL-II; IGF-II appeared to increase the mRNA levels of rPLP-A. No significant effect was detected with GM-CSF (Figure 4). The effect of the same growth factor at different concentrations also appeared to be different. These preliminary experiments indicated that the optimal concentration for both EGF and TGFα was 10 ng/ml, for CSF-1, 1 ng/ml and for IGF-II, 5 ng/ml. Further experiments investigated the effects of these growth factors in more detail.

2.2 The effects of EGF, TGFα, CSF-1 and IGF-II

The standard experiments were focused on the effects of EGF, TGFα, CSF-1 and IGF-II on rPL-I , rPL-II and rPLP-A mRNA expression. Rcho cells were grown and maintained as described. Two different times after plating were studied: day 7 when rPL-I expression was increasing and rPL-II was barely detectable, and day 14 when expression of both PLs were close to peak values. Cells were given SF-NCTC 135 for 24 hours, then given SF-NCTC 135 with and without growth factors for 3 days. Two μg of total RNA was loaded onto a slot blot and hybridized with rPL-I, rPL-II and rPLP-A cDNA probes. The results in Figure 5. showed that TGFα (10 ng/ml) produced a 3-fold increase in rPL-I mRNA levels ($p<0.05$), a 2-fold decrease in rPL-II mRNA levels ($p<0.05$), but no significant effect on rPLP-A mRNA levels at both times. No significant effect was seen with EGF, CSF-1 and IGF-II. The day 14 data above, the previous optimization data, and the following dose response data were normalized by comparing to SF-NCTC control samples in each experiment. The results were that TGFα (10 ng/ml) increased rPL-I mRNA levels by 3.5 fold and decreased rPL-II mRNA levels by 2.5 fold.

Figure 5 →

Fig.5 The effects of TGFα on rPL-I and rPL-II mRNA expression.

Rcho cultures were grown and maintained as described. Two different times (day 7 and day 14) after plating were studied. Cells were washed with PBS and maintained in SF-NCTC for 24 hours, then given growth factors (EGF/TGFα 10ng/ml, CSF-1 1 ng/ml, IGF-II 5ng/ml) with SF-NCTC 135 for 3 days. Control plates were given equal volumes of PBS/BSA in SF-NCTC 135. The total RNAs were isolated from individual plates, 2 μg for each plate were loaded onto a slot blot and hybridized with nick-translated rPL-I and rPL-II cDNA probes. The relative absorbance units were analyzed using the PhotoFinish and Scanplot programs. All the relative absorbance units of mRNA levels were corrected by comparison to a G3PD cDNA probe loading image. The student's t-test in SigmaStart program was used for statistical analysis. Each column of data (mean ± SD) presents 4 separate plates.

A: the effect of EGF, TGFα, CSF-1 and IGF-II on rPL-I mRNA expression.

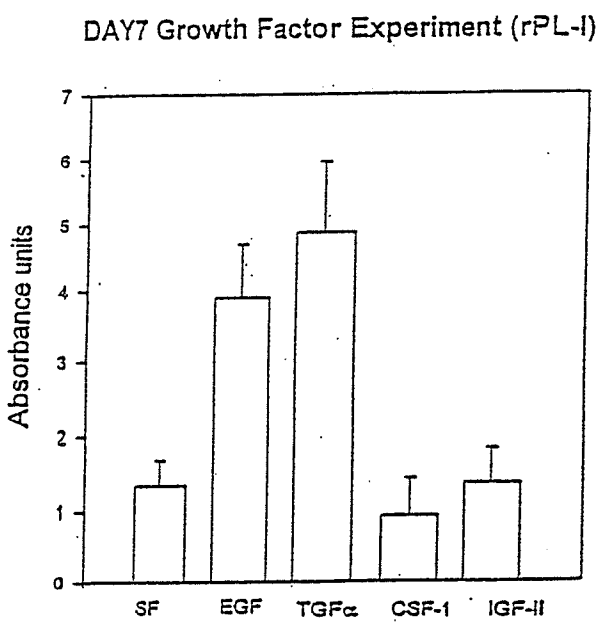
B: the effect of EGF, TGFα, CSF-1 and IGF-II on rPL-II mRNA expression.

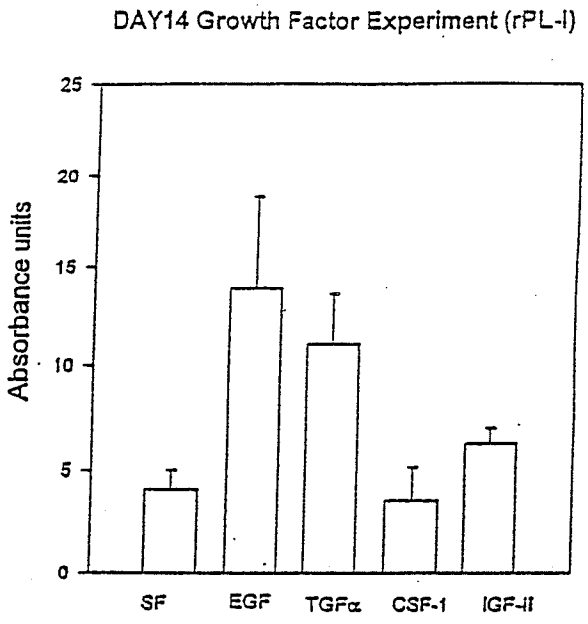
Figure 6 →

Fig.6 TGFα dose response effects on rPL-I and rPL-II mRNA expression.

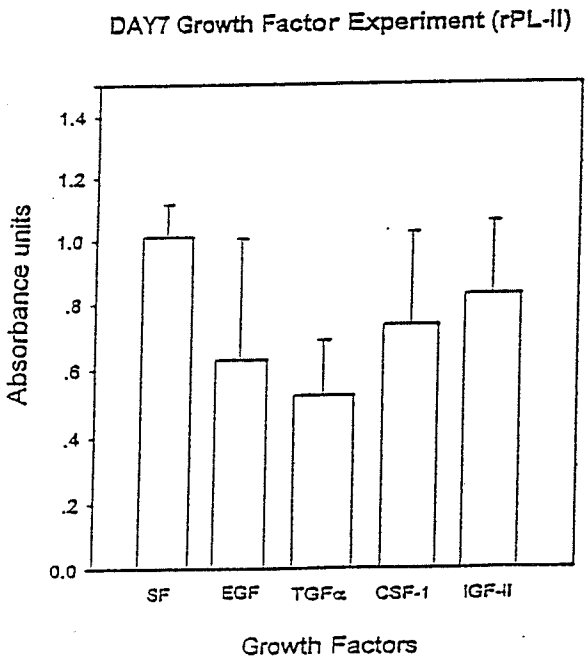
0.1, 1.0 and 10 ng/ml TGFα were used to investigate the optimal TGF α concentration for the effect on rat placental lactogen mRNA expression in Rcho cells. All the methods were as in Fig. 5. The experiment was done 14 days after plating. Each group of data (mean ± SD) represents 6 separate plates.

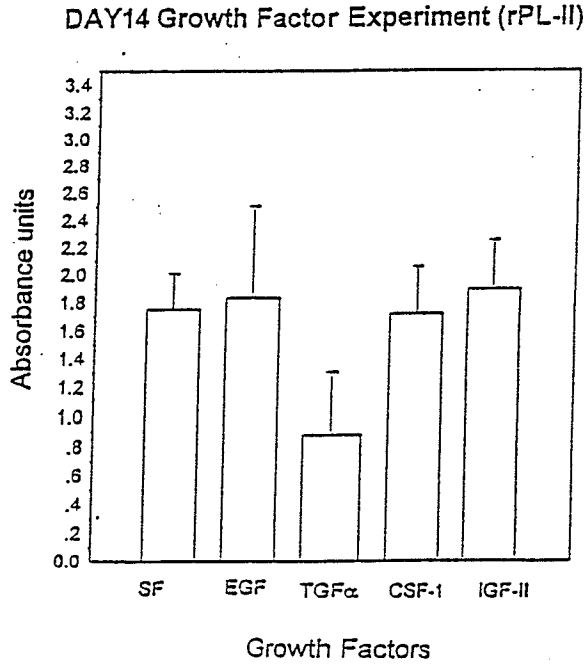
A


DAY7 Growth Factor Experiment (rPL-I)
Absorbance units
0
1
2
3
4
5
6
7
SF
EGF
TGFα
CSF-1
IGF-II


DAY14 Growth Factor Experiment (rPL-I)
Absorbance units
0
5
10
15
20
25
SF
EGF
TGFα
CSF-1
IGF-II

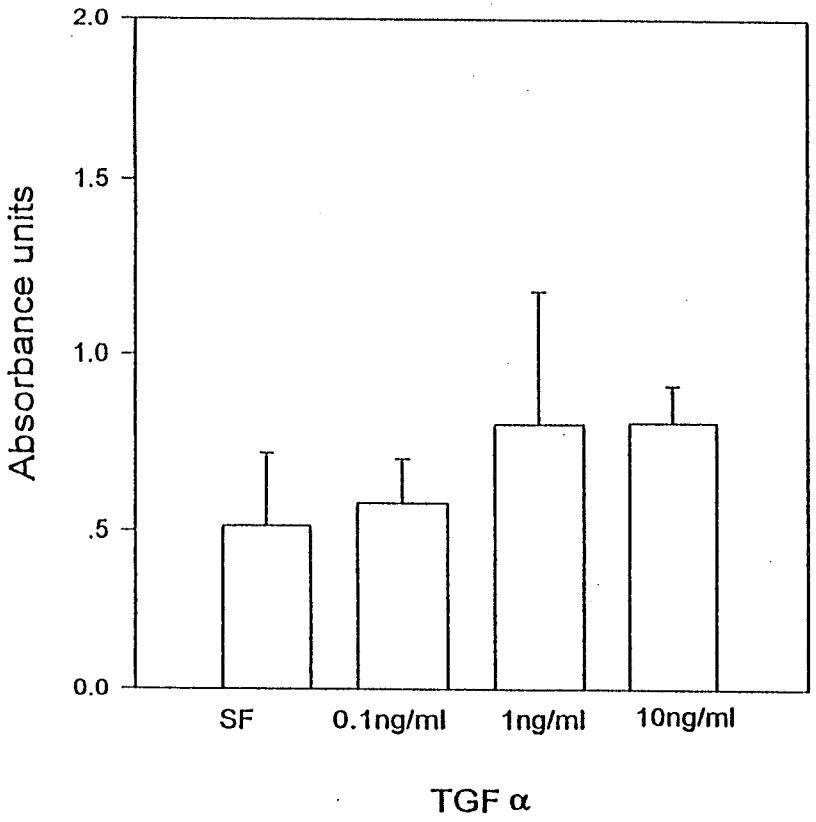
B


DAY7 Growth Factor Experiment (rPL-II)
Absorbance units
0.0
.2
.4
.6
.8
1.0
1.2
1.4
SF
EGF
TGFα
CSF-1
IGF-II
Growth Factors


DAY14 Growth Factor Experiment (rPL-II)
Absorbance units
0.0
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
SF
EGF
TGFα
CSF-1
IGF-II
Growth Factors

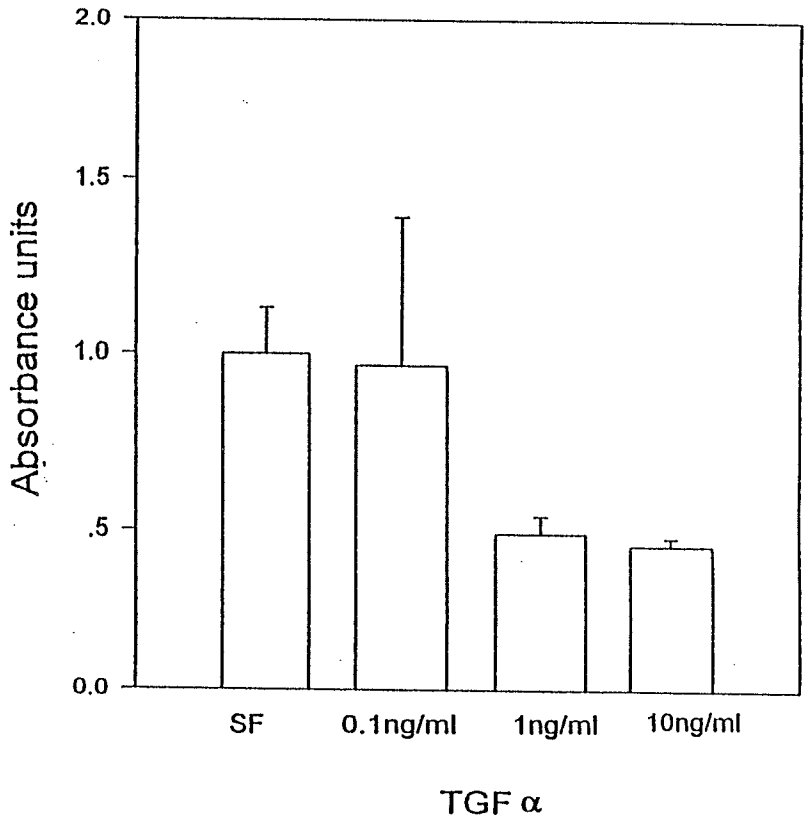
A

rPL-I



B

rPL-II



All the data indicate that TGFα positively regulates rPL-I mRNA levels but negatively regulates rPL-II mRNA expression in Rcho cells, which correlates with previous studies showing that EGF stimulates the secretion of mouse PL-I and inhibits mouse PL-II in primary placental cell cultures (Yamaguchi *et al.*1992).

2.3 TGFα dose response effects on rPL-I and rPL-II mRNA expression

The optimal concentration range of TGFα for the cell proliferation has been reported as 0.1-10 ng/ml according to the manufacturer's information (GIBCO). The concentrations of 0.1, 1.0, 10 ng/ml of TGFα were tested on rPL-I and rPL-II mRNA expression. TGFα showed a significant stimulatory effect on rPL-I only at 10 ng/ml ($p<0.05$), but a significant inhibitory effect on rPL-II at both 1 ng/ml and 10 ng/ml ($p<0.05$) (Figure 6), indicating that 10 ng/ml TGFα is optimal to detect the effects on rPL-I and rPL-II mRNA expression.

3. THE EXPRESSION OF EGF, CSF-1 AND IGF-II RECEPTORS IN RCHO CELLS

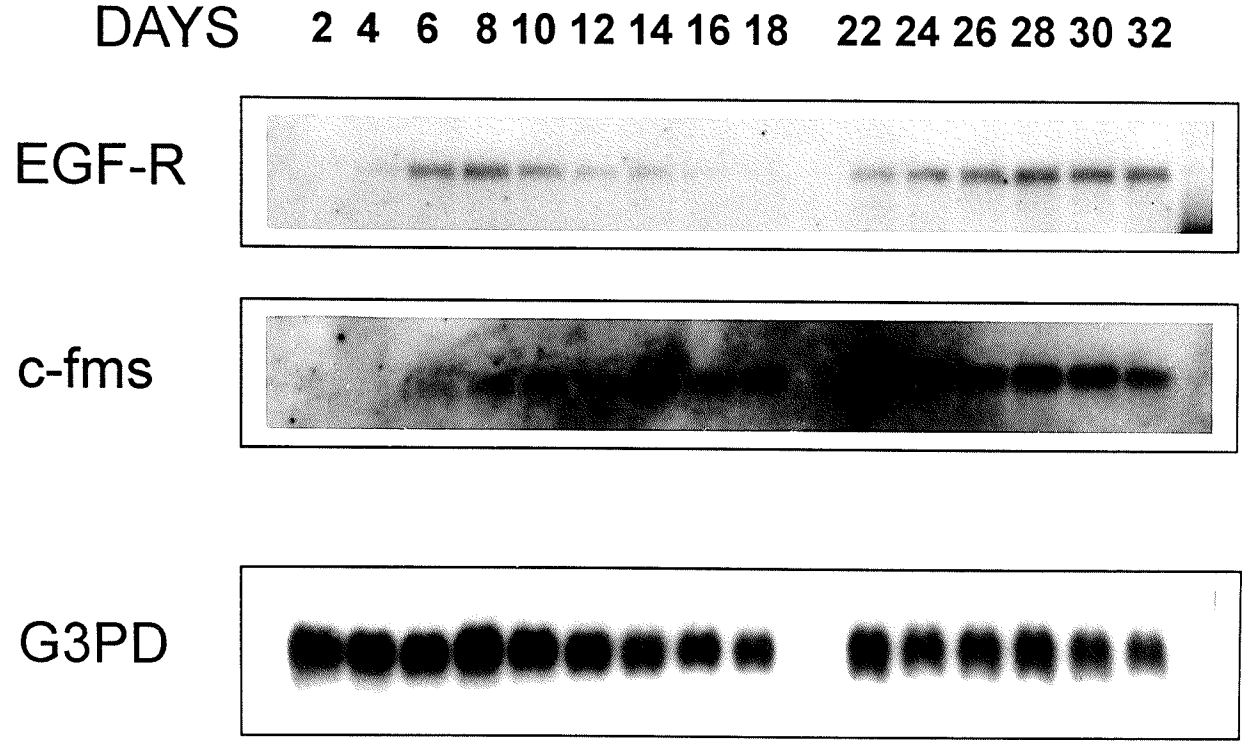
The expression patterns of EGF, CSF-1 and IGF-II receptor mRNAs were investigated in Rcho cells grown from 2 to 32 days (Figure 7). The same RNA blot was used as for the rPL-I expression study .

Rat EGF receptor mRNA shows one major (9.6 Kb) and two minor (6.5 and 5.0 Kb) species of mRNA (Petch *et al.* 1990). For detecting EGF receptor mRNA, the rat EGF receptor cDNA probe was used. A 9.6 Kb mRNA band was detected on the blot, perhaps because of low levels of expression the minor bands were not seen. The

Figure 7 →

Fig. 7 The expression of EGF receptor and *c-fms* in Rcho cells.

The same blot hybridized to rPL-I in Figure 3 was hybridized to nick-translated rat EGF receptor, mouse c-fms and rat IGF-II receptor cDNA probes. A 9.6 Kb EGF-receptor and a 4.0 Kb c-fms mRNA band were detected. No specific hybridization was seen with rat IGF-II receptor. The exposure time for EGF receptor was 14 days, *c-fms* was 8 days, and IGF-II was 14 days. G3PD mRNA was used as a loading control.


DAYS 2 4 6 8 10 12 14 16 18 22 24 26 28 30 32
EGF-R
c-fms
G3PD

expression of EGF-receptor at day 6 -10 and day 24 - 32 was high, but the expression between day 12 - 18 was low. This result is similar to that seen in mouse placenta where it has been reported that EGF receptors are in lower quantities at day 14 than at either day 10 and day 17 (Smith & Talamantes 1986).

For detecting CSF-1 receptor mRNA, the mouse *c-fms* cDNA probe was used. A 4.0 Kb rat c-fms mRNA band was detected (Borycki *et al.* 1993). A constant level of c-fms mRNA was seen between day 8 to day 32.

No specific hybridization was detected for rat IGF-II receptor mRNA.

4. ANALYSIS OF THE 5'-FLANKING REGIONS OF rPL-I, rPL-II and rPLP-A GENES

4.1 Construction of hybrid plasmids

4.1.1 Restriction enzyme mapping of rPL-II genomic clone

A 6.6 Kb genomic rPL-II DNA fragment (HindIII/HindIII) including approximately 4.5 Kb 5'-flanking region and three exons had been previously cloned into the vector pVZ1 (6.6 Kb rPL-II pVZ1) and partially restriction enzyme mapped (Dr.M.L.Duckworth). A variety of restriction endonucleases were used to try to locate further useful restriction enzyme sites for subcloning, especially unique sites at 5' untranslated region. Most enzymes that were tested did not cut (NotI, XhoI, ClaI, NsiI, NheI, BssHI) or cut more frequently than was useful. A unique SacI site was identified at ~3.3 Kb (Figure 8).

4.1.2 Subcloning strategies

rPL-I luciferase constructs

Maps of the initial rPL-I genomic clone and constructs are shown in Figure 8A.

The rPL-I 5' flanking reporter constructs were produced as follows:

The initial clone was prepared from an EcoRI/SacI restriction enzyme digestion fragment containing 5'-flanking sequence of an rPL-I genomic clone, then subcloned into these sites in pGEM7Z to form clone *7bES* (Dr.M.C.Robertson).

1. An XhoI/HphI (blunt-ended with Klenow DNA polymerase) *7bES* fragment from approximately -1.4 Kb to +12 bp was cloned into the XhoI and SmaI sites of the luciferase vector pXP1(Nordeen 1988) to form **-1.4rPLIp.Luc** (Dr.M.L.Duckworth).

2. **-1.4rPLIp.Luc** clone was cut with BglII and religated to form **-300rPLIp.Luc**.

rPL-II luciferase constructs

Maps of rPL-II initial genomic clone and constructs are shown in Figure 8B.

The rPL-II 5' flanking reporter constructs were produced as follows:

1. The starting clone was 6.6 Kb rPL-II pVZ1. A PvuII/PvuII fragment of rPL-II 5' flanking DNA from approximately -900 bp to +66 bp in 6.6 Kb rPL-II pVZ1 was cloned into the SmaI site of pBluescript II SK (pBsp) in a 3' to 5' direction to form clone *-900rPLIIpBsp*.

A HindIII/BamHI fragment of clone *-900rPLIIpBsp* was ligated into the HindIII and BglII cut luciferase vector pXP2 (Nordeen 1988) to form **-900rPLIIp.Luc**.

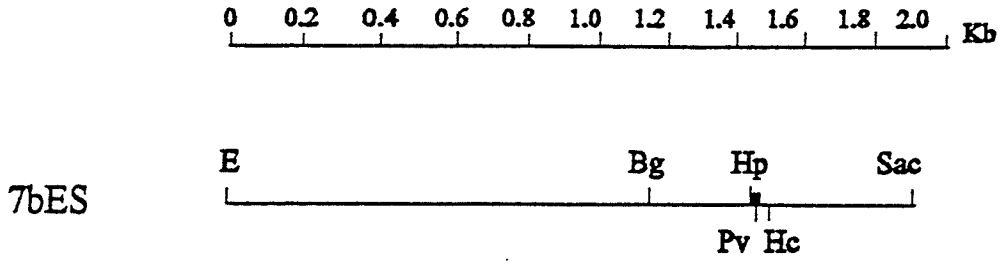
Figure 8 →

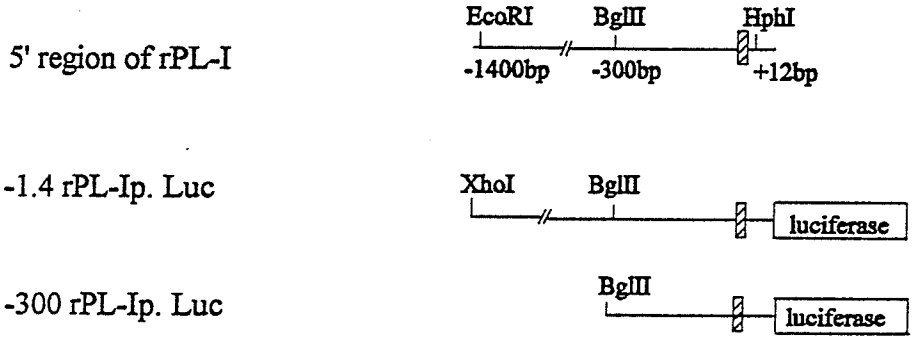
Fig.8 rPLI and rPL-II genomic clones and hybrid luciferase constructs.

On the top of A and B are the restriction enzyme maps of the initial genomic clones of rPL-I (7bES) and rPL-II (6.6 Kb rPL-II pVZ1). Hd = HindIII, Xb = XbaI, Bg = BglII, Sac = SacI, E = EcoRI, Pv = PvuII, Ev =EcoRV, Bs =BstNI, Ps = PstI, Sa = Sau3A, Rs = RsaI, Hc = Hinc II, Hp = HphI. The solid boxes represent exons.

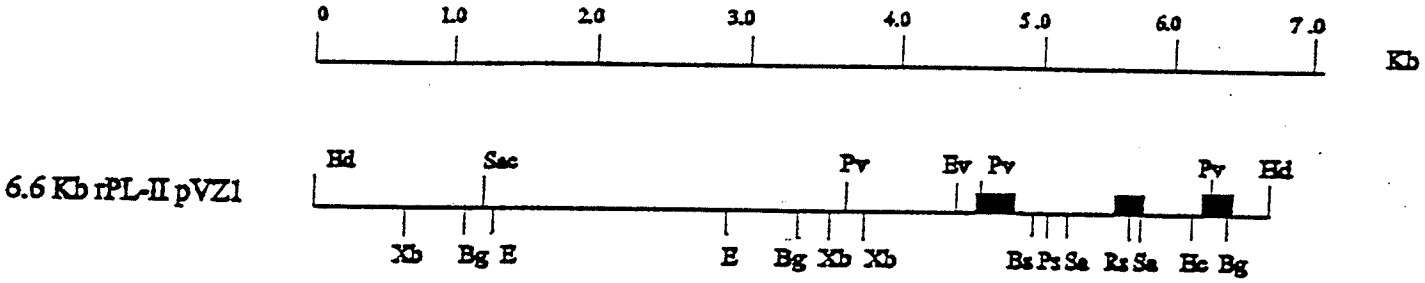
On the bottom of A and B are the maps of rPL-I and rPL-II luciferase constructs. The TATA box is represented by the hatched box. The transcription start site is at position +1. The two rPL-I constructs were -1.4 Kb and -300 bp to + 12 bp. The three rPL-II constructs were -4.5 Kb, -3.3 Kb and -900 bp to +66 bp.

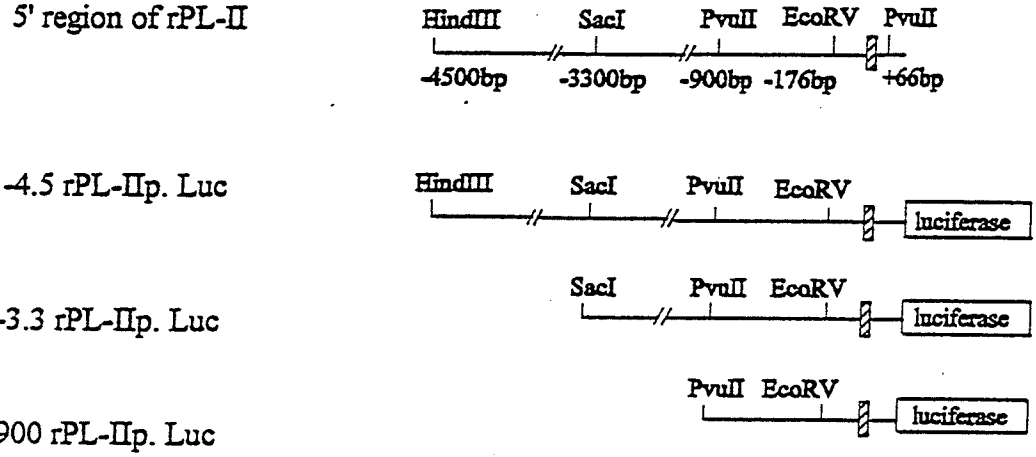
A


0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0 Kb
7bES
E
Bg
Hp
Sac
Pv Hc


5' region of rPL-I
EcoRI
BglII
HphI
-1400bp
-300bp
+12bp
-1.4 rPL-Ip. Luc
XhoI
BglII
luciferase
-300 rPL-Ip. Luc
BglII
luciferase

B


0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
Kb
6.6 Kb rPL-II pVZ1
Hd
Sac
Pv
Ev
Pv
Pv
Hd
Xb
Bg
E
E
Bg
Xb
Xb
Bs
Ps
Sa
Rs
Sa
Hc
Bg


5' region of rPL-II
HindIII
SacI
PvuII
EcoRV
PvuII
-4500bp
-3300bp
-900bp
-176bp
+66bp
-4.5 rPL-IIp. Luc
HindIII
SacI
PvuII
EcoRV
luciferase
-3.3 rPL-IIp. Luc
SacI
PvuII
EcoRV
luciferase
-900 rPL-IIp. Luc
PvuII
EcoRV
luciferase

2. A rPL-II 5' flanking HindIII/EcoRV fragment from approximately -4.5 Kb to -116 bp was ligated to the -900rPLIIpBsp clone cut with HindIII and EcoRV to form clone *-4.5rPLIIpBsp*.

A HindIII/BamHI fragment from *-4.5rPL-IIpBsp* was cloned into the HindIII and BglII sites of pXP2 to form **-4.5rPLIIp.Luc**.

A SacI/BamHI fragment from *-4.5rPL-IIBsp* was cloned into the SacI and BglII sites of pXP2 to form **-3.3rPLIIp.Luc**.

rPLP-A luciferase constructs

Vuille *et al.* (1993) tested the CAT activity of -4.6 Kb and -975 bp of rPLP-A 5'-flanking DNA in *cat* reporter constructs. The rPLP-A luciferase constructs used in this thesis were the same fragments subcloned into luciferase vectors (pXP1 or pXP2) (Dr.M.L.Duckworth).

4.2 Reporter gene activity of the hybrid plasmids in Rcho and GC cells

4.2.1 Transfection optimization

In Rcho cells:

There are reports in the literature of the use of both lipofection (Shida *et al.* 1992) and calcium phosphate (Vuille *et al.*1993) to transfect Rcho cells. Lipofection is considered to be more efficient than the calcium phosphate method (GIBCO). In the lipofection gene transfer procedure, DNA is incorporated into artificial lipid vesicles - liposomes, which fuse with the cell membrane, delivering their contents directly into the cytoplasm. Since the rPL-II expression was quite low, initially the lipofection method was employed. To optimize the transfection condition, different amounts of DNA (1 μg,

Table 3→

Table 3 A transfection optimization.

The Rcho cells were grown and maintained as described to day 14 after plating. CMVp. Luc was used as a optimization plasmid. The lipofection gene transfer was done in 35 mm plates according to GIBCO BRL instructions, and the calcium phosphate gene transfer was done in 10 cm plates according to the protocol in Howley *et al.*(1983). The data presented in Table 3A and 3B were from single plates. The calcium phosphate data (Table 3C) were from duplicate plates (mean ± SEM).

Table 3A Luciferase activity of different amounts of plasmid and lipofection reagents.

Amount of CMVp.Luc(μg) vs vol of reagents (μl)		Luciferase activity (light units/mg protein)×10^2 lipofectin	lipofectamine
1μg:	3	91	1180
	6	907	130880
	12	763464	1439090
	24	322572	3828000
2μg:	3	218	480
	6		5470
	12	4400	3227350
	24	4176940	5171220
4μg:	3	140	2060
	6	12190	680
	12	530	470630
	24	32860	2792310

Table 3B The luciferase activity of 2 μg of plasmid with different volumes of lipofection reagents

Vol of reagents(μl)	Luciferase activity (light units/mg protein)×10^2	
	lipofectin	lipofectamine
10	6	2303
12	16	7155
14	63	1146
16	85	5636
18	212	22526
20	363	23407
22	421	14736
24	129	22216

Table 3C The luciferase activity of different amounts of CMVp.Luc by calcium phosphate method

Amounts of CMVp.Luc (μg/plate)	Luciferase activity (light units/mg protein) ×10^6
5	10.9 ± 1.4
10	103.9 ± 16.5
15	40.4 ± 5.3

2 μg and 4 μg of supercoiled CMVp.Luc) and different volumes of lipofectin or lipofectamine (3, 6, 12 and 24 μl) were used in 35 mm plates. The luciferase activity results showed that lipofectamine gave higher luciferase activity than lipofectin (Table 3), suggesting better transfection efficiencies. Two μg of DNA with 24μl of lipofectamine appeared to be optimal (Table 3A). A further test of 2 μg of DNA with 10, 12, 14, 16, 18, 20, 22 and 24 μl of lipofectamine, showed that 2 μg of DNA and 20 μl of lipofectamine appeared to be optimal (Table 3B). There was no transfection efficiency control in above preliminary experiments and the CMV.Luc DNA used in the two experiments was not from the same preparation. The differences between the two experiments in Table 3A and 3B might due to the different quality of DNA resulting in different plasmid uptake.

The calcium phosphate method was also tested. The method was as Howley *et al.* (1983). For optimization, 5 μg, 10 μg and 15 μg of CMVp.Luc were transferred into 10 cm plate Rcho cultures. The results indicated that 10 μg of DNA in a 10 cm plate was optimal (Table 3C). Since our results using the calcium phosphate method gave similar transfection efficiency as the lipofection method, all the transfection studies described below were done using the calcium phosphate method with 10μg of DNA per 10 cm plate.

GC cells:

The rat pituitary GC cell line was used in this study as a non-placental control cell line to study tissue specific expression of rat prolactin-like proteins. These cells are able to be transfected by calcium phosphate method (Dr. P.Cattini's pers. comm.). In this

study, GC cells were tested with 10 μg CMVp.Luc by the calcium phosphate method (Howley *et al.* 1983). The GC cells were grown to 40% to 50% confluence. One group was given a 24 h gene transfer, then 24 h for gene expression; the other group was given a 6 h gene transfer, a 20% glycerol shock for 2 min, and 48 h for gene expression. The luciferase activity of the glycerol shocked group was approximately 3.5 fold higher than the non shocked group. This glycerol shock protocol was used in all GC transfections.

4.2.2 Comparison of reporter activity of different constructs

To identify the 5'-flanking regions of rPL-I, rPL-II and rPLP-A genes that contain potential placental specific expression elements, Rcho cells were routinely cultured for 14 days after plating; GC cells were grown to 40% to 50% confluence usually 2 days after plating. Ten μg each of rPL-I, rPL-II and rPLP-A 5'-flanking DNA luciferase plasmids were transiently transfected into Rcho and GC cells. The luciferase vectors pXP1/pXP2 (-p.Luc) were used as promoterless controls and CMVp.Luc. was used as a positive control. All the samples were co-transfected with 1 μg of CMVp.cat as a plasmid uptake control. Data are shown in Table 4. In Rcho cells both the -1.4 Kb and -300 bp rPL-I constructs showed increased luciferase activity of approximately 100 fold ($p<0.001$); the -4.5Kb and -3.3Kb rPL-II constructs showed increased luciferase activity of approximately 2.5 fold ($p<0.05$); the -4.6 Kb and -975 bp rPLP-A constructs showed increased luciferase activity 386 and 116 fold ($P<0.001$), compared with promoterless controls. In GC cells none of these constructs showed significant luciferase activity compared with promoterless controls. Since the expression of rPL-II was much lower than rPL-I in Rcho cultures, it

Table 4 →

Table 4 Hybrid rPL-I, rPL-II and rPLP-A expression in Rcho and GC cells.

The Rcho cells and GC cells were transiently transfected using calcium phosphate with rPL-I, rPL-II and rPLP-A 5'-flanking DNA constructs, promoterless control pXP1/pXP2 (-p.Luc) or the positive control CMVp.Luc. 10 μg of plasmid DNA were used for each 10 cm plate. Promoter activity is represented as luciferase activity in light units/mg cytoplasmic proteins (mean ± SEM). Each value is from a pool of two separate experiments; total sample numbers were 6 to 8. All values were corrected for plasmid uptake by cotransfection with CMVp.cat (1μg per plate).

Table 4 Hybrid rPL-I, rPL-II and rPLP-A expression in Rcho and GC cells.

Hybrid gene constructs	Luciferase Activity (light units/ mg protein)×10^2	
	In Rcho cells	In GC cells
-p. Luc	8.3 ± 1.0	1.8 ± 0.3
-1.4rPL-Ip. Luc	807.0 ± 118.0	5.0 ± 0.2
-300rPL-Ip. Luc	1,050.0 ± 201.0	3.8 ± 0.9
CMVp. Luc	172,000.0 ± 29,700.0	24,200.0 ± 4,080.0
non-transfected cells	4.9 ± 0.7	3.3 ± 0.9
-p. Luc	36.0 ± 8.8	13.0 ± 4.1
-4.5rPL-IIp. Luc	107.0 ± 12.0	21.0 ± 6.0
-3.3rPL-IIp. Luc	82.0 ± 15.0	4.3 ± 1.9
-900rPL-IIp. Luc	13.0 ± 2.8	3.9 ± 1.8
CMVp. Luc	327,000.0 ± 28,500.0	16,900.0 ± 2,450.0
-p. Luc	8.3 ± 1.0	1.8 ± 0.3
-4.6rPLP-Ap. Luc	3203.7 ± 360.9	3.5 ± 0.8
-975rPLP-Ap. Luc	964.6 ± 476.4	5.5 ± 1.5
CMVp. Luc	172,000.0 ± 29,700.0	24,200.0 ± 4,080.0
non-transfected cells	4.9 ± 0.7	3.3 ± 0.9

was not perhaps unexpected that the luciferase activity of the hybrid rPL-II plasmids was lower than that of hybrid rPL-I plasmids. No significant luciferase activity was detected with the -900 bp rPL-II construct, which correlates with the published data using the same fragment with *the cat* reporter gene (Vuille *et al.* 1993).

Overall, the data suggest that the -300 bp 5'-flanking region of rPL-I appears to contain enough information to specify placental specific expression. Placental specific regulatory element (s) of rPL-II might be located between -3.3 Kb and -900 bp of the 5'-flanking region of rPL-II. The -975 bp 5'-flanking region of rPLP-A is capable of determining placental specific expression, but there might be enhancer(s) between -4.6 Kb and -975 bp of 5'-flanking region.

5. TGFα EFFECTS ON HYBRID rPL-I and rPL-II PLASMIDS

To address the mechanisms by which TGFα could be having an effect on rat placental lactogen mRNA levels, further experiments were carried out to assess the potential roles of gene transcription and mRNA stability.

The rPL-I and rPL-II luciferase constructs that showed expression in Rcho cells were transfected into day 14 Rcho cultures as usual. During the gene expression period, the cells were given SF-NCTC with and without TGFα (10 ng/ml). Cells were harvested 48 hours after the start of gene transfer. Luciferase, CAT and protein assay were performed. TGFα increased luciferase activity of both rPL-I 5'-flanking constructs (-1.4 Kb and -300 bp) by approximately 2 fold ($p<0.001$) and both 5'-flanking rPL-II constructs (-4.5 Kb and -3.3 Kb) by approximately 5 fold ($p<0.05$) compared with the control group

Table 5 →

Table 5 TGFα effects on hybrid rPL-I and rPL-II gene promoter activity.

The constructs that showed expression in the Rcho cells (-1.4 Kb and -300 bp rPL-I, -4.5kb and -3.3Kb rPL-II) were transiently transfected into Rcho cells asusual. During gene expression cells were given TGFα (10 ng/ml) in SF-NCTC 135. The control cultures were given equal volume of PBS/BSA in SF-NCTC135 medium. Cells were harvested 48 hours after transfection. The luciferase light units were corrected for transfection efficiency by co-transfection of CMVp.*cat* and measurement of CAT activity. Each value (mean ± SEM) is from a pool of two (rPL-II) or three (rPL-I) separate experiments; total sample numbers are 6 to 12.

To test for potential effect on luciferase mRNA or protein, CMVp.Luc and pT81Luc were tested with TGFα. TGFα (10 ng/ml) did not show any significant effect on luciferase activity of CMVp.Luc. and pT81Luc at either 5 or 10 µg/10 cm plate compared with minus TGFα control ($p>0.05$). The data (mean ± SEM) were from the duplicate plates.

Table 5 TGFα effects on hybrid rPL-I and rPL-II gene promoter activity

Hybrid gene constructs	Luciferase Activity (light units/ mg protein)×10^2	
	Control	TGFα
-p. Luc	22.0 ± 8.1	11.0 ± 1.7
-1.4 rPL-Ip. Luc	1,060.0 ± 191.0	2,360.0 ± 289.0
-300rPL-Ip. Luc	741.0 ± 75.0	1,480.0 ± 152.0
-p. Luc	0	0
-4.5rPL-IIp. Luc	37.0 ± 7.3	210.0 ± 55.0
-3.3rPL-IIp. Luc	83.0 ± 28.0	390.0 ± 96.0
-p.Luc	0	0
CMVp.Luc (5μg/plate)	34,430 ± 21,500	84,500 ± 27,210
(10μg/plate)	78,870 ± 34,120	59,110 ± 17,770
pT81Luc (5μg/plate)	0	4.5 ± 4.5
(10μg/plate)	7.1 ± 5.7	4.1 ± 4.1

(Table 5). The data also indicate that the effect of TGFα could be detected in 24 h.

To eliminate the possibility that TGFα was having its effects on luciferase mRNA and/or luciferase protein stability, TGFα effects on the luciferase activity of Rcho cells transfected with CMVp.Luc and pT81Luc were tested. CMVp.Luc contains the strong cytomegalovirus promoter, while pT81Luc has only a minimal 81 bp herpes simplex I thymidine kinase promoter, with no additional regulatory elements. Any changes in luciferase activity expressed by pT81Luc in the presence of TGFα would suggest effects on luciferase mRNA or protein. The data in Table 5 indicate that TGFα (10 ng/ml) did not have any significant effect on either CMVp.Luc or pT81Luc. luciferase activity as compared with a control without TGFα ($p>0.05$). This suggests that TGFα has no direct effect on the stability of the reporter gene luciferase mRNA or protein.

Taken together, the effects of TGFα on rat placental lactogen genes and viral luciferase constructs, suggest that the effects of TGFα on rPL-I and rPL-II mRNA expression are, at least in part, at the level of gene transcription.

DISCUSSION

DISCUSSION

The results in this thesis have confirmed that Rcho cells express trophoblast giant cell specific proteins - rPL-I, rPL-II and rPLP-A in specific temporal patterns (Figure 3. and Hamlin *et al.* 1994) which indicates this cell line is a rich source of placental specific transcription factors. The rPL-II has been shown mainly to be expressed during the second half of the experimental period (Figure 3) which is similar to that seen in the rat placenta in which rPL-II is exclusively expressed during the second half of the pregnancy (Duckworth *et al.* 1993). Furthermore it has been seen that the temporal expression pattern of rat EGF receptors in Rcho cells is similar to that seen in mouse placenta (Figure 7). This evidence demonstrates that the Rcho cell line is a valuable system in which to study the trophoblast giant cell mRNA expression. The observation that high cell density and the absence of growth stimulation (deceasing serum) facilitates trophoblast giant cell formation suggests that the Rcho cell line is a controllable *in vitro* model. The transfection data demonstrate that the Rcho cell line is a transfectable cell system for the identification of the *cis*-acting sequences responsible for placental specific expression of these genes, and a potentially rich source of the *trans*-acting protein factors which bind to these sequences.

Like many *in vitro* model systems, however, the Rcho cell line also has its limitations. In Rcho cultures, rPL-I and rPLP-A were expressed at high or low levels throughout the entire culture period (Figure 3), which is unlike the placenta where the expression of rPL-I and rPLP-A does not overlap - rPL-I is only expressed from day 7 to day 13 and rPLP-A is only expressed from day 14 to term exclusively. Rcho cells

express all rat PRL family of proteins expressed by giant cells (Hamlin *et al.* 1994), but there is no expression of spongiotrophoblast specific rPLP-B in Rcho cells (Duckworth *et al.* 1983), which indicates that the small cells in Rcho culture are not spongiotrophoblast cells. Taken together, these data suggest that the Rcho cell cells do not have the potential to develop into spongiotrophoblasts, but are already committed to a giant cell identity.

In rat placenta, there is a switch in expression between rPL-I and rPL-II around midpregnancy - rPL-I is expressed from day 7 to day 13, and rPL-II from day 11 to term (Duckworth *et al.* 1993). The expression patterns of these proteins in Rcho cells showed similar kinetics of expression to that seen in the rat placenta. Rat PL-I mRNA was expressed as soon as giant cells differentiated in the cultures (day2) and decreased after day 22, while significant levels of rPL-II were barely detectable until about day 14 and were maintained at least to day 32 (the termination of the cultures (Figure 3). This shift *in vitro* suggests that earlier Rcho cell cultures may contain some positive transcriptional regulators for rPL-I but no sufficient levels of positive transcriptional regulators for rPL-II, and the opposite condition in the later cultures.

Yamaguchi *et al.* (1992) have reported that EGF stimulates mPL-I secretion and inhibits mPL-II secretion in primary mouse placental cultures. In our growth factor experiment, TGFα increased the expression of rPL-I mRNA levels but decreased rPL-II mRNA levels (Figure 5), which is similar to the reported data, suggesting that the differences of EGF on secretion could be at least partly due to effects on mRNA levels. The opposite effects of TGFα on rPL-I and rPL-II data suggest that TGFα may play a

role in the switch of rPL-I and rPL-II gene expression at midpregnancy. TGFα mRNA is present in rat decidua where the highest concentration is in the region adjacent to the embryo, and is lower or undetectable in placenta (Han *et al.* 1987). EGF receptors, to which TGFα binds, are expressed in mouse placenta (Adamson & Meek 1984). In humans it has been shown that they are expressed in syncytiotrophoblasts which are the source of placental lactogen (Maruo *et al.* 1987). Both TGFα and its receptors are present in the uteroplacental units, suggesting that TGFα might affect rat placenta by a paracrine mechanism.

EGF and TGFα are members of the EGF family that bind to the same EGF receptor (Pike *et al.* 1982). Theoretically EGF should have the same effects as TGFα, but we observed the human TGFα but not mouse EGF have significant effects on rat PL mRNAs. This inconsistency may be a result of the differences of EGF or EGF receptors among the species (human versus mouse versus rat) or in the methods employed *(in vitro* and *in vivo*). In this study the human TGFα and mouse EGF were used. The protein sequences of human TGFα and rat TGFα share 96% homology (Zurfluh *et al.* 1990), but the protein sequences of mouse EGF and rat EGF share only 77% homology (Simpson *et al.* 1985). The lower homology of mouse EGF to rat EGF might result in the ineffective binding of mouse EGF to the rat EGF receptor. There are reports from others which also showed an inconsistency. It has been reported that EGF but not TGFα may be involved in the estrogen-induced cell growth *in vitro* (Adachi *et al.* 1995), and there is evidence that the binding affinity of EGF and TGFα to EGF receptors is differentially affected by a low pH environment. It has been speculated that the ligands of the EGF

family, which are internalized bound to the EGF receptor, undergo different intracellular processing that may account for differences in cellular response (French *et al.* 1995).

The data in this thesis show that TGFα positively regulates rPL-I mRNA and negatively regulates rPL-II mRNA expression in Rcho cells (Figure 5). The level of control could be at the level of gene transcription and/or mRNA stability. In the investigation of the molecular mechanisms of TGFα effects, it was demonstrated that TGFα influenced the promoter activity of rPL-I and rPL-II genes; it was also shown that TGFα had no significant effect on the luciferase activity of viral promoter luciferase constructs, suggesting that TGFα has no effect on the luciferase protein itself (Table 5). These data together suggest that TGFα regulates rat placental lactogen genes, at least in part, at the level of gene transcription. The effect of TGFα on rPL-I mRNA expression (3 fold increase) correlates with the effect on the promoter activity of a hybrid rPL-I reporter constructs (2 fold increase), suggesting that the effect of TGFα on rPL-I expression could be at the level of transcription. The effect of TGFα on rPL-II mRNA expression (2 fold decrease) is opposite to that on promoter activity of hybrid rPL-II reporter constructs (5 fold increase), suggesting that the effect of TGFα on rPL-II expression could be more complex. One important point here is that, in the effects of TGFα on mRNA levels, the effect of TGFα on the entire endogenous PL gene was detected; but in the promoter activity analysis the effects of TGFα on a limited 5'-flanking fragment were tested. These 5'-flanking fragments might not contain all the regulatory elements.

For further studies of the regulation of TGFα of the rat PRL family of genes, the following approaches could be considered. A transcription assay (nuclear run on) and further mRNA stability studies would help us come to a better understanding of the molecular mechanisms of TGFα action on rat placental lactogen genes. Structural studies (such as gel-shifts, footprinting and further sequence analysis) could lead to the identification of TGFα response element(s).

The transfection data indicated that the -300 bp 5'-flanking region of rPL-I, -3.3 Kb 5'-flanking region of rPL-II and -975 bp 5'-flanking region of rPLP-A are sufficient to specify placental specific expression in Rcho cells (Table 4). The rPL-I result is similar to that seen in the mouse where -274 bp of mPL-I 5'-flanking DNA was showed to be sufficient to direct the placental specific expression (Shida *et al.* 1993) . In our study the -900 bp of rPL-II 5'-flanking DNA is shown not to have enough information to specify placental specific expression, which correlates with the published data using the same fragment with *a cat* reporter gene (Vuille *et al.* 1993). The luciferase reporter that was used is considered to be more sensitive than the *cat* reporter gene, but we were still not able to detect any activity. These data indicate that the element(s) required for placental trophoblast giant cell expression might be located within the -3.3 Kb to -900 bp of rPL-II gene 5'-flanking region. This is similar to that seen in the mouse where the elements to specify trophoblast giant cell expression of mPL-II are located within the -2700 bp to -569 bp 5'-flanking fragment of mouse PL-II (Shida *et al.* 1992). Shida *et al.* (1992) were not able to detect CAT activity of a -2.7 Kb rPL-II.*cat* construct in Rcho cells. Our data showed that 2.5 fold luciferase activity increase of the -4.5 Kb and -3.3

Kb rPL-II luciferase constructs. Considering the luciferase reporter is more sensitive than the *cat* reporter, the CAT assay might not be sensitive enough to detect the low activity. A previous study using a *cat* reporter showed that -975 bp of rPLP-A 5'-flanking region is sufficient to specify placental specific expression although a construct containing -4.6 Kb of 5'-flanking gene gave higher expression levels (Vuille *et al.*1993). The same results were shown in our experiments with the luciferase reporter constructs.

In the mouse within the -274 bp to +1 of mPL-I 5'-flanking DNA, two AP-1 and GATA-2 and GATA-3 binding sites were shown to be required for maximal expression (Shida *et al.* 1993, Ng *et al.* 1994). In a transgenic mouse model, GATA-3 was also shown to be involved in the expression of mPL-I (Ma et al. 1995). Dr.M.C.Robertson has sequenced -620 to +100 bp of rPL-I (unpublished data), Dr.M.L.Duckworth and the former students in our laboratory P.Shah, and N.Quan have analyzed -758 to +65 bp of rPL-II (unpublished data). There are two AP-1 sites in the -300 bp 5'-flanking region of rPL-I and at least one AP-1 site in the -620 bp 5'-flanking region of rPL-II at the same locations as mouse, which may play an important role in placental specific expression of rPL-I and/or rPL-II. AP-1 sites are the targets of Jun-Jun homodimers and Jun-Fos heterodimers (Curran & Franza 1988). The AP-1 transcription factors are considered immediate response genes and are thought to be involved in a wide range of transcriptional regulatory processes linked to cellular proliferation and differentiation (Johnson *et al.* 1993). Many growth factors (including EGF) are thought to participate in a signalling cascade affecting the AP-1 complex composed of the Jun and Fos proteins. TGFα might act on rPL-I by interacting with Jun/Fos complex at AP-1 sites. In order to

determine whether AP-1 sites are the only sequences required for the TGFα effects, structural analyses (such as gel-shifts or footprinting) will need to be done.

Differentiation of the extraembryonic trophoblast trophectoderm into the various trophoblast cell types is expected to involve a coordinated program of gene expression dictated by trophoblast specific transcription factors or trophoblast-specific combinations of transcription factors. Recently it has been reported that the basic-helix-loop-helix transcription factors Mash-2 and Hxt are strongly expressed in the extraembryonic trophoblast lineage. In mouse, Mash-2 is found throughout preimplantation development, but is highly expressed later only in the ectoplacental cone, the chorion and their derivatives in the placenta. Mash-2 $^{-/-}$ embryos die from placental failure at 10 days post-coitum. In mutant placentaes, spongiotrophoblast cells and their precursors are absent while giant cells are still present (Guillemot *et al.* 1994). Hxt is preferentially expressed in extraembryonic cells that become the placenta (Cross *et al.* 1994a). Overexpression of Hxt in Rcho-1 trophoblast cells induced their differentiation, whereas the HLH negative regulator Id-1 was inhibitory (Cross *et al.* 1994a). Genetic evidence indicates that basic-helix-loop-helix (bHLH) transcription factors function as cell-lineage determinants in skeletal muscle development in mammals (MyoD, myogenin, mrf-4, myf-5, etc.) and in mesoderm and neuronal cell differentiation in Drosophila (achaete-scute) (Olson 1990 & 1992, Jan and Jan 1993). For example, myogenin is a skeletal muscle-specific transcription factor that can activate myogenesis when introduced into a variety of nonmuscle cell types. Myogenin shares homology with MyoD and other myogenic regulatory factors within a basic region and a HLH motif that mediate DNA

binding and dimerization, respectively (Schwarz *et al.* 1992). Cell specific bHLH factors have also been identified in other mammalian cell types, whereas it is likely they function as regulators of lineage commitment and differentiation. The evidence described above suggesting that bHLH proteins Mash-2 and Hxt are related to trophoblast cell differentiation poses the question whether they might be important transcriptional regulators of the rat placental prolactin family of genes.

For further characterization of the *cis*-acting element(s) of the rat PRL family of genes, more detailed functional studies (such as transfection), to narrow down the 5'-flanking sequences involved in placental specific expression, and mutations or deletions would provide more information about the tissue specific genetic elements. The structural studies (such as gel-shifts, footprinting and further sequence analysis) would assist in the identification of transcription factors that are involved in trophoblast gene expression and their binding sites. A gel-shift survey with the trophoblast cell lineage related transcription factors like Mash-2 and Hxt might provide some information of the possible involvement of these transcription factors in the of expression of the placental specific rat PRL family of genes. Further, footprinting studies and sequence analysis could be used to identify the *cis*-acting elements. The sequence information of the *cis*-acting elements could lead to identification of the binding transcription factor. Once the transcription factor has been identified, functional studies could be approached by developing a transcription factor transfected Rcho cell line, then studying the expression of endogenous gene or 5'-flanking reporter constructs of rat PRL family proteins. The level of expression of rat PRL genes might be different between the transfected cell line and no-transfected

Rcho cells. Transcription factor deficient transgenic mouse models could be considered as an *in vivo* model system to examine the role of the placental specific transcription factors.

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