IDENTIFICATION AND CHARACTERIZATION OF CIS- AND TRANS-ACTING FACTORS INVOLVED IN THE EXPRESSION OF THE RAT PLACENTAL LACTOGEN II GENE

BY

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Yuxiang Sun

A Thesis/Practicum 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 loving parents Mr. Zhaoli Sun / Mrs. Fenglan Li,

and

my dear daughter Grace Sun

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If I have seen further, it is by standing of the shoulders of Giants.

Sir Isaac Newton

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ABSTRACT

The rat and mouse placental lactogen I and II genes (PLI and PLII) are members of a large family of prolactin-related proteins that are expressed in a developmentally specific manner by the placenta during pregnancy. There is an intriguing switch in expression between PL-I and PL-II at midpregnancy; the factors that control this switch are unknown. To understand the mechanisms of the switch, we need to understand the regulation of each of these genes. PLI and PLII are expressed in the placental trophoblast giant cells according to different but overlapping developmental patterns, suggesting there may be both common and unique factors which regulate the expression of these genes. Members of the GATA and AP-1 (Jun/Fos) families of transcription factors have been shown to be important for high level expression of the mouse PLI gene. DNA binding sites for these transcription factors are conserved in the 5' flanking region of the rat PLI gene (rPLI), suggesting a similar regulation. Little is known about the regulation of PLII gene. The work in this thesis focuses on the molecular mechanisms of rat PLII (rPLII) gene regulation.

The rat choriocarcinoma cell line, Rcho, which differentiates in culture into the trophoblast giant cell type, has proven to be a good model system for studying gene expression in this cell. Differentiated Rcho cells express rPLII, as well as rPLI and some other members of the prolactin gene family. This cell line was used extensively for our rPLII gene regulation studies.

Transfection studies with the luciferase reporter gene indicated a 3031 bp rPLII 5' flanking sequence DNA is active in the Rcho cell line, but not in the non-placental pituitary GC cell line. Transgenic mouse studies also indicated that this fragment was efficient in targeting reporter gene expression to the placenta *in vivo*. Deletion analysis of this 3031 bp 5' flanking fragment showed that the proximal 1435 bp of rPLII 5' flanking DNA is required for minimal

expression of the reporter gene in Rcho cells. Two negative regulatory regions were identified between -3031 to -2838 and -1729 and -1435. Most importantly, a fragment between -2838 to -1729 (an EcoRI fragment) appeared to be essential for maintaining the level of expression seen with the entire 3031 bp rPLII 5' flanking DNA. This region was shown to contain placental-specific enhancing sequences that functioned in both Rcho and human choriocarcinoma cell lines, but had no activity in pituitary GC cells. Further detailed deletion analysis of this EcoRI fragment using a heterologous thymidine kinase (TK) promoter localized a 65 bp enhancer region between -1794 to -1729.

DNAse I protection analysis of this 65 bp enhancer fragment identified two regions which were protected by placental and Rcho nuclear extracts from DNAse I digestion, but not by GC nuclear extracts. Sequence analysis showed that the two protected regions corresponded to putative binding sites for the Ets and AP-1 families of transcription factors. Site-directed mutagenesis of the individual binding sites led to a partial loss of enhancing activity; a double Ets/AP-1 mutation led to a complete loss of activity. Electrophoretic mobility shift assays (EMSA) with nuclear extracts and *in vitro* translated proteins further showed that c-Jun and c-Fos, bound the AP-1 site as heterodimers. The Ets family consists of at least 35 members. Since Ets1 and Ets2 had previously been shown to be involved in trophoblast gene regulation, their expression in placenta and Rcho cells was tested. Northern analysis detected Ets2 but not Ets1 mRNA in both rat placenta and Rcho cells. Although overexpression of Ets2 in Rcho cells increased the luciferase activity of the enhancer, Ets2 did not bind the rPLII Ets sequence in EMSA, suggesting that another Ets member may responsible for direct effects, while Ets2 may affect the enhancer function indirectly. Unlike the PLI gene, two putative GATA sites on the rPLII-enhancing fragment were shown not to be in functional in Rcho cells by mutagenesis

studies in combination with gene transfer. These data suggested that, even though PLI and PLII are expressed by the same placental cell type, the molecular mechanisms of gene regulation may be quite different.

This study is the first to identify a specific regulatory element on the rPLII gene. The identification of the Ets family member that interacts with the rPLII enhancer and investigation of other potential regulatory elements on the rPLII gene will lead to a better understanding of the genetic factors involved in complete developmental regulation of rPLII.

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LIST OF ABBREVIATIONS

Units of measure

cm	centimeter
min	minute
h	hour
^0C	degrees centigrade
µl	microliter
ml	milliliter
ng	nanogram
µg	microgram
mg	milligram
µM	micromolar
mM	millimolar
cpm	counts per minute
rpm	revolutions per minute
v/v	volume per volume
bp	base pairs
Kb	kilobases
µCi	microCurie

Reagents and media

BSA	bovine serum albumin
FBS	fetal bovine serum
CsCl	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
DTT	dithiothreitol
PMSF	phenylmethylsulfonylfluoride

Proteins

PRL	prolactin
PL	placental lactogen
CAT	chloramphenicol acetyltransferase
Luc	luciferase
AP-1	activator protein-1

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
dNTP	deoxyribonucleotide triphosphate
G3PD	Glyceraldehyde-3-phosphate dehydrogenase
CMV	cytomegalovirus
TK	herpes simplex I thymidine kinase
SF	serum free
OD	optical density
UV	ultraviolet
PCR	polymerase chain reaction
pcv	packed cell volume

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. A mammalian embryo cannot develop without the placenta. The placenta undergoes dramatic structural reorganization during pregnancy in order to match functionally the requirements of the embryonic/fetal and maternal development (Ohlsson 1989, Cross et al. 1994).

1. RODENT PLACENTA

1.1 Structure and development

In rodents, the egg, after fertilization in the oviduct, undergoes a series of symmetrical cell divisions to create a mass of 2, 4 and 8 cells called blastomeres, enclosed within a specialized membrane, the *zona pellucida*. As cleavage proceeds to the 16 cell stage, compaction takes place - blastomeres become flattened and cell-cell contact increases. The formation of the blastocyst is the first differentiation event that occurs after compaction of the morula, resulting in the generation of two distinct lineages the tropectoderm and inner cell mass. Cells in the outer layer of the blastocyst, called the trophectoderm, become distinguishable from the undifferentiated embryonic stem cells in the inner cell mass (ICM). Trophectoderm cells are the progenitors of the trophoblast cells in the placenta, and development of the trophoblast cell lineage is the first differentiation event during mammalian embryogenesis. The inner cell mass goes on to differentiate mainly into the embryo proper and the allantoic placental structures (Gardner 1983, Rossant et al. 1986).

Implantation occurs soon after the blastocyst hatches from the *zona pellucida* (day 5

in rat; day 0 of gestation is when sperm is detected in the vagina). During 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 stroma cells termed "decidualization" (DeFeo 1967). After implantation, the polar trophectoderm located at the implantation site proliferates to form the ectoplacental cone, which is the primary structure of placenta. Embryo development doesn't begin until the first placental structures have formed (Davies and Glasser 1968).

A schematic diagram of placental trophoblast cells at different developmental stages during pregnancy in the rat is shown in Figure 1. The rodent possesses two placental structures at different times during pregnancy - the choriovitelline placenta and the chorioallantoic placenta. The choriovitelline placenta surrounding the early embryo develops first, and is associated with decidual cells termed the *decidua capsularis*. The choriovitelline placenta is comprised of a layer of mural trophoblast giant cells adherent to a prominent basement membrane, called Reichert's membrane (Davies and Glasser 1968, Soares et al. 1991).

The choriovitelline placenta eventually degenerates and is efficiently replaced by the chorioallantoic placenta at midpregnancy. The chorioallantoic placenta develops from polar trophoblast giant cells of the ectoplacental cone and chorioallantoic mesoderm from the ICM. The outermost trophoblasts of the ectoplacental cone differentiate into secondary trophoblast giant cells which lie on the outer edge of the placenta, forming the interface with maternal cells of the *decidua basalis* (Davies and Glasser 1968).

The mature rodent chorioallantoic placenta consists of two well-defined zones: the junctional or basal zone and the labyrinth zone. 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 the maternal blood supply while the labyrinth zone is supplied by both maternal and fetal blood supplies. The labyrinth is the location of maternal-fetal nutrient exchange(Davies and Glasser 1968, Soares et al. 1991). This mature placental structure continues to grow and develop throughout the second half of the pregnancy.

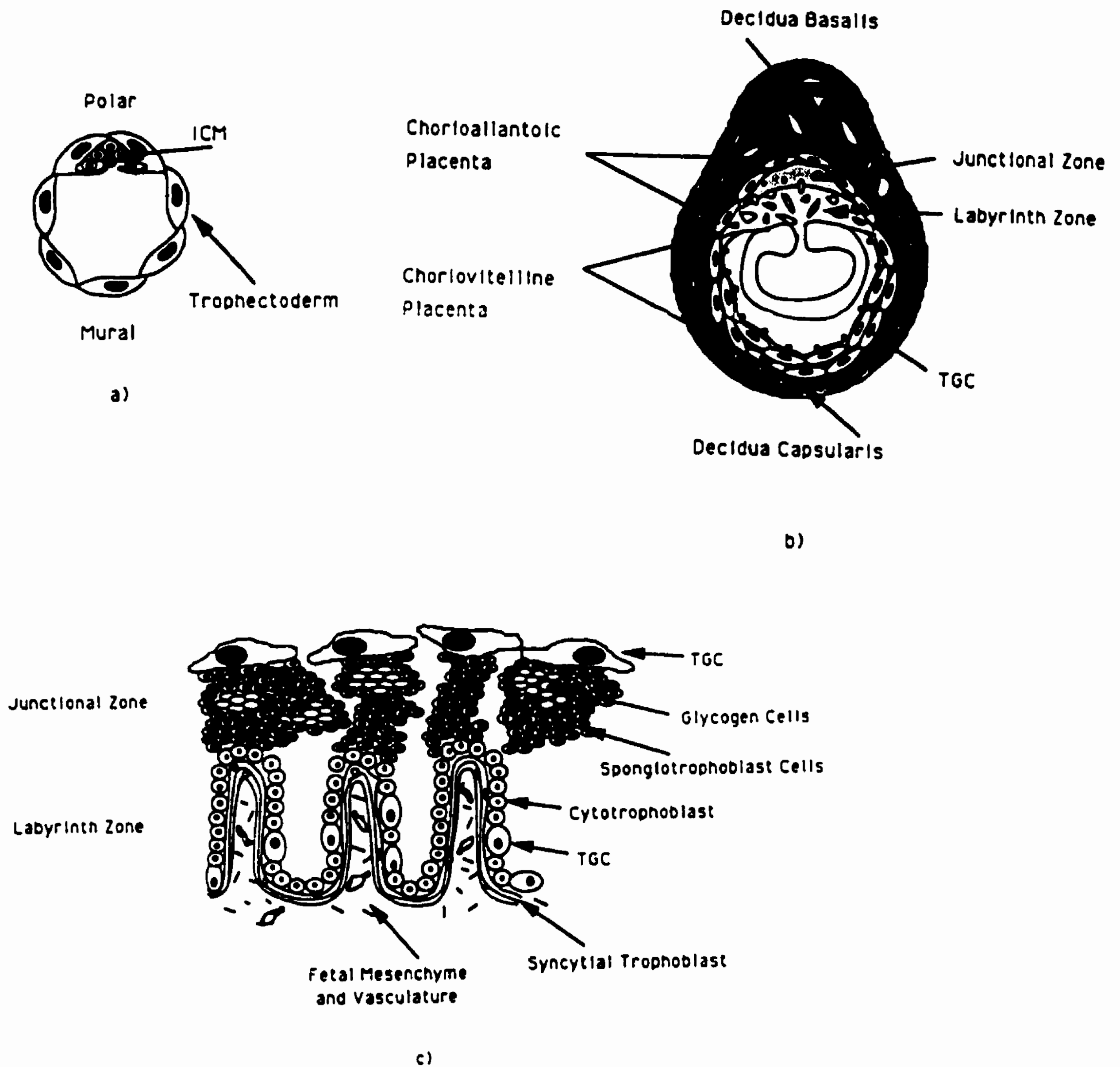


Fig. 1 Schematic diagrams representing the organization of trophoblast cells at three different developmental phases during pregnancy in the rat. a) blastocyst; b) midgestation conceptus; c) portion of late gestation chorioallantoic placenta. ICM, Inner cell mass; TGC trophoblast giant cell (Soares et al. 1991)

1.2 Clinical significance of placental defects

The placenta establishes functional connections that are critical for embryonic survival. Implantation and development of the placenta occur in a stepwise manner; a defect at any step can be critical for embryonic survival (Cross et al. 1994).

The structure of the human placenta is different from that of rodent placenta. The trophoblast invasion of human placenta is much more significant than in rodents (Strickland and Richards 1992). After implantation of the blastocyst on the wall of uterus, trophoblast cells begin to form small, fingerlike projections called microvilli, which extend into the maternal endometrium. As the invasion proceeds, the trophoblast cells differentiate into two layers: the outer layer of fused multinucleated syncytiotrophoblast cells, which lead the advance into the endometrium, and the inner layer of flat cytotrophoblast cells, that form a complex system of projections which eventually push through the syncytiotrophoblast layer into the pools of maternal blood. Trophoblasts anchor the conceptus to the decidua and tap a blood supply from maternal blood (Beaconsfield et al. 1980).

It has been estimated that about one-third of normal human pregnancies end in spontaneous abortion, some at very early developmental stages. More than twenty percent of such abortions are estimated to occur before pregnancy is detected (Wilco et al. 1988). A defect in placental vascular connections results in preeclampsia, a disease of pregnancy with significant morbidity and mortality to both mother and fetus (Friedman et al. 1991, Roberts et al. 1991). Currently, the approaches for diagnosis and treatment of diseases of pregnancy are limited because of our limited understanding of placental development. It is very difficult to study this developmental process in humans. Recent discoveries from targeted mutagenesis of mouse genes may give some general insights into placental development.

There are several mouse models that affected at different stages of placental development.

1.3 Mouse models of placental defects

1.3.1 Implantation

The uterus undergoes dramatic developmental changes during the preimplantation period. Uterine changes are controlled by the ovarian steroid hormones estrogen and progesterone (Beaconsfield et al. 1980, White et al. 1994). Estrogen triggers uterine epithelium to secrete leukemia inhibitory growth factor (LIF) (Shen and Leder 1991, Stewart et al. 1992). LIF appears to have an essential role in triggering events required to initiate implantation. In LIF null mice normal embryos fail to implant (Stewart et al. 1992).

1.3.2 Trophoblast invasion

Trophoblast invasion anchors the placenta to the uterine wall. Trophoblasts traverse the uterine epithelium and invade the decidua, the inner section of the myometrium and the maternal arteries to effect successful implantation (Strickland and Richards 1992). Trophoblasts produce proteinases that degrade the extracelluar matrix (ECM). For example metalloproteinases, such as stromelysin, collagenase and gelatinase B (matrix metalloproteinase 9, MMP-9) are produced by trophoblast cells and are required for both mouse and human trophoblast invasiveness (Librach et al. 1991). It has been shown that changes in their synthesis correlate with gestation-related changes in trophoblast invasive behavior. In the human cytotrophoblast, production and activation of MMP9 peak during the first trimester, coinciding with maximal invasive behaviour *in vivo* (Behrendtsen et al. 1992, Librach et al. 1991). Recently, the targeted mutation of the transcription factor Ets2 gene has been shown to result in death of homozygous mouse embryos before day 8.5 of

embryonic development. The embryonic death is due to the deficient expression of MMP-9 which results in failed implantation (Yamamoto et al. 1998).

In addition to producing proteinases that degrade the extracelluar matrix, trophoblasts also change their adhesive properties during invasion. Integrins of extracellular matrix are important for cell adhesion, differentiation, and migration (Wassarman 1995). Homozygous loss of β1 integrin expression in mice is lethal during early postimplantation development. The β1 integrin null embryos at embryonic day 4.5 showed collapsed blastocysts. Although the trophoblast cells penetrated the uterine epithelium, extensive invasion of the decidua was not observed and blastocysts had degenerated extensively by embryonic day 5.5 (Stephens et al. 1995).

1.3.3 Fusion of allantois

Vascular cell adhesion molecule-1 (VCAM-1), which was originally identified on vascular endothelium cells, is a member of the immunoglobulin (Ig) superfamily and functions as a cytokine-inducible cell surface protein capable of mediating adhesion to cells that express alpha 4 integrins. In homozygous null VCAM-1 mice, the allantois failed to fuse to the chorion at day 8.5 of gestation, resulting in abnormal placental development and embryonic death (Gurtner et al.1995). These experiments suggested that cell-cell interactions between these tissues are critical in chorioallantoic placental development.

1.3.4 Vasculature development

Another critical step in placental development in humans is the establishment of a hybrid vasculature, where the fetal trophoblast replace endothelial cells in blood vessels and are in direct contact with maternal blood. The vasculature transports nutrients and gases. A major roadblock to placental development is that the capacity of the placenta does not meet

the cardiovascular demands of the embryo during its progressive growth (Beaconsfield et al. 1980, Cross et al 1994). JunB is an immediate early gene product and a member of the AP-1 transcription factor family (Lee et al. 1987). Lack of JunB causes embryonic lethality between embryonic day 8.5 and day 10.0. The retardation of growth and eventual death are caused by the inability of establishing proper vascular interactions with the maternal circulation. JunB appears to be involved in multiple signalling pathways regulating genes involved in the establishment of a proper fetal and maternal circulatory system. In trophoblasts, the lack of JunB causes decreased expression of gelatinase B, resulting in a defective neovascularization of the decidua (Schorpp-Kistner et al 1999).

In humans, abnormalities in the vascular connections result in preeclampsia (Friedman et al. 1991, Roberts et al. 1991), in which cytotrophoblast invasion is shallow and uterine arteriole invasion is nearly absent (Zhou et al. 1993). So far there is no good mouse model for preeclampsia, but it is known that cytotrophoblasts isolated from preeclamptic placenta do not show the integrin switch that is a characteristic of normal trophoblast differentiation, and express no gelatinase B. These studies suggest that defects in adhesion molecules and proteinases might be the underlying causes of preeclampsia (Lim et al. 1997).

1.3.5 Maintenance of the trophoblast cell populations.

During placental development, the maintenance of the trophoblast cell population is critical for the continuation of pregnancy. There are now several mouse models with decreased trophoblast cell populations. Hepatocyte growth factor/scatter factor (HGF/SF) acts as a mitogen, motogen and morphogen for a variety of cultured cells. Homozygous mutant mouse embryos with a targeted disruption of the HGF/SF gene die before birth. These embryos have severely impaired placentas with markedly reduced numbers of

labyrinthine trophoblast cells, and an entire disorganized labyrinth layer (Uehara et al. 1995, Schmidt et al. 1995). The growth of trophoblast cells is stimulated by HGF/SF *in vitro*, andthe HGF/SF activity is released by the allantois in primary culture of normal but not mutant embryos. These findings suggest that HGF/SF is an essential mediator of allantoic mesenchyme-trophoblastic epithelia interaction required for placental organogenesis (Uehara et al. 1995).

αv integrins have been implicated in many developmental processes (Wassarman 1995). Eighty percent of the αv integrin null embryos died in mid-gestation, showing poor development of the labyrinthine layer and poor interdigitation of fetal and maternal vessels (Bader et al. 1998), which suggest the αv integrin may have impacts on both trophoblast cell population and vasculature formation.

It has also been reported that mice lacking the epidermal growth factor receptor (EGFR) die at mid-gestation *in utero* due to a defect in the spongiotrophoblast layer of the placenta (Sibilia and Wagner 1995, Threadgill et al. 1995).

Recently two basic helix-loop-helix transcription factors, Hand 1 and Mash 2, have been shown to be essential for determination of placental trophoblast cell types. These factors will be discussed in more detail in the following section.

1.4 Gene regulation of trophoblast differentiation

The trophoblast cell lineage is the first to differentiate during mammalian embryogenesis (Rossant et al. 1986). Until recently little was known about the transcriptional regulation of trophoblast differentiation. The identification of the basic helix-loop-helix transcription factors (bHLH) Mash-2 (Guillemot et al. 1994) and Hand-1

(also called as Hxt, Cross et al. 1995; eHAND, Srivastava et al. 1995; Thing-1, Hollenberg et al. 1995) has provided a breakthrough in our understanding of the events that regulate the development of trophoblast-specific cell lineages.

Targeted disruption of Mash-2 and Hand-1 genes in mice have given the first insights into the control of trophoblast differentiation. Mash-2, initially identified in a preneuronal cell line (Johnson et al 1990), is expressed in large amounts in spongiotrophoblast cells but not in trophoblast giant cells (Guillemot et al 1994). Mutation of the Mash-2 gene results in a diminished spongiotrophoblast layer and an enlarged giant cell layer in basal zone of placenta (Guillemot et al 1994). Mash-2 null embryos die from placental failure at embryonic day 12.

An interaction screening for bHLH factors expressed in blastocysts resulted in the identification of another bHLH factor, Hxt, which is expressed in trophoblast giant cells (Cross et al. 1995). This factor was also found to be expressed in the heart and neural-crest cell derivatives by two other laboratories (Srivastava et al. 1995, Hollenberg et al. 1995). Several observations suggest that Hand-1 regulates trophoblast giant cell formation. Hand-1 induces the commitment of cells to differentiate into trophoblast giant cells, as shown by injection of Hand-1 into uncommitted blastomeres of two cell mouse embryos, which directed their development into trophoblast giant cells in blastocysts (Cross et al 1995). Overexpression of Hand-1 in Rcho cells reduces proliferation and promotes differentiation to giant cells (Cross et al 1995). Development of homozygous Hand-1 mutant embryos was arrested by embryonic day 7.5 of gestation with defects in trophoblast giant cell differentiation (Riley et al. 1998, Firulli et al. 1998).

It is interesting to note that members of the basic helix-loop-helix (bHLH) family of

transcription factors are important cell lineage determinants in many cell types. Genetic evidence indicates that bHLH transcription factors function as cell-lineage determinants in skeletal muscle development (MyoD, myogenin, mrf-4, myf-5, etc), heart development and neuronal cell differentiation (Olson 1990 and 1992, Riley et al. 1998, Jan and Jan 1993).

In rodents, the balance between proliferation of trophoblasts and differentiation into nonproliferative trophoblast giant cells appear to be regulated by these trophoblast-specific bHLH transcription factors, as well as by the HLH factors, such as the Id proteins (Id) (Barone et al. 1994), which lack a basic domain. The Id proteins dimerize with members of the bHLH protein family, but due to the absence of the basic region, the resulting heterodimers cannot bind DNA. Id proteins can therefore negatively regulate activity of bHLH transcription factors (Barone et al. 1994). The expression of Id-1 (Evans and O'Brien 1993, Cross et al 1994a) and Id-2 (Cross et al. 1995, Janatpour et al 1994) is high in proliferative trophoblast Rcho cells and is down regulated during differentiation. Ectopic expression of Id-1 reduces the ability of Rcho cells to differentiate (Cross et al 1995); this is similar to the activity of Id-1 in regulating the differentiation of the other cell lineages (Barone et al 1994).

Although the bHLH factors Hand-1 and Mash-2 appear to play an important role in placental development, no target genes have been identified for these transcription factors. Only a few transcription factors have been identified as important for placental gene expression. Activator protein-2 (AP-2) and members of GATA family of transcription factors have been identified as necessary for the placental expression of human chorionic gonadotropin α and β subunit genes (Steger et al 1994, Johnson et al 1997). The TEF family of transcription factors has been implicated in the expression of human placental

lactogens (Jacquemin et al 1997, Jiang et al 1997). None of these transcription factors, however, are expressed exclusively in the placenta, suggesting that protein-protein interactions between different combinations of transcription factors and co-activator proteins will be important for defining placental specificity.

Understanding the genetic control of placental gene regulation and identifying factors which regulate placental cell-specific gene expression are important to our understanding of placental development. We have been studying the regulation of a large family of prolactin (PRL) - like family genes that are expressed according to specific developmental programs in the rat placenta during pregnancy.

2. RAT PLACENTAL PROLACTIN FAMILY OF HORMONES

2.1 Historical review of gene family

Human placental lactogen (hPL, also called chorionic somatomammotropin, hCS) was first characterized as a placental hormone uniquely expressed during pregnancy. Human PL, which is produced by syncytiocytotrophoblast cells at the maternal-fetal interface of the placenta, stimulates lipolysis, directs maternal metabolism, and enhances fetal growth (Walker et al. 1991). Human PL structurally and immunologically is related to human growth hormone (GH) (Josimovich and McLaren 1962).

Pioneering studies in rodents showed that hypophysectomy performed on a pregnant mouse or rat before mid-gestation resulted in abortion; after midterm the outcome of pregnancy was unaffected (Pencharz and Long 1931, Selye 1933, Astwood and Greep 1938). Pituitary PRL is critical for the continued function of the *corpus luteum* and the development of mammary gland during pregnancy (Greep 1974). These early studies

suggested that PRL is essential in the earlier stages of pregnancy, but that the proteins from tissues other than pituitary, most likely the placenta, are able to take over the role of PRL after that time.

In 1975, Kelly et al. discovered that there were two peaks of lactogenic activity in pregnant rat serum and placental extracts (Kelly et al. 1975). The first peak, designated rat placental lactogen I (rPLI), was detected from day 8 to day 14 of the pregnancy; the second peak, designated rat placental lactogen II (rPLII), was detected from day 12 to term of the placenta. Robertson and Friesen (1981) further characterized these two placental lactogens, and found that rPLI had a molecular weight of 40,000 and rPLII had a molecular weight of 20,000. Although both would bind the prolactin receptor, the antibody to rPLII did not cross-react with rPLI (Robertson and Friesen 1981).

With the advance of recombinant DNA technology, this simple picture changed dramatically. In 1986, Duckworth et al isolated rPLII cDNA (Duckworth et al. 1986a), the first non-human placental lactogen to be cloned. Interestingly, unlike the human, its sequence was more closely related to PRL than to GH. During the characterization of rPLII cDNA, two further prolactin-like proteins (PLP), termed rPLP-A and rPLP-B, were identified. As these family members were characterized at the cDNA, protein, and genomic levels, more members were discovered. Several recently identified PLP members were identified by searching for closely related sequences in expressed sequence tag (EST) databases with cDNA sequences of known PRL family members. To date, 17 members of the PRL gene family expressed in the rat placenta and/or maternal decidua have been cloned; these are listed in Table 1.

Hiraoka et al.(1999) cloned a decidua protein called "rPLP-I", which is the same as

rPLP-J identified by Toft and Linzer, 1999. Ishibashi et al. (1999) cloned rPLP-I, rPLP-J (same as Linzer's rPLP-J), rPLP-K, rPLP-L from a rat placental library; only rPLP-I has been shown to have expression in a tissue other than placenta, in this instance, the testis. The time of expression and expressing cell types in placenta for rPLP-I have not yet been determined. Most recently, two new rat PRL family members, rat prolactin-like protein F (rPLP-F) and rat prolferin-related protein (rPLF-RP) have been identified (Sahgal et al. 2000). These new members show considerable sequence homology to the mouse PLP-F and PLF-RP proteins (Lin et al. 1997a; Linzer and Nathans 1985) and their presence in rat reinforces the contention that these proteins have important functions during pregnancy in rodents.

Table 1. The cloned rat placental/decidual PRL family members

PRL family member*	Reference
rPLII	Duckworth et al. 1986a
rPLP-A	Duckworth et al. 1986b
rPLP-B	Duckworth et al. 1988
rPLI	Robertson et al 1990
rPL-Iv	Robertson et al. 1991
rPLP-C	Deb et al. 1991a
d/tPRP	Roby et al. 1993
rPLP-Cv	Dai et al. 1996
rPLP-D	Iwatsuki et al. 1996
rPLP-E	Soares et al. 1998; Iwatsuki K and Shiota K, unpublished data
rPLP-H	Iwatsuki et al. 1998
rPLP-I	Ishibashi et al. 1999
rPLP-J	rPLP-J by Toft and Linzer 1999 ; Ishibashi et al. 1999
rPLP-K	Ishibashi et al. 1999
rPLP-L	Ishibashi et al. 1999
rPLP-F	Sahgal et al. 2000
rPLF-RP	Sahgal et al. 2000

*rat placental lactogen (rPL); rat PRL-like protein (rPLP); rat decidual/trophoblast prolactin-related protein (d/tPLP).

2.2 Developmental expression of rat PRL family proteins

The placental PRL family is characterized by distinct cell and temporal patterns of expression. Time of expression and expressing cell types are summarized in Table 2. The ancestral member of the family, PRL, is expressed in lactrotroph cells of the anterior pituitary (Li 1978); other family members are expressed in placenta and/or the maternal antimesometial decidua.

The expression of rPLI is initiated shortly after implantation in mural trophoblast giant cells of the implanting blastocyst and later extends to the secondary giant cells in the junctional zone of the chorioallantoic placentas (Soares et al. 1985, Duckworth et al. 1993). Within a 48 h period between day 11 and 12 of gestation (in rat, day 1 of gestation was defined as the day sperm was observed in the vagina) rPLII expression is initiated and rPLI expression is terminated (Faria et al. 1990a, Duckworth et al. 1993).

Rat PLII is initially expressed exclusively in trophoblast giant cells of the basal zone; by day 14, trophoblast giant cells of the labyrinth zone also acquire the capability to express rPLII (Campbell et al. 1989, Duckworth *et al.* 1990). The labyrinth becomes the major source of the rPLII protein in late pregnancy (Duckworth et al. 1993). During the overlapping period of rPLI and rPLII expression, the same trophoblast giant cells in junctional zone are responsible for the production of both hormones (Duckworth et al. 1993). The mechanisms responsible for the placental lactogen midpregnancy switch in expression are unknown.

Rat rPL-Iv, rPLP-A, rPLP-C, rPLP-Cv, rPLP-D, rPLP-E, and rPLP-H are restricted to the basal zone of the chorioallantoic placenta. Expression of these PRL-related hormones, which is initiated just after midgestation, is confined to spongiotrophoblast cells

and, to a much lesser extent, to trophoblast giant cells of the basal zone. Levels increase as gestation advances (Robertson et al. 1991, Campbell et al. 1989, Duckworth et al. 1990, Deb et al. 1991 a,b,c, Soares et al. 1998).

In placenta rPLP-B is restricted to the spongiotrophoblast cells, and represents a specific marker for these cells (Duckworth et al. 1990, 1993). Earlier in pregnancy rPLP-B is also expressed in the antimesometrial decidual tissue of the pregnant and pseudopregnant rat (Croze et al. 1990).

Rat d/tPRP is expressed in antimesometrial decidua during early gestation, and then in spongiotrophoblasts and giant cells of basal zone during the second half of pregnancy (Roby et al 1993, Gu et al. 1994). The recently described rPLP-J is exclusively expressed in decidua (Toft and Linzer 1999). Temporally, most of the placental members are expressed during the second half of the pregnancy.

The most recently identified rat family members, rPLP-F and rPLF-RP (Sahgal et al. 2000) show different expression patterns from those of the mouse. Murine PLP-F is expressed only in spongiotrophoblasts, while the rat homologue is expressed mainly by the basal zone giant cells with low level expression from spongiotrophoblasts only late in pregnancy. The rat PLF-RP is first expressed in cells in the invading ectoplacental cone and later exclusively within the labryrinth. The murine PLF-RP is expressed in the giant cell layer of the midgestation chorioallantoic and choriovitelline placentas and later within the trophoblast giant cell and spongiotrophoblast layers of the junctional zone (Colosi et al. 1988).

Very recently Prigent-Tessier et al. (1999) have shown that PRL itself is also expressed in the antimesometrial decidua of the pregnant rat. Expression of PRL in decidua

appears to be regulated differently from pituitary expression suggesting tissue-specific regulation of the rPRL gene, possibly from an alternative promoter as has been shown for the human PRL gene (DiMattia et al. 1990).

Table 2 Expression patterns of the rat prolactin family of proteins

Protein	Time of expression*	Expressing cell types
Prolactin	Embryonic day 9 - 11	Antimesometrial decidua
	Postnatal	Pituitary lactotroph
rPLI	Day 7 - 12	Trophoblast giant cells
rPLII	Day 11 - term	Trophoblast giant cells
rPLIv	Day 14 - term	Spongiotrophoblasts and trophoblast giant cells
rPLP-A	Day 14 - term	Spongiotrophoblasts and trophoblast giant cells
rPLP-B	Day 7 - 13	Antimesometrial decidua
	Day 13 - term	Spongiotrophoblasts
rPLP-C	Day 14 - term	Spongiotrophoblasts and trophoblast giant cells
rPLP-Cv	Day 14 - term	Spongiotrophoblasts and trophoblast giant cells
rPLP-D	Day 14 - term	Spongiotrophoblasts and trophoblast giant cells
rPLP-E	Day 14 - term	Spongiotrophoblasts and trophoblast giant cells
rPLP-H	Day 14 - term	Spongiotrophoblasts and trophoblast giant cells
rPLP-J	Day 7 - 10	Decidual cells
d/tPRP	Day 7 - 13	Antimesometrial decidua
	Day 13 - 16	Spongiotrophoblasts and trophoblast giant cells

*Day 1 is defined as the day sperm was observed in the vagina.
Duckworth et al. 1993, Soares et al. 1998, Toft and Linzer 1999.

2.3 PRL family in other species

Most of the placental/decidual PRL family of proteins described in the rat have also been identified in the mouse (Jackson et al. 1986, Lin et al. 1997a,b, Colosi, et al. 1987,1988, Orwig et al. 1997a) and their developmental expression patterns in rat and mouse are very similar. Proliferin (PLF) (Linzer et al. 1985) and proliferin-related protein (PLF-RP) (Linzer and Nathans 1985) were the first placental members of this gene family to be described in the mouse and until recently neither had been identified in the rat. As mentioned, Sahgal et al. (2000) have now reported on the isolation of a rat cDNA clone with considerable structural similarity to PLF-RP. Double immunofluorescence staining and *in situ* hybridization demonstrated that the same trophoblast giant cells express mPLI, mPLII, and PLF simultaneously at midpregnancy (Yamaguchi et al. 1994, Lee et al. 1988). A further protein that has still only been detected in the mouse is PLP-Cα, which like the very similar protein PLP-C, is expressed in both the spongiotrophoblasts and giant cells of the mid- to late term placenta.

A family of PRL-like proteins are also present in cow (Schuler et al. 1987, 1988, Kessler et al. 1989), sheep (Colosi et al. 1989, Kenn et al. 1999), and hamster (Southard et al. 1989, Jones and Renegar 1994, Barnes and Renegar 1996). The genes for all identified placental PRL-like proteins have been localized to the same chromosome as PRL: rat, chromosome 17 (Duckworth et al. 1993), mouse, chromosome 13 (Jackson-Grusby et al. 1988, Dai et al. 1998), cow, chromosome 22 (Dietz et al. 1992). In human, no placental PRL family members have been identified, but the growth hormone-like hPL is contiguous with the GH gene on chromosome 17 (George et al. 1981). The human PRL gene is located on chromosome 6 (Owerbach et al. 1981).

The precise arrangement of each gene along its respective chromosome has not been

reported. Our laboratory has characterized a P1 genomic clone that links rPLII and rPLP-B together on a 70 Kb fragment (unpublished data). All known members of the mouse family, except proliferin, are located on a single 700 Kb YAC clone (Lin et al 1997), which suggests that the PRL family of genes are likely linked in a single locus. Members of PRL family genes have probably arisen by gene duplication as has been proposed for the human GH/PL family (Niall 1982). Interestingly, primates are the only group so far identified that have evolved a family of proteins expressed in placenta that are related to GH rather than PRL.

2.4 Structural features of the gene family

All members of the PRL family have a greater than 30% identity to rat PRL at the amino acid level and have highly conserved cysteine and tryptophan residues within the protein. All members of the PRL family have at least four highly conserved cysteine residues. These conserved characteristics suggest that the proteins will have similar folding patterns. It is interesting to note, however, that only PLI, PLII and PLIv have been shown to bind the prolactin receptor (Kelly et al. 1975, Robertson et al. 1981, Cohick et al. 1996). Additional structural information has been obtained by comparing amino acid sequences of the family members. In some cases there are differences between the proteins that may lead to differences in receptor binding. For example, exon 3 of the all members of the PLP-C subfamily encodes a segment rich in aromatic amino acids called an "aromatic domain" (Dai et al. 1996, 1998).

All uteroplacental PRL family members, except PLII and possibly PLP-Cv, appear to receive some type of carbohydrate modification. PRL family members expressed by

spongiotrophoblast cells possess distinct glycosylation patterns. These patterns are cell type- and protein-dependent (Manzella et al. 1997). There also appears to be species differences between rat and mouse regarding glycosylation patterns. Rat PLP-A has two putative N-linked glycosylation sites, generating 29 and 33 KDa glycosylated forms of the protein (Deb et al. 1989, Muller et al. 1998). The mouse has only a single N-linked glycosylation site that is associated with a 29 KDa glycosylated protein.

Two exon/intron organizations have been described for this family in rat and mouse. A 5 exon/4 intron structure has been found in PRL (Cooke et al. 1986), PLII (Shida et al 1992, Shah et al. 1998) and proliferin (Connor et al. 1989); a 6 exon/5 intron structure has described for PLP-Cv (Dai et al. 1996a), PLP-Cα (Dai et al. 1998) and d/tPRP (Orwig et al. 1997b).

2.5 Function

2.5.1 Prolactin

Prolactin (PRL) has been implicated in numerous physiological and developmental processes (Reviewed by Bole-Feysotet al. 1998). In particular, in rodents it plays a key role in the establishment and maintenance of pregnancy. Early in pregnancy beginning at day 2 after mating, pituitary PRL is secreted as diurnal and nocturnal surges which are essential for establishing and maintaining pregnancy until midgestation (Freeman and Neill 1972, Smith et al. 1975).

Pituitary PRL directly stimulates the growth and development of the mammary glands and stimulates milk protein synthesis (Thordarson and Talamantes 1988, Forsyth 1988). PRL regulates the development of the mammary gland at three stages in the

reproductive life history of females. The first stage is mammary gland organogenesis, during which PRL contributes to the maturation of the mammary glands from a primary ductal system, which grows from terminal end buds, to the fully mature non-pregnant gland. During pregnancy PRL, placental lactogens, and progesterone stimulate the expansion and physiological differentiation of the lobuloalveolar system from the lobular buds. After delivery, PRL in the context of falling progesterone stimulates the final induction of milk protein gene expression and lactation (reviewed in Horseman et al. 1999).

PRL is not only essential for mammary gland development, it also plays an important role in ovary development. Studies have shown that PRL is a necessary participant in the extension of the functional lifespan of the *corpus luteum* of the estrous cycle (Morishige et al. 1974) and that it stimulates the luteal progesterone production, which is essential for maintaining pregnancy (Matsuyama et al. 1990). PRL also has a direct effect on developmental competence and maturation of oocytes (Yoshimura et al. 1991).

In the PRL null mouse model, PRL-deficient males are fertile and produce offspring with normal Mendelian gender and genotype ratios when they are mated with heterozygous females. PRL deficiency in female mice causes infertility, but does not prevent spontaneous maternal behaviour when challenged with foster pups. Mammary glands of PRL null female mice develop a ductal tree which is characteristic of the normal virgin adult mammary gland, but ducts fail to develop lobular decorations. The lymphocyte cells do not appear to be affected since myelopoiesis and primary lymphopoiesis are unaltered in the PRL null mice (Horseman et al. 1997).

Mice carrying a germ-line null mutation of the PRL receptor gene show more severe defects than the PRL null mice. Homozygous females are sterile because of a complete

failure of embryonic implantation. The mutant mice show multiple reproductive abnormalities, including irregular estrous cycles, reduced fertility rates, defective preimplantation embryonic development, and lack of pseudopregnancy. Half of the homozygous males are infertile or show reduced fertility. Even heterozygous females show almost complete failure of lactation that appears to be caused by greatly reduced mammary gland development after their first pregnancy; this failure to lactate appears to be overcome in subsequent pregnancies (Ormandy et al. 1997).

Recently studies of prolactin receptor null mice indicated that prolactin affects mammary morphogenesis in two different ways. PRL controls ductal side branching and terminal end bud regression in virgin animals via indirect mechanisms. It also acts directly on the mammary epithelium to produce lobuloalveolar development during pregnancy (Brisken et al. 1999). Studies of pup-directed maternal behaviour in PRL receptor null mice indicate that the PRL receptor is also a regulator of maternal behaviour. Both homozygous and heterozygous PRL receptor mutant females show a profound deficiency in maternal behaviour when challenged with foster pups (Lucas et al. 1998). This is a different response from the normal behaviour of the PRL null mice placed in similar circumstances. Taken together with the more severe mammary gland defects found in PRL receptor null mice, these findings suggest that other PRL receptor ligands also have important roles in pregnancy.

2.5.2 Other PRL family members

Although the developmental expression of the placental/decidual members of the PRL family proteins is tightly regulated, little is known about their function. There are *in vitro* data that show that PLI and PLII have similar mammotrophic and luteotrophic effects

to PRL in primary mammary epithelial cell cultures. The PLs stimulate various stages of mammary epithelial cell growth and differentiation (Thordarson et al. 1986, Thordarson and Talamantes 1987) and promote the biosynthesis of progesterone by the *corpus luteum* (Galosy and Talamantes 1995, Thordarson et al. 1997). It has been demonstrated that PLI and PLII bind to PRL receptors (Robertson and Friesen 1981) and activate its signal pathways. As described above, the defects of PRL receptor null mice appear to be more severe than those of the PRL null mice, suggesting that the other PRL receptor ligands, such as the PLs, may play important roles in mammary gland development during pregnancy.

Although PRL plays a crucial role during pregnancy the PRL surges decline at midpregnancy (Freeman and Neill 1972, Smith et al. 1975). Hypophysectomy of a pregnant rat before day 12 of gestation results in abortion, but after midterm the outcome of pregnancy is unaffected (Astwood and Greep 1938). These data suggest that pituitary hormones are essential in the earlier stages of pregnancy but that the placental proteins are able to take over the role of PRL after that time. The rat placental PRL proteins, in particular PLI and PLII, are obvious candidates to replace the functions of the rat pituitary PRL. It has been speculated they may also have a role in regulating the decline of the PRL surges, since they are expressed during pregnancy mainly after PRL secretion is shut down (Yogev and Terkel 1978, 1980; Voogt 1980).

PLI and PLII are expressed at specific times during pregnancy suggesting that they have specific roles. The rat choriocarcinoma cell line, Rcho, when transplanted under the kidney capsule of female rats, has been shown to produce rPLI but no other PRL family members (Faria et al. 1990b). It has been reported that proteins secreted from implanted Rcho cells could inhibit the synthesis of pituitary PRL *in vivo* (Tomogane et al. 1993),

suggesting that rPLI itself may inhibit PRL release via effects on the hypothalamus.

Mouse PLII has been reported to be present in the fetal serum from day 14 (Ogren and Talamantes 1988). Functional rPL-II binding has been revealed in the fetal adrenal cortex, renal tubules, small intestinal villi, pancreatic islets and hepatic parenchyma (Royster et al. 1995). In rodents, expression of the PRL receptor mRNA and protein is widespread throughout the fetus during development (Tzeng and Linzer 1997). Prolactin itself, however, is not expressed in the fetus until close to birth (Gash et al. 1982, Aubert et al. 1985, Tonh et al. 1989), suggesting that other PRL receptor binding protein (s) function through these PRL receptors. These data together suggest that PLII, which binds PRL receptors, may have a vital role in fetal growth and development (Tzeng and Linzer 1997). An *in vitro* study has shown that both rat and mouse placental lactogens have effects on islet B cell division and insulin secretion (Brelje et al. 1993), suggesting PLs may potentially have a role in insulin metabolism.

The biological functions of two of the mouse PRL family members have been relatively well-studied. Proliferin (PLF) and proliferin-related protein (PLF-R) are potent regulators of angiogenesis; proliferin stimulates and proliferin-related protein inhibits endothelial cell migration in cell culture and neovascularization *in vivo* (Jackson et al.1994). The mouse placenta secretes an angiogenic activity during the middle of pregnancy that corresponds mainly to PLF, and later in gestation releases anti-angiogenic activity that corresponds to PLF-R. PLF binds to capillary endothelial cells, suggesting that it may stimulate the reorganization and growth of maternal blood vessels in decidual tissue at the implantation site. PLF-R may act to slow down vessel growth in response to proliferin and other angiogenic factors, and generate a barrier zone that prevents maternal blood vessels

from over-growing (Jackson et al. 1994).

PLF has also been detected in both the maternal and fetal compartments. Mouse PLF binds to insulin-like IGF II/mannose 6-phosphate receptor (Lee and Nathans 1988), and this receptor is required for PLF-induced angiogenesis and PLF binding to specific cells in the fetus (Volpert et al. 1996, Jackson and Linzer 1997). The major binding sites for PLF have been detected in developing fetal vertebral and vascular structures. PLF immunoreactivity has also been observed in association with the yolk sac (Jackson and Linzer 1997).

No specific receptors have yet been identified for other members of the prolactin family. It has been reported that recombinant rPL-Iv is able to compete with ovine PRL for rat ovarian and liver PRL receptors, but the binding affinity is much lower than that of PLI and PLII. (Cohick et al. 1995). Using an alkaline phosphatase-tagging strategy, it has been shown that rPLP-A specifically interacts with a population of natural killer (NK) lymphocytes within the mesometrial compartment of decidua from pregnant and pseudopregnant rats (Muller et al. 1999). Another member of the mouse PRL family, PLP-E, targets megakaryocytes through an unidentified specific cell receptor and induces megakaryocyte differentiation (Lin and Linzer, 1999). These associations with cells in the hematopoietic lineage suggest some interesting new functions for members of the PRL family during development.

Although the biological functions of the placental members of the PRL gene family are not yet clear, the specific cellular and temporal expression of these genes suggest that they must play special roles during pregnancy. The unique expression patterns of this gene family make it a valuable resource to study placental-specific gene regulation.

3. REGULATION OF RAT PROLACTIN FAMILY GENES

3.1 General review of transcriptional regulation of eukaryotic genes

DNA is transcribed into a pre-mRNA containing exon (coding) and intron (noncoding) sequences. RNA processing of this pre-mRNA includes the removal of introns and addition of a poly A tail resulting in a mature mRNA. The mRNA is either translated into protein or degraded. Subsequent post-translational processing of protein includes modification and degradation of the protein. A gene can be regulated at the levels of transcription, RNA processing, translation or post-translational processing (Reviewed by Grunstein 1990; Jackson 1991). Transcriptional regulation plays a key role in the regulation of many genes.

Eukaryotic protein-coding genes are activated and regulated at the level of transcription through two general classes of DNA elements and their protein binding factors: 1) common core promoter elements (proximal to the transcription start site) interact with RNA polymerase II and general transcription factors - together these are called the general transcription machinery or preinitiation complex (PIC). 2) sequence-specific transcription factors and cofactors interact with gene-specific regulatory elements (distal to the core promoter) and modulate the function of the general transcription machinery by looping mechanisms (reviewed by Roeder 1998).

The general transcription factors that interact with core promoters are ubiquitous, have intrinsic ability to initiate transcription and are involved in the basal transcription process. The regulatory transcription factors that interact with distal regulatory elements (enhancer, repressor or locus control regions) are gene-, cell- and/or stage-specific.

Together transcription factors with cofactors regulate the rate of transcription of each gene in response to various developmental and environmental signals by affecting the general transcription machinery (Reviewed by Roeder 1998).

3.2 Prolactin gene regulation

The pituitary-specific expression of prolactin (PRL) and growth hormone is regulated by the POU and homeodomain DNA binding factor, Pit-1/GHF-1. Pit-1 has been shown to essential for the lactotroph cell lineage, as well as the somatotroph and thyrotroph cell lineages (Li et al. 1990, Sornson et al. 1996). In addition to Pit-1, regulation of PRL gene transcription in lactotroph cells also requires co-operative interactions between the pituitary-specific Pit-1 and members of the Ets transcription factor family (Bradford et al. 1997).

The Ets transcription factor family consists of more than 35 members (Wasylyk et al. 1998). Members are defined by a highly conserved 85 amino acid DNA binding domain known as the Ets binding domain (EBD) that recognizes a consensus core DNA sequence, A/CGGAA/T (Wasylyk et al. 1993, 1998). The Ets family of transcription factors function as either transcriptional activators or repressors. As targets of the Ras-MAPK signaling pathway, Ets proteins function as critical nuclear integrators of ubiquitous signaling cascades. To direct signals to specific target genes, Ets proteins interact with other transcription factors that promote the binding of Ets proteins to composite Ras-responsive elements (Wasylyk et al. 1998, Sharrocks et al. 1999).

Ets proteins bind to DNA as monomers, but protein-protein interactions appear to be important for their function (Wasylyk et al. 1991, Butticè et al. 1996, Basuyaux et al. 1997).

In the case of the PRL gene Ets-1 physically interacts with Pit-1 to fully reconstitute proximal PRL promoter activity. The Ets-1/Pit-1 synergy requires a composite Ets-1/Pit-1 *cis*-element and is dependent on an Ets-1-specific protein domain. Ets-2 has no direct effect on the PRL promoter (Bradford et al. 1997). Interestingly overexpression of recombinant Ets-2 repressor factor (ERF) inhibits PRL promoter activity by interfering with the Ets-1/Pit-1 interaction (Day et al. 1998). These results underscore the importance of transcription factors that are distinct from, but interact with, homeodomain proteins to establish lineage-specific gene expression.

EGF increases PRL gene expression in pituitary GH4 cells (Murdoch et al. 1982). The EGF response element of the PRL promoter is a recognition sequence for the Ets transcription factors, and Ets factors have been shown to bind this element (Jacob et al. 1999). Expression of the DNA-binding domain of c-Ets-1, which acts as a dominant negative inhibitor of Ets transcription factors, reduces EGF-increased reporter gene expression from the PRL promoter. Thus, EGF may signal through Ets transcription factors to activate PRL gene transcription (Jacob et al. 1999).

Other studies have suggested that transcription factor(s) besides Pit-1 are also involved in the regulation of PRL promoter activity. CCAAT/enhancer-binding protein α (Jacob et al. 1999) and PRL regulatory element binding protein (PREB) (Fliss et al. 1999) have been shown to regulate PRL gene expression. The newly identified PREB protein contains two proline and glutamine-rich potential transactivation domains. Although it contains no apparent DNA-binding motif, it exhibits sequence-specific binding to a site called 1P, which is not identical to that for Pit-1 (Fliss et al. 1999).

The other PRL family members are expressed primarily in placenta. Gene

regulation studies were difficult because only primary placental cells were available. In 1989 Verstuyf et al. developed the rat choriocarcinoma cell line, Rcho, providing a new tool for the study of regulation in trophoblast-specific genes.

3.3 Rat choriocarcinoma cell line

The rat choriocarcinoma cell line, Rcho, was derived from a transplantable rat choriocarcinoma tumour (Verstuyf et al.1989). This cell line is composed of pure trophoblast cells that grow as a mixture of cell types: undifferentiated small cells and differentiated giant cells. The small cells, that grow in clusters, multiply and differentiate into trophoblast giant cells in cultures, while the terminally differentiated giant cells lose the ability to proliferate. The morphology of the Rcho giant cells is very similar to placental giant cells (Verstuyf et al. 1990). M. Soares' laboratory rederived the cell line from tumours, calling it Rcho-1. This line has very similar characteristics to the original Rcho line (Faria and Soares 1991). I will not make a distinction between these lines in this thesis.

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 rPLI, but not rPLII and rPLP-A (Faria et al. 1990b). In culture, the Rcho cell line has been shown to produce rPLI, rPLII, rPLP-A, rPLP-C (Faria and Soares 1991, Duckworth et al. 1990, 1993, Hamlin et al. 1994), rPLP-D (Iwatsuki et al 1996), rPLP-H (Iwatsuki et al. 1998) and rPL-Iv (Dai et al. 1996). All genes are expressed in the differentiated trophoblast giant cell population.

Rat PLP-B, which is spongiotrophoblast specific, is not expressed in Rcho cells (Faria and Soares 1991). These observations suggest that the undifferentiated Rcho cells do

not have the potential to develop into spongiotrophoblasts, but are already committed to a giant cell identity (Faria and Soares 1991, Duckworth et al. 1993, Hamlin et al. 1994). The Rcho cell line provides a unique *in vitro* model to study the regulation of trophoblast giant cell genes during trophoblast differentiation. Gene regulation studies for several placental members of the rat and mouse PRL gene families have been conducted in this cell line.

3.4 Regulation of other PRL family members

Rat PLI is expressed in Rcho cells as soon as cultures differentiate (Duckworth et al. 1993, Sun et al. 1998). Shida et al. (1993) have reported that the mouse PLI gene promoter extending 274 bp 5' from the transcription start site contains all of the elements necessary for maximal expression of mPLI in Rcho cells. It has been shown that two AP-1 binding and two GATA-binding sites are required for the maximal reporter expression of this 274 bp mPLI promoter in Rcho cells (Shida et al.1993, Ng. et al.1994). We have found that a similar regulatory region appeared to be important in rPLI promoter activity (Sun et al.1998).

The AP-1 transcription factors consist of related but distinct protein factors Jun and Fos, which share the common property of binding to the DNA sequence TGANTCA/CA, termed the TPA responsive element (TRE) or activator protein-1 (AP-1) binding site (Lee et al. 1987a). These factors bind DNA either as Jun-Fos heterodimers or as Jun-Jun homodimers, and the specificity of the binding is determined by the interaction with other transcription factors (Ransone and Verma 1990, Distel and Spiegelman 1990, Vogt and Bos 1990). Jun and Fos are ubiquitous transcription factors and they have been shown to be involved in the regulation of many genes (Lee et al. 1987b, Curran et al. 1988, Ransone and

Verma 1990).

GATA factors are another family of transcription factors that have been implicated in the regulation of the PRL gene family. They contain two Zn^{++} finger domains that are essential for DNA binding. Although all GATA proteins bind DNA sequences containing a core consensus element, there are overlapping but distinct sequence preferences among the different family members (Bockamp et al. 1994). GATA-2 and GATA-3 are expressed in placental trophoblast giant cells, with peak mRNA levels at mid-gestation. The importance of GATA2/3 factors in mPLI expression has been demonstrated by their ability to induce transcription from the 274 bp mPLI promoter when transfected into non-trophoblast (fibroblast) cells (Ng et al. 1993, 1994). In stably transfected Rcho cells it has been observed that the expression from this promoter increases as the percentage of differentiated giant cells in culture increases (Shida et al.1993), suggesting that the factors which interact with this promoter may only be present in trophoblast giant cells.

In the rat, rPLP-A is specifically expressed in giant cells and spongiotrophoblasts of the junctional zone (Duckworth et al. 1990). Vuille et al. (1993) showed that a fragment from -975 of the 5'-flanking region of rPLP-A is sufficient to specify placental expression of a *cat* reporter gene in Rcho cells, but additional enhancing elements are present between -4600 and -975 in the rPLP-A 5'-flanking region.

Rat PLP-Cv is highly expressed in spongiotrophoblasts and to a much lesser extent in basal zone trophoblast giant cells (Dai et al. 1996). Promoter constructs containing sequences within -4500 to -149 of 5'-flanking DNA showed significant activity in Rcho cells; the region located between -149 and -124 was found to be essential for activation. Spongiotrophoblast cells required additional rPLP-Cv 5'-flanking DNA to be functional. A

region located between -2518 and -2242 of the rPLP-Cv gene significantly enhanced the activity of the minimal promoter in primary spongiotrophoblast cell cultures. These data suggest that the DNA sequences important for the rPLP-Cv promoter activation are different in trophoblast giant cells as compared to spongiotrophoblast cells (Dai et al. 1999).

The decidual/trophoblast PRL-related gene (d/tPRP) is expressed by decidual antimesometrial cells, spongiotrophoblasts and trophoblast giant cells (Roby et al. 1993, Gu et al. 1994, Orwig et al. 1997b). It has been reported that decidual and trophoblast giant cell-specific expression of rat d/tPRP is regulated by a 3.96 Kb 5'-flanking region (Orwig et al. 1997b).

Mouse proliferin (PLF) is highly expressed in trophoblast giant cells immediately after implantation; expression continues through the second half of the gestation at reduced levels of production (Lee et al. 1988). Transcription from a cloned PLF promoter is inducible by phorbol esters, and this induction involves a region of 31 bp 5' flanking DNA that includes an AP-1 site. The mutation of the AP-1 abolishes phorbol ester induction, and the transfer of this 31-bp sequence to a site upstream of a minimal promoter is sufficient to confer phorbol-ester responsiveness. In contrast, glucocorticoids repress PLF transcription, and the repression is dependent on the glucocorticoid receptor, which binds to the PLF promoter in the 31-bp phorbol ester inducible region (Mordacq and Linzer 1989). The binding site for the glucocorticoid receptor in the PLF gene promoter has been reported to function as a composite glucocorticoid response element when fused to a minimal promoter. The ability of this element to repress PLF promoter activity is position-dependent (Hoeppner et al. 1995).

In GATA-2 null mice, placentas continued to express some mPLI mRNA, but there

was a markedly greater reduction (5-6 fold) in PLF gene expression. A null mutation of GATA-3 had a similar effect on the levels of PLI and PLF mRNAs reducing both to 50% of that in the wild-type placenta. These results suggest that GATA-2 and GATA-3 are important regulators of expression of at least some trophoblast giant cell-specific genes, and reveal a difference in the effect of these factors in regulating the synthesis of related placental hormones (Ma et al. 1997).

The role of GATA factors in PLII gene regulation could not be studied in these null mice as they died due to other defects before PLII expression. In the transgenic mouse, however, it has been 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 mPLII gene (Shida et al. 1992). No specific *cis*- or *trans*-factors were identified in this study.

In sheep, trophoblast cell-specific transactivation of a reporter gene was conferred by the proximal 1.1 Kb of 5' flanking sequence of the ovine placental lactogen (oPL). Maximal activation in both human (BeWo) and rat (Rcho) choriocarcinoma cell lines resided within the proximal 383 bp of oPL gene 5'-flanking sequence. Two functional GATA sequences were identified, and a previously undefined element (GAGGAG) was shown to be required for full promoter activation. In addition, an AP-2 site and an E-box were identified but not functionally tested (Liang et al. 1999).

The cell-specific and temporal-specific expression of the PRL genes make this family an important resource to study trophoblast specific gene regulation. Although we understand a great deal about pituitary prolactin gene regulation, we know relatively little about the regulation of the placental members of the PRL genes. From information

accumulated so far, it is unlikely that, as for Pit-1 in the pituitary, there will be one major factor that is responsible for trophoblast gene expression. The PRL gene family will provide tools to study gene regulation in several trophoblast/maternal cell types at different times during pregnancy.

RATIONALE, HYPOTHESIS AND RESEARCH OBJECTIVES

Rationale: Placental PRL gene family is expressed according to specific temporal and cellular expression patterns, making this family a unique resource to study the placental specific gene regulation. Among these members, PLI is expressed during the first half of the pregnancy while PLII is expressed only in mid to late term pregnancy. Their expression patterns overlap for about two days at mid-pregnancy. During this time the same trophoblast giant cells express both mRNAs (Duckworth et al. 1993). An intriguing question is the identity of the factors in trophoblast giant cells that regulate this tightly controlled temporal switch between PLI and PLII.

To answer this question, we need a good understanding of the regulation of each of these genes. GATA and AP-1 factors are required for the maximal expression of mPLI (Shida et al. 1993, Ng et al. 1993, 1994). A functional rPLI promoter has similar DNA binding sites to those in mPLI (Sun et al. 1998), which suggests that these genes are regulated by the same factors. Little, however, is known about the transcriptional regulation of the rPLII gene. My project focuses on the molecular mechanisms of rPLII regulation.

Hypothesis: Specific transcription factors are involved in placental specific expression of rat placental lactogen II gene.

The aim of my project is to identify the factors that regulate the placental specific expression of rPLII, by identifying the *cis*-acting (DNA sequences) and *trans*-acting (their binding proteins) factors that are involved in rPLII transcriptional regulation in placental giant cells. To accomplish this goal, I have the following objectives.

1. **To characterize the role of the 5' flanking region of the rPLII gene in placental expression.**
2. **To identify *cis*-acting elements involved in placental expression.**
3. **To identify and characterize the transcription factors which interact with the *cis*-acting elements.**

MATERIALS AND METHODS

1. CHEMICALS, REAGENTS, CELL LINES AND CLONES

Purchased materials:

GIBCO Life Technologies, Burlington, Ontario, Canada: RPMI-1640 medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin, phosphate buffered saline/calcium and magnesium free (PBS/CMF), trypsin/EDTA, HEPES buffer, NUNC culture dishes and flasks, agarose, NACS columns, custom-synthesized oligonucleotides, Taq DNA polymerase.

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

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

Amersham Pharmacia Biotech in Canada, Baie d'Urfe, Quebec: NICK™ columns, DNA markers (lambda DNA and φx 174 DNA), restriction endonucleases, Klenow DNA polymerase, T4 DNA ligase, nick translation DNA labelling kits, random prime DNA labelling kits, ^{3}H-Acetyl CoA, SEQUENASE DNA sequencing kit.

Promega Corporation, Madison, Wisconsin, USA: Wizard miniprep DNA purification kits, luciferase assay reagents, reporter lysis buffer (5X), some restriction endonucleases, fmol DNA Cycle Sequencing System, TNT^{R} SP6/T7-coupled reticulocyte lysate system, DNAse I (RQ).

Santa Cruz Biotechnology, Santa Cruz, California, USA: AP-1 consensus oligonucleotide, AP-1, and Ets2 antisera.

Qiagen, Mississauga, Ontario: Qiagen plasmid maxi kit and QIA prep spin miniprep kit.

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

Boehringer Mannheim, Laval, Quebec: calf intestinal phosphatase.

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

Fisher Scientific, Nepean, Ontario: Scintilene, Nitroplus membrane, guanidine isothiocyanate and general laboratory chemicals.

Mandel Scientific, Guelph, Ontario: ^{32}PdCTP, ^{35}SdATP, ^{32}PγATP.

Intersciences Inc., Markham, Ontario: Kodak XAR film.

MILLIPORE Corporation, Bedford, Massachusetts, USA: Ultrafree-MC 0.45 μm filter units.

Gift materials:

The rat choriocarcinoma Rcho cell line (Verstuyf et al. 1990) was kindly provided by Drs. A. Verstuyf and M. Vandeputte, Rega Institute for Medical Research, Catholic University of Louvain, Louvain, Belgium. The rat pituitary GC (West et al. 1987, Lefevre et al. 1987, Ingraham et al. 1988), human choriocarcinoma JEG (Kohler and Bridson, 1971) and JAR cell lines (Pattillo et al. 1971) were gifts from Dr. P. A. Cattini, Department of Physiology, University of Manitoba, Winnipeg, MB, Canada.

The clones and vectors were generously provided as follows:

Luciferase vector pXP2 (Nordeen 1988), a cytomegalovirus promoter/luciferase vector (CMVp.luc) and an 81 bp herpes simplex thymidine kinase promoter/luciferase vector, pT81luc (Nordeen 1988) from Dr. R.J. Matusik, Department of Urologic Surgery, Vanderbilt University, Nashville, TN, USA.

The vector pcDNA3 (INVITROGEN, Carlsbad, CA) and a cytomegalovirus promoter/chloramphenicol acetyltransferase construct (CMVp.*cat*) from Dr. R.P.C. Shiu, Department of Physiology, University of Manitoba, Winnipeg, MB, Canada.

Rat c-*Jun* and rat c-Fos cDNA clones (Rauscher et al. 1988) from Dr. T. Curran, St Jude Children's Research Hospital, Memphis, TN, USA.

The cDNA expression clones of hEts1, hEts2 (Watson et al. 1988), hElf-R (Aryee et al. 1998), and hELK-1 (Yang et al. 1998a,b) from Dr. P.A. Cattini. Elf-R and ELK-1 were originally gifts to Dr. P. A. Cattini from Dr. H. Kovar, Children's Cancer Research Institute (CCRI), St. Anna Kinderspital, Vienna, Austria; and Dr. A. D. Sharrocks, Department of Biochemistry and Genetics, The Medical School, University of Newcastle upon Tyne, United Kingdom respectively.

Human ESX (Chang et al. 1997, Neve et al. 1998) cDNA expression clone, Dr. C.C. Benz, Cancer Research Institute and Division of Oncology-Hematology, University of California at San Francisco, CA., USA.

Special equipment

Access to the TROPIX™ luminometer (BIO/CAN Scientific, Mississauga, Ontario) was kindly provided by Dr P. A. Cattini.

2. ISOLATION AND ANALYSIS OF PLASMID DNA

2.1 Plasmid transformation

Competent *Escherichia coli* 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 (Ausubel et al. 1994).

2.2 Large scale plasmid preparation

Transformed *Escherichia coli* cultures were grown in LB medium in the presence of ampicillin (Ausubel et al. 1989). Large scale preparations of plasmid DNA were carried out by alkaline/SDS lysis followed by centrifugation on a cesium chloride gradient as described in Sambrook et al. (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 sample at 260 nm using the formula: $1OD_{260}$ = 50 µg/ml DNA. The plasmid samples were stored at -20°C or -70°C.

2.3 Restriction enzyme mapping

A 6.6 Kb rPLII *HindIII* genomic DNA fragment, including approximately 4.5 Kb 5' flanking region and three exons had been previously cloned into the vector pVZ1 in our laboratory. The pVZ1 vector was modified from M13 Bluescribe plus by S. Henikoff to contain extra cloning sites at the 3' end of the poly linker. A variety of restriction enzymes were used to completely digest this clone. The sizes of the different bands were estimated using *HindIII*-digested lambda DNA and φx 174 DNA as markers. A unique *SacI* site was

identified at approximately -3000. A restriction enzyme map was developed as shown in Figure 2; established sites were used for subcloning.

2.4 Isolation of DNA fragments

Agarose gel electrophoresis was used to separate restriction enzyme-digested DNA fragments. The DNA band of interest was cut out of the gel, and put inside a piece of Spectrapor dialysis membrane (cut-off 6000-8000 MW^2) with 400 µl of 4X Tris/Acetate/EDTA buffer (AGB). The gel and buffer were laid between the electodes of a Biorad minigel apparatus in a small amount of buffer and a current was applied until the DNA was removed from the gel fragment as visualized under ultraviolet light. The DNA-containing buffer was collected, extracted with phenol/chloroform and precipitated with ethanol. Some DNA fragments were also isolated using Ultrafree-MC MLLIPORE filters, according to the supplier's protocol.

2.5 Subcloning of DNA fragments into plasmid vectors

To clone fragments into a vector for sequencing or reporter gene expression, ligations were performed as follows: For blunt-end and single enzyme digested fragments, the vector was treated with calf intestinal alkaline phosphatase (18 u) after restriction enzyme digestion to reduce self religation. Ligation reactions were carried out in 10 µl reactions containing 1µl of 10X ligation buffer (New England Biolabs), 1 µl of T4 DNA ligase, 20-50 ng of digested vector and approximately 3 fold molar excess of insert DNA. Ligation reactions were incubated at room temperature overnight, and heat denatured for 15 min at 65 °C; the final volume was brought up to 25 µl with TE pH 7.5. Five µl of the

ligation mix were used for transformation of 100 µl of competent cells. The transformation was performed by a standard method as outlined in Ausubel et al. (1989).

Clone selection: The resulting colonies were tested for the presence of insert by a small scale plasmid preparation using the alkaline lysis method as described in Sambrook et al. (1989), followed by a diagnostic restriction enzyme digestion.

2.6 Enzymatic DNA sequencing

All the new clones created in this thesis were purified using Wizard Minipreps and sequenced by either a SEQUENASE DNA sequencing kit or fmol DNA Cycle Sequencing System to check the boundaries for correct ligation. For clones constructed for sequencing, pBluescript SK (pBsp) was used as the vector and T3 and T7 primers were used as sequencing primers. For luciferase reporter clones, a luciferase primer, GLprimer2 (Promega), located at the very beginning of and complementary to the coding strand was used. The rPLII 5' flanking DNA was completely sequenced from a +64 *Pvu*II site in the first exon to -3031 at a *Sac*I site.

3. CONSTRUCTION OF HYBRID PLASMIDS

3.1 Subcloning strategies for the native rPLII 5' flanking DNA /luciferase constructs

-765 rPLIIp.Luc: The starting clone was 6.6 Kb rPLII pVZ1. A *Pvu*II/*Pvu*II fragment of rPLII 5' flanking DNA from -765 to +64 (Figure 2) was cloned into the *Sma*I site of pBluescript II SK (pBsp) in a 3' to 5' direction to form clone -765 rPLIIpBsp. A *Hind*III/*Bam*HI fragment of clone -765rPLIIpBsp was ligated into the *Hind*III and *Bgl*II cut luciferase vector, pXP2 (Nordeen 1988), to form -765 rPLIIp. Luc.

-4.5 rPLIIp.Luc: An rPLII 5' flanking *Hind*III/*Eco*RV fragment from approximately -4500 to -118 (Figure 2) was ligated to the -765rPLIIpBsp clone, cut with *Hind*III (in vector) and *Eco*RV (-118 in rPLII clone) to form clone 4.5rPLIIpBsp. A *Hind*III/*Bam*HI fragment from -4.5rPLIIp.Bsp was cloned into the *Hind*III and *Bgl*II sites of pXP2 to form -4.5 rPLIIp.Luc.

All the luciferase reporter clones below are shown in Figure 4 on page 64.

-3031 rPLIIp.Luc: A *Sac*I/*Bam*HI fragment from -4.5rPLIIpBsp was cloned into the *Sac*I and *Bgl*II sites of pXP2 to form -3031 rPLIIp.Luc.

-1729 rPLIIp.Luc: An *Eco*RI/*Bam*HI fragment (including -1729 to +64 of the rPLII gene) from -4.5 rPLIIpBsp was recloned into the *Eco*RI/*Bam*HI sites of pBsp to form clone -1729rPLIIpBsp. A *Hind*III/*Bam*HI fragment of -1729rPLIIpBsp was cloned into the *Hind*III/*Bgl*II sites of pXP2 to form -1729 rPLIIp. Luc.

-1435 rPLIIp.Luc: -1729rPLIIp. Luc was digested with *Bam*HI/*Bgl*II to remove the sequences between -1729 and -1435, then religated to form -1435 rPLIIp.Luc.

-118rPLIIp.Luc: An *Eco*RV/*Bam*HI fragment from -765rPLIIpBsp was cloned into the *Sma*I/*Bgl*II sites of pXP2 to form -118rPLIIp. Luc.

ΔErPLII.pluc: To produce an rPLII 5' flanking clone deleted for the 1109 bp *Eco*RI fragment, the -4.5rPLIIpBsp clone was digested with *Eco*RI and religated, this clone was further digested with *Sac*I/*Bam*HI and the fragment cloned into the *Sac*I/*Bgl*II sites of pXP2. This clone contains the sequences from -3031 to -2838 and -1729 to +64.

3.2 Subcloning strategies for *Eco*RI fragment/luciferase constructs

Further deletion analysis of the -2838 to -1729 *Eco*RI fragment was carried out to identify the placental-specific enhancing elements. Fragments were cloned in the luciferase vector pT81luc (Nordeen 1988), which contains a minimal 81 bp herpes simplex thymidine kinase promoter. The clones were constructed as follows: the rPLII 5' flanking *Eco*RI fragment was first cloned in both the 5' to 3' (E/E_F) and 3' to 5' (E/E_R) directions into pBluescript SK and orientation was determined by sequencing. The 5' to 3' clone E/E_F was digested with *Hind*III/*Sma*I and ligated into the *Hind*III/*Sma*I sites of pT81luc to form E/E_FTKpluc. The 3' to 5' clone E/E_R was digested with *Bam*HI/*Eco*RV and ligated into the *Bam*HI/*Sma*I sites of pT81luc to form E/E_RTKpluc.

3.3 Subcloning strategies for *Eco*RI sub-fragments /luciferase constructs

For all constructs, E/E_FTKpluc was used as the starting clone and pT81luc was used as the vector. These luciferase su-clones are shown in Figure 8 on page 73.

E/E_FTKpluc was first digested with *Hind*III/*Dra*I/*Sac*I which divided the *Eco*RI fragment into 3 subfragments. The *Hind*III/*Dra*I fragment, from -2838 to -2726 (F1), was cloned into *Hind*III/*Sma*I cut vector; the *Dra*I fragment, from -2726 to -2405 (F2), was cloned into *Sma*I cut vector; the *Dra*I/*Sac*I fragment, from -2405 to -1729 (F3), was cloned into *Sma*I/*Sac*I cut vector.

The F3TKpluc clone was further digested with *Bsa*BI and either *Hind*III or *Sac*I. The 5' *Hind*III/*Bsa*BI fragment, from -2405 to -2058 (F4), was cloned into *Hind*III/*Sma*I cut vector; the 3' *Bsa*BI/SacI fragment, from -2058 to -1729 (F5), was cloned into *Sma*I/*Sac*I cut vector.

The F5TKpluc was further digested with *Stu*I and either *Hind*III or *Sac*I. The 5' *Hind*III/*Stu*I fragment, from -2058 to -1880 (F6), was cloned into *Hind*III/*Sma*I cut vector; the 3' *Stu*I-*Sac*I fragment, from -1880 to -1729 (F7), was cloned into *Sma*I/*Sac*I cut vector.

The construction of the F7 TKpluc clone produced a polylinker with 2 *Bam*HI sites one on either side of the F7 fragment. This *Bam*HI fragment was isolated from F7TKpluc, digested with *Hph*I and blunt-ended with Klenow DNA polymerase. This blunt-ended fragment was further digested with *Hind*III and *Sca*I. The 5' *Hind*III/*Hph*I fragment, from -1880 to -1794 (F8), was cloned into *Hind*III/*Sma*I cut vector; the 3' *Hph*I/*Sac*I fragment, from -1794 to -1729 (F9), was cloned into *Sma*I/*Sac*I cut vector.

4. CELL LINES AND CULTURE CONDITIONS

Cell lines

The rat choriocarcinoma cell line, Rcho, was routinely grown for 4 days after plating in RPMI-1640 medium with 20% fetal bovine serum (FBS) supplemented with 50 μM β-mercaptoethanol, 1 mM sodium pyruvate, 50 u/ml of penicillin and 50 μg/ml of streptomycin (20% FBS-RPMI 1640). For the routine maintenance of the cell line, the cells were always in 20% FBS-RPMI 1640 medium and were passaged at greater than 90% confluence every 2 - 3 days. To promote differentiation for expression experiments, cultures were shifted into NCTC-135 medium (Shida et al. 1993) with 10% FBS supplemented as above (10% FBS-NCTC 135), and maintained in this medium for the length of the experiment.

Human choriocarcinoma JEG and JAR cells were routinely grown in RPMI-1640 medium with 10% fetal bovine serum (FBS) supplemented with 50 u/ml of penicillin, 50 μg/ml of streptomycin and 500 μM glutamine.

The rat anterior pituitary tumour GC cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS supplemented with 0.4 mM L-glutamine, 50 u/ml of penicillin and 50 μg/ml of streptomycin (10% FBS-DMEM). Cells were passaged at less than 80% confluence to prevent cells from lifting.

For routine maintenance of tissue culture cell lines, medium was changed every other day. To passage the cells, the growth medium was aspirated and the plate surfaces were rinsed with PBS; 0.05% trypsin/0.53 mM EDTA solution (3 ml/10 cm plate) was added and incubated for 1-2 min at 37°C. The serum-containing medium was added to wash off cells and stop the effect of trypsin. Cells were collected by centrifuging for 3 min at 800 rpm at room temperature. All cell cultures were incubated in a 37°C incubator containing 5% CO_2. Cultures were routinely maintained on 10 cm plates. When cells were grown for nuclear extract preparation, the cells were grown on 15 cm plates.

5. TRANSIENT TRANSFECTIONS

5.1 Standard transfection experiments.

For all cultures, transfections were carried out routinely in 10 cm plates. Ten μg of supercoiled luciferase reporter clone or vector were transiently transfected into the cultures using a calcium phosphate procedure 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 24 h, followed by gene expression for 24 h. For Rcho cultures, the cells were grown

to 6 or 14 days after plating when the majority of the cells were differentiated. Gene transfer was carried out in 10% FBS-NCTC 135 medium, then replaced with 10% FBS-RPMI 1640 growth medium. The Rcho cells were harvested 48 h after the start of gene transfer.

The JEG and JAR cells were also switched in to 10% FBS-NCTC 135 during the 24 hours gene transfer, then switched back to 10% FBS-RPMI1640 for a further 24 h to allow gene expression. The cells were harvested 48 h after the start of gene transfer.

Pituitary GC cells were transfected when cultures were 30 to 40% confluent. A two minute 20% glycerol shock was given 6 h after the initiation of gene transfer (Cattini et al. 1986). Cultures were washed twice with PBS and given 10% FBS-DMEM. The GC cells were harvested 48 h after the glycerol shock.

5.2. Overexpression experiments

Five µg of the different hybrid expression plasmids were cotransfected with 10 µg of luciferase vector into day 6 Rcho cells. The total amount of transfected plasmid was kept constant at 25 µg or 15 µg in the different experiments. All samples were co-transfected with 1 µg CMVp.*cat* for determination of plasmid uptake. Other procedures were the same as for the standard transfections.

6. REPORTER GENE ASSAY ANALYSIS

6.1 Preparation of extracts

Cultures were rinsed with calcium and magnesium free PBS, then lysed with Promega reporter lysis buffer according to the supplier's protocol. This reporter lysis buffer is suitable for both luciferase and CAT assays, and luciferase protein is more stable in this buffer than 100 mM Tris pH7.8/0.1% TritonX100 buffer. The luciferase assay was performed immediately and the remaining cell extracts were frozen at -70°C until used for protein and CAT assays.

6.2 Luciferase assays

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

6.3 Chloramphenicol acetyltransferase (CAT) assays

The CAT activity was measured by a two-phase fluor diffusion assay (Ausubel et al. 1989). Briefly, 200 μg of cell extract were added to a 7 ml scintillation vial with sufficient cell lysis buffer (100 mM Tris pH7.8/0.1% TritonX100) to give a total volume of 200 μl. The solution was heated for 15 min at 65°C to inactivate some CAT inhibitors, then cooled to room temperature. A reaction mix (75 μl), containing 2 μl of [^{3}H] acetyl-CoA (0.5 μCi/assay), 50 μl of 5 mM chloramphenicol, dissolved in water, 7.5 μl of 1 M Tris/HCl pH 7.8, and 15.5 μl of water were added. The reaction mixture was carefully overlaid with 3 ml

of organic-phase 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/mg protein.

6.4 Quantitation of Proteins

The samples for a standard protein curve were prepared using 1-20 μg BSA. Five μl of 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 according to the manufacturer's instructions. The mixtures were incubated at room temperature for at least 10 min. The OD was measured at 595 nm wavelength.

7. NUCLEAR EXTRACT PREPARATION

7.1 Tissue and cell preparations

Rat placentae were collected at day 16 to day 18, and the labyrinth region was dissected and frozen immediately on dry ice. Frozen placentae were grounded to fine powder in a mortar and pestle placed on dry ice. The volume of the tissue powder was measured for determining the volume of buffers used for preparing nuclear extracts.

Rcho cells were grown for 6 days in twenty 15 cm plates. To enrich for the transcription factors expressed in differentiated giant cells, the small undifferentiated cells were first removed by a short 0.25% trypsin pretreatment before giant cells were collected. GC cells were grown to confluence in twenty 15 cm plates. Rcho giant cells and GC cells were finally collected using PBS without Mg^{++} and Ca^{++}/1mM EDTA and centrifuged at

3000 rpm for 10 min at room temperature. The volume of cell pellets was carefully measured. Pellets were frozen at -70^{0}C until used.

7.2 Isolation procedures

Nuclear extracts were isolated from placental tissue and cell lines according published protocols (Dignam et al. 1983) with slight modifications. Cells were thawed completely on ice (~10 minutes), then resuspended gently with 5 packed cell volumes (pcv) of ice cold buffer A to swell the cells. An International HN-S desktop centrifuge with a swing-out rotor was used in the first few steps. The suspended cells were homogenized in a 40 ml loose homogenizer, then centrifuged at 3000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 2 pcv of buffer A, then transferred to a 15 ml Dounce homogenizer. The cells were gently broken by 10 strokes with a B pestle at first; more strokes were given when necessary. The homogenization was monitored under the microscope to make sure most cells were lysed and uniform nuclei could be seen in a streaming cytoplasm. This nuclear suspension was transferred to a 30 ml Oakridge tube and centrifuged at 3000 rpm for 15 min. The supernatant was carefully decanted. The loose pellets were further centrifuged at 14,500 rpm for 20 min at 4°C with a JA-20 rotor in a Beckman J2-21 centrifuge. This step removes any remaining cytoplasm and medium from the cell pellets that may contain proteases that will interfere the quality of the nuclear extracts. The supernatant was decanted, and the nuclei were resuspended in 1/2 pcv of buffer C and transferred to 10 ml Oakridge tubes. The mixture was gently mixed on a rocking platform Nutator at 4°C for 30 min. The mixture was then centrifuged at 15000 rpm for 30 min at 4°C with a JA21 rotor in a Beckman J2-21 centrifuge. The supernatant was

transferred to Spectrapor dialysis tubing (6000-8000 MW[2] cut-off) and dialysed against buffer D at 100X volume of the nuclear extract supernatant for 3 hours at 4°C. The insoluble materials were removed by centrifuging at 15,000 rpm for 30 min in a JA21 rotor. Protein concentration of the supernatant was determined by Bio-Rad assay, and frozen in 20 - 30 μl aliquots at -70°C.

7.3 Buffers

The buffers used in this assay are as follows:

Buffer A: 10 mM HEPES-KOH pH 7.9

1.5 mM $MgCl_2$

10 mM KCl

Just before use 0.5 mM DTT, 1 mM PMSF and 2 μg/ml aprotinin were added.

Buffer C: 20 mM HEPES-KOH pH 7.9

25% (v/v) glycerol

0.42 M NaCl

1.5 mM $MgCl_2$

0.2 mM EDTA

Just before use 0.5 mM DTT and 1 mM PMSF were added.

Buffer D: 20 mM HEPES-KOH pH 7.9

20% (v/v) glycerol

0.1 M KCl

0.2 mM EDTA

Just before use 0.5 mM DTT and 1 mM PMSF were added.

8. DNASE I PROTECTION ASSAYS

DNAse I protection assays of the 329 bp rPLII 5' flanking *BsaBI/EcoRI* fragment (F5) were carried out according to standard protocols (Allegretto et al. 1990). The F5TKpluc clone was digested at an *XhoI* site in the pT81luc polylinker and treated with calf intestinal phosphatase. The fragment was labelled at the 5' end of anti-sense strand with T4 polynucleotide kinase and $^{32}P\gamma ATP$ (Ausubel et al. 1989), then released by *HindIII* digestion. This probe fragment was purified by agarose gel electrophoresis, followed by electroelution and ethanol precipitation. Initially 10 - 20 μg of plasmid DNA was digested and the molarity of the DNA was calculated. The loss of DNA at each gel purification was estimated as 50%, the final molarity of DNA was used to estimate the amount of labelled probe. Binding reactions were carried out in a final volume of 20 μl containing approximately 20,000 cpm (estimated 5-10 fmoles) of fragment and 0.5 μg dI:dC. Increasing amounts of placental labyrinth and Rcho nuclear extracts (10 to 80 μg) or GC nuclear extracts (20 and 40 μg) were incubated with the probe on ice for 15 min followed by digestion with 0.05 units of DNAse I for 90 seconds at 26°C. After phenol/chloroform extraction and precipitation, the digested products were fractionated on a denaturing 6% polyacrylamide/urea gel. Maxam-Gilbert G and G+A sequencing reactions of the end-labelled DNA (Ausubel et al. 1989), run on the same gel, were used to identify specific DNAse I protected regions. The gels were dried and exposed to Kodak XAR film for autoradiography.

The probe DNA was searched for transcription factor binding sites using the TFMATRIX transcription factor binding profile database, and TFSEARCH ver.1.3 (c)1995 Yutaka Akiyama (Kyoto Univ.).

9. PCR SITE-DIRECTED MUTAGENESIS

When sequences within or near the DNAse I protected region were analyzed for transcription factor binding sites, consensus or near-consensus binding sites were found for Ets, AP-1 and GATA factors. To elucidate the functional importance of the DNAse I protected sequences, a PCR mutagenesis strategy was used to mutate these putative binding sites. The rPLII 5' flanking *StuI/Eco*RI fragment (F7) was cloned into the *SmaI/Sac*I sites of pBspKS. Three mutagenic primers were synthesized as follows:

mEts: GCGCGAGCTCGAATTCAAGCCCTACT**gaa**TGTTTACCCTTGAGCA

mAP-1: GCGCGAGCTCGAATTCAAGCCCTACTTCCTGTTTACCCTT**ctag**AA ATAACCCTGGGAAATG

mGATA: GCGCGAGCTCGAATTCAAGCCCTACTTCCTGTTTACCCTTGAGCA AATAACCCTGGAAATGCGTAAAACACAT**cgg**TACATA**cg**TTACTCACC

Sequences are shown 5'->3'; mEts mutates a consensus Ets binding site, mAP-1 mutates a putative AP-1 site and mGATA mutates two putative GATA sites, all shown in lower case. In addition to the mutated sequences, each primer included a *Sac*I site and a four base pair GCGC extension at the 3' end of the primers (underlined) to facilitate directional cloning.

The PCR reactions were according to the standard protocol in Current Protocols in Molecular Biology (1989). The PCR reaction mixes include: 1X PCR buffer (GIBCO

BRL), 25 pmoles of each dNTP, 100 ng DNA template, 10 pmoles each of M13 reverse primer and one of the mutagenic primers (mEts, mAP-1 or mGATA), 2.5u Taq DNA polymerase (GIBCO BRL) in a total volume of 50µl. A 45°C annealing temperature was used to suit both the M13 reverse primer and mutant primers. PCR was performed for 25 cycles to reduce errors. PCR fragments were isolated by agarose gel electrophoresis and electroelution, then digested with HindIII/SacI and cloned into these sites in pBspKS for sequencing to confirm that only the expected mutations had been introduced. The correctly mutated *Hind*III/*Sac*I fragment was cloned into *Hind*III/*Sac*I digested pT81luc for testing in transfection assays.

The double Ets/AP-1 mutant was generated by PCR using the mAP-1 primer with the mEts.pBsp clone. PCR product was cloned into pBspKS as described above for the single mutant to facilitate sequencing, followed by cloning of the correct fragment into pT81luc.

10. RNA ANALYSIS

10.1 Total RNA isolation

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.

10.2 RNA blotting

For RNA blots, 30 μg of total RNA was mixed with 1.5 μl 10X RNA running buffer (0.2M MOPS, 50mM sodium acetate, 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 10X loading buffer was then added. The samples were separated by electrophoresis on 1.2% agarose/2.2 M formaldehyde gels with ethidium bromide according to the standard protocol in Current Protocols in Molecular Biology (Ausubel et al. 1989). 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 membrane. The next day, the blots were soaked briefly in 2X SSC to reduce salt. The membranes were air-dried and baked for 2h at 80°C under vacuum.

10.3 Probe labelling

Labelling of cDNA probes with ^{32}P-dCTP: All nick translation or random prime DNA labelling 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™ 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 adding the probe to the hybridization solution, it was boiled for 5 min and cooled rapidly on ice to separate the two DNA strands.

10.4 Hybridization

The RNA blots were prehybridized at least 2 h at 42°C in 50% (v/v) deionized formamide, 4X SSC, 0.4X Denhardt's solution (0.4X DH = 0.08% each of BSA, Ficoll 400 and polyvinylpyrrolidone 360), 10mM Na_2HPO_4 pH 7.4, 0.1% SDS, 1mM EDTA pH 8.0, 250 μg/ml sonicated salmon sperm DNA .

Hybridizations were performed in the same solution, containing ^{32}P-labelled cDNA probes for 16-20 h at 42°C. The membranes were washed twice in 2X SSC and 0.1% SDS for 15 min at 65°C. An extra wash in 0.2X SSC and 0.1% SDS for 15 min at 65°C was performed if necessary. For re-use, blots were stripped in boiling 0.1X SSC and 0.1% SDS for 3 min.

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

11. ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSA)

The 65 bp F9 fragment which had been cloned into the *Sma*I/*Eco*RI sites of pBspKS was cut out with *Eco*RI, electroeluted from an agarose gel, ethanol precipitated and end-labelled by a fill-in reaction with Klenow fragment and dATP^{32} as described in Current Protocols in Molecular Biology (Ausubel, 1989).

Recombinant rat c-Jun, rat c-Fos, hEts1, hEts2 and hESX were synthesized using an *in vitro* transcription/translation-coupled reticulocyte lysate system according to the supplier's protocol (Promega). SP6 polymerase was used for rat c-Jun and rat c-Fos, and T7 polymerase was used for hEts1, hEts2 and hESX. Nuclear extracts were made as described

above. Four μl each of the *in vitro* translated mixtures, 20 μg of rat placental nuclear extract or 5 μg of GC extract were used in a binding reaction (appropriate amounts determined by titration). Reactions were carried out in a volume of 20 or 40 μl with binding buffer containing 20 mM HEPES buffer, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 5mM $MgCl_2$, 1 mM PMSF, 0.5 μg dI:dC. Specific or non-specific competitors were added and incubated for 15 minutes before the labelled probe; Jun or Ets2 antisera were added 45 minutes before the probe. 10 to 20 fmoles of labelled probe (1-2 x 10^4 dpm) was added and incubated for a further 30 minutes. All binding reactions were at room temperature. Complexes were separated by electrophoresis on 4% non-denaturing polyacrylamide gels. Gels were dried and exposed for autoradiography. The specific oligonucleotide competitors used were as follows (coding strand):

AP-1 consensus: CGCTTGATGACTCAGCCGGAA

Ets2 consensus (Stacey et al. 1995): CTAGGACCGGAAGTGGGAGT

mAP-1/mEts: CCAGGGTTATTTctagAAGGGTAAACAttcAGTAGGGCTTG

12. STATISTICS

All the statistics were done by an unpaired student's t-test in the SigmaStart program (Jandel Scientific). The definition of statistical significance was $p < 0.05$ or $p<0.001$.

RESULTS

1. RAT PLII GENE 5' FLANKING DNA SEQUENCE ANALYSIS

1.1 Gene mapping

An rPLII genomic clone, GCI, was previously isolated in our laboratory from a rat liver lambda bacteriophage library (Shah et al. 1998). The GCI clone is approximately 18.5 Kb in length and contains the entire rPLII coding region as well as 5' and 3' flanking sequences. A 6.6 Kb *Hind*III fragment, including approximately 4.5 Kb of 5' flanking region and three exons from GCI, was previously subcloned into the vector pVZ1. A variety of restriction enzymes were used. Several diagnostic enzyme sites were identified. The restriction enzyme map of GCI is shown in Figure 2.

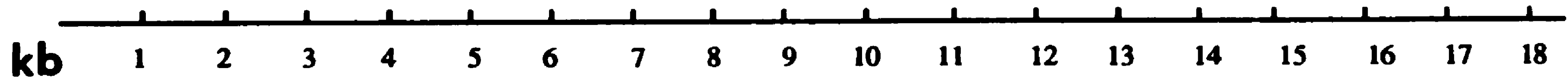
1.2 Sequencing 3 Kb of 5' flanking DNA

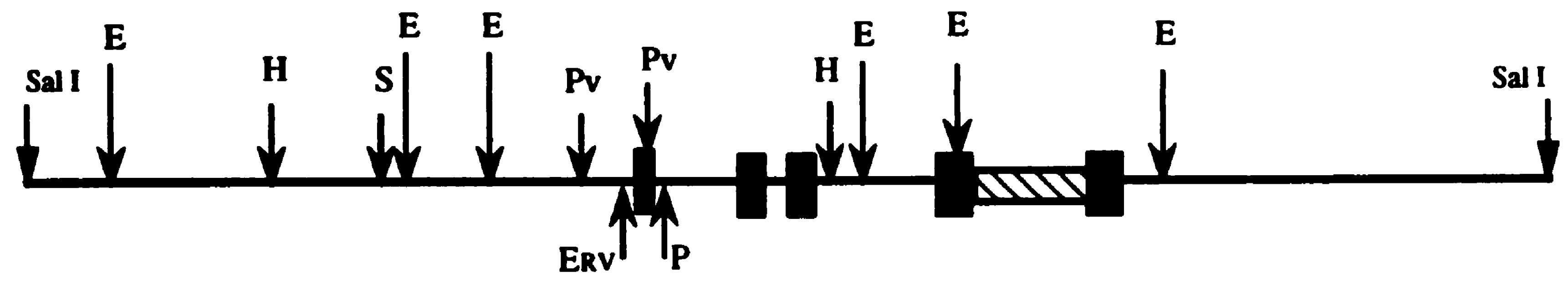
The transcription start site of the rPLII gene was previously determined by primer extension (Shah et al. 1998). To sequence the rPLII 5' flanking DNA fragment between a *Sac*I site at approximately -3000 and the transcription start site, restriction enzyme fragments were subcloned into pBluescript SK vector. An approximately 1.1 Kb *Eco*RI fragment was further subcloned into pBluescript SK vector, and the two ends of the fragment were sequenced. Using this established sequence, a pair of sequencing primers (Forward: TTCAATCGGGTAGACAGGT; Reverse: GGTGATTTAGTTCTTTGGGG) were designed which allowed the entire *Eco*RI fragment to be sequenced. The Genbank database accession number for this rPLII 5' flanking sequence and exon 1 is AF026294.

Figure 2

Figure 2. Restriction enzyme map of rPLII genomic clone, GCI.

The scale above the restriction map is shown in Kilobases (Kb). The entire genomic DNA in the GCI clone is about 18 Kb. Five black boxes represent exons. The hatched box represents a region containing Alu repetitive sequences. *Sal*I sites at both ends of the clone flank the λEMBL3 cloning cassette. Restriction enzyme sites are described as follows: E - *Eco*RI, H - *Hind*III, S - *Sac*I, Pv - *Pvu*II, E_{RV} -*Eco*RV, P - *Pst*I.


kb
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18


Sal I
E
H
S
E
E
Pv
Pv
ERV
P
H
E
E
E
Sal I

The sequence around the transcription start site is shown in Figure 3. The TATA box is located at -29 to -34 upstream of the transcription start site. The *PvuII* site at +64 in exon 1 was used as the 3' end in all native promoter constructs tested.

2. FUNCTIONAL ANALYSIS OF THE NATIVE 5' FLANKING SEQUENCES

2.1 Transfection studies

To characterize the role of the 5' flanking region of the rPLII gene in placental expression, three rPLII 5' flanking DNA fragments from approximately -4500, -3031 and -765 to +64 were subcloned into the luciferase reporter vector, pXP2, and transiently transfected into the rat choriocarcinoma Rcho cell line and the rat pituitary GC cell line (West et al. 1987, Lefevre et al.1987, Ingraham et al. 1988). The GC cells represented a non-placental cell type. The results are shown in Figure 4. The luciferase vector, pXP2, was used as a promoterless control. Rcho cells were transfected at day 14, and harvested at day 16. This time represents a period during which rPLII mRNA is highly expressed in Rcho cells (Sun et al. 1998). The cell lysates were assayed as outlined in Materials and Methods. Both the -4500 and -3031 constructs, but not the -765 construct, showed significantly higher luciferase activity than the control ($P<0.05$) in Rcho cells. The activities of the -4500 and -3031 fragments were comparable and not significantly different ($P>0.05$). These results indicated that the region between -765 to +64 did not contain the necessary information to direct luciferase expression in placental Rcho cells. The -3031 to +64 fragment was, however, sufficient to induce a similar level of expression to the -4500 to +64 fragment in placental Rcho cells. None of the fragments tested were able to induce significant levels of expression in pituitary GC cells.

Figure 3

Figure 3. Features of the rPLII gene in the vicinity of the transcription start site.

Transcribed regions of the gene are shown in uppercase letters; the translated sequences of first exon are indicated in single letter codes; intron sequences are shown in lowercase letters. The transcription start site is shown as a bent arrow; this site was determined by primer extension (Shah et. al. 1998). A putative TATA box is shown in an open box. The *Pvu*II site in exon I was used in all rPLII 5' flanking reporter constructs.

tggacactcttgcaaactgtaaaacaattcatgcagaaacaggactgtaacaagagtaaggaatattttatacttccta
cagggatatcatctagtaacctttaccttgacaaaacacatacttccctgtgcctggtgacctgcatgaccttggggaga

transcription start

atctgttagtatatatggcaggtgtagaggctgagggaaggcagcACTCAGGGAGCAGAAGCCAGTCTAGTGCTGTCTTC

PvuII

primer extension

ACGTCCGAGAGAACTCCTCAAAG **ATG CAG CTG TCT TTG ACT CAA CCA TGC TTC T** gtaagtagctctgc

M Q L S L T Q P C F

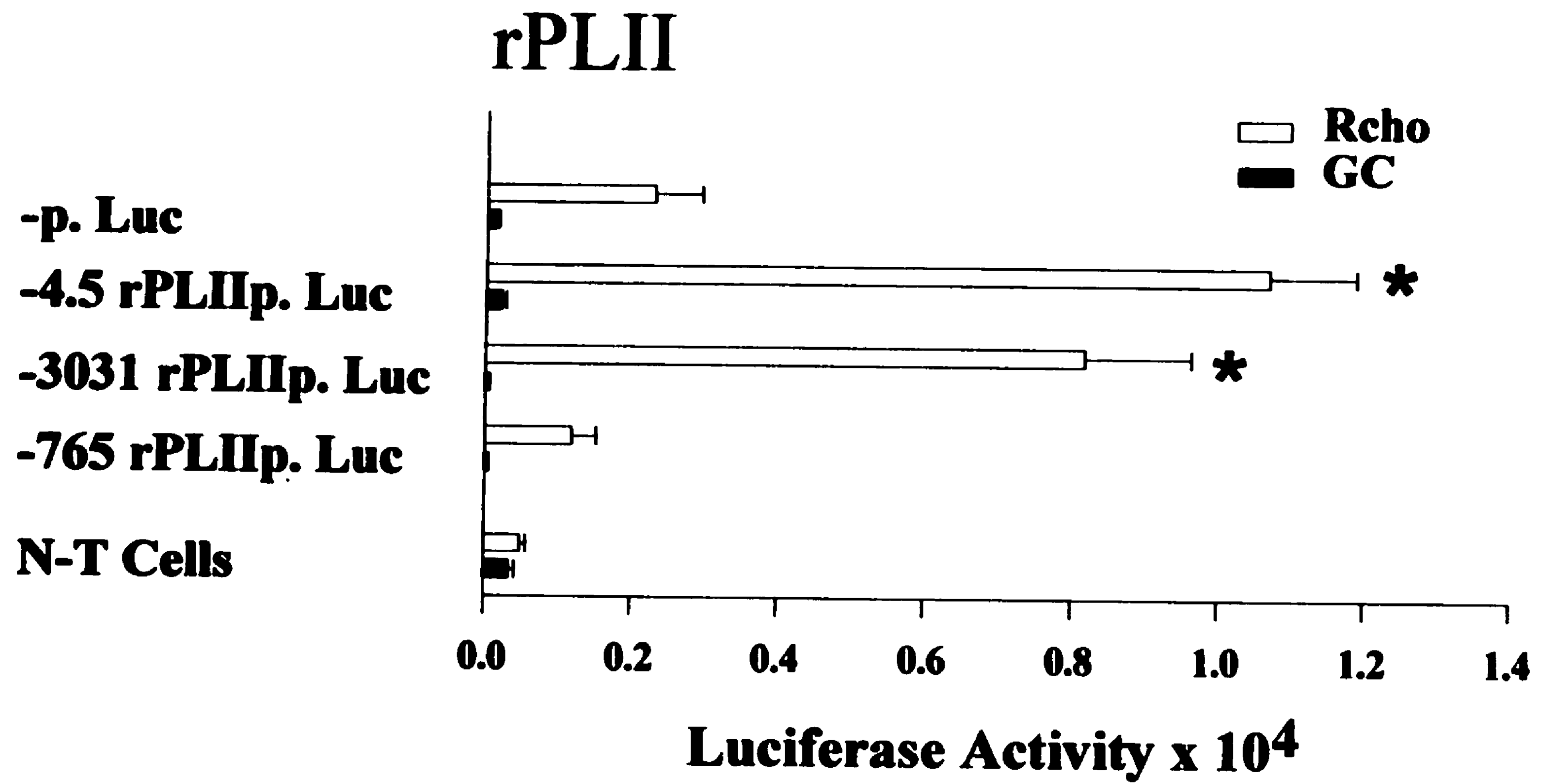
agccatactgcgcttcattactatgctttctcatgatgattgtttgcatcaaatacagtctttgttgctttctgtgtgct

gtagtatttggagaactgttgctttccatgactaaccaagatataagtccacattgaactgagaattagaattt......

Figure 4

Figure 4. Expression of rPLII 5' flanking hybrid luciferase constructs in Rcho and GC cells.

Luciferase reporter constructs containing appoximately 4500bp, 3031 bp, or 765bp of 5' flanking DNA were cloned into pXP2. All the constructs end at the *Pvu*II site at +64. These constructs were transiently transfected into differentiated rat trophoblast Rcho cells or rat pituitary GC cells. The Rcho cells were transfected at day 14 after plating and harvested at day16. The GC cells were transfected at 30% to 40% confluence, and harvested 48 hours later when the cultures were close to confluence. The final luciferase activity is presented as light units per mg protein (mean$\pm$SEM). All values are corrected for plasmid uptake as described in Materials and Methods. -pLuc denotes the promoterless vector, pXP2; N-T cells denote non-transfected cells. Results are from four separate experiments (n = 12 for each construct; n = 6 for nontransfected cells). Statistical significance ($P<0.05$) is presented as *.


rPLII
Rcho
GC
-p. Luc
-4.5 rPLIIp. Luc
-3031 rPLIIp. Luc
-765 rPLIIp. Luc
N-T Cells
*
*
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Luciferase Activity x 10⁴

2.2 Transgenic studies

To test whether the -3031 to +64 region was sufficient to target reporter gene expression specifically to the placenta, the -3031 rPLIIp. Luc construct was used to develop F0 (transient) transgenic embryos. A fragment containing the -3031 to +64 rPLII DNA/luciferase reporter gene was excised free of vector sequences by a *SacI/PstI* digestion. The fragment was isolated from an agarose gel, further purified on a NACS column and concentrated by ethanol precipitation.

Insert was injected at a final concentration of 3 ng/µl into the pronuclei of one cell CD1 mouse embryos according to standard protocols (Hogan et al. 1994). Injected embryos were replaced into the oviducts of day 1 pseudopregnant CD1 females. The recipient females were sacrificed between day 14 and day 16 of pregnancy. F0 placenta and fetuses were collected, and the fetuses divided into head, thorax and abdomen regions. The transgenic placental/fetal units were identified by Southern blot; nine out of forty-five were transgenic.

The luciferase expression was measured in placenta and the three fetal regions of identified transgenic fetuses. All transgenic placenta showed some level of luciferase expression. The results are summarized in Table 3 (Shah et al. 1998). Only three fetuses showed luciferase expression in all three body regions, and only one of these at a high level. There appeared to be no correlation between level of expression and copy number (Southern data not shown). This transgenic study indicated that the sequences within the -3031 bp 5' flanking region could target expression to the placenta although the ectopic expression

Table 3

Table 3. Luciferase activity in transgenic placentas and fetuses

Transgenic fetus/placenta [a]	Day of pregnancy [b]	Placenta	Luciferase activity (units/mg protein x 10^3) [c] Head	Thorax	Abdomen
P1	day 14	33.6	nd	nd	nd
P2		0.77	0.3	0.2	0.76
P3	day 15	3.6	nd	nd	nd
P4		70.6	0.07	0.04	nd
P5*		0.13	nd	nd	nd
P6		2.8	nd	nd	nd
P7		0.9	3.3	nd	nd
P8	day 16	5.2	nd	nd	nd
P9		68.5	447	759	1186

[a] Transgenic fetal/placental units were identified by Southern blot hybridization. Nine out of forty-five were transgenic.

*indicates a fetus which appeared to carry < 1 copy of the transgene, potentially a mosaic.

[b] Fetal/placental tissue was collected on days 14 to 16 of pregnancy where day 0 is the day of mating. mPLII is highly expressed during this period.

[c] All transgenic placenta showed varying amounts of luciferase activity as measured in relative light units/mg protein. Fetuses were divided into three regions which were tested individually for luciferase expression. "nd" indicates no detectable luciferase activity.

suggested that there are other regulatory elements located outside of the 3031 bp 5' flanking region that will also be important for placental-specific expression.

These *in vitro* and *in vivo* functional studies both indicated, however, that the 3031 bp rPLII 5' flanking fragment was sufficient to direct luciferase reporter expression in placental cells, but not in the non-placental cells, suggesting this region contained sequences important for placental-specific expression (Shah et al. 1998).

3. IDENTIFICATION OF AN ENHANCER REGION IN THE rPLII 5' FLANKING DNA

3.1 Deletion analysis of native promoter

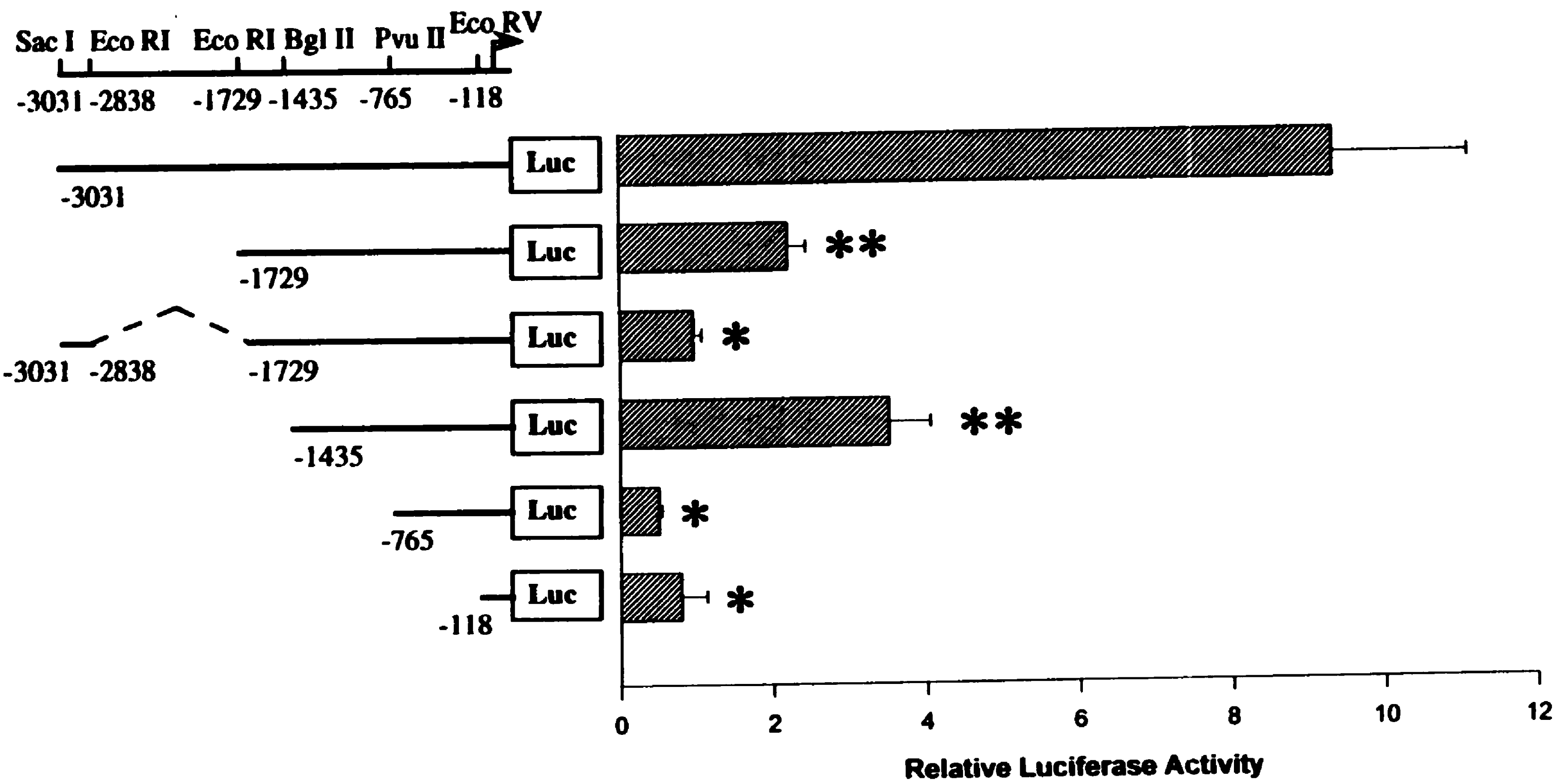
To identify the region(s) within the 3031 bp 5' flanking DNA sequence important for expression in placental giant cells, a series of subfragments of 5' flanking DNA (-1729, -1435, -118 to +64) were cloned into the luciferase reporter vector, pXP2. A deletion construct was also developed by removing an *Eco*RI fragment (-2838 to -1729) from -3031rPLIIp.Luc. These constructs were transfected into Rcho cells at day 14 after plating. A map of the luciferase constructs and the transfection data are shown in Figure 5.

Both the -1729 and -1435 constructs produced significantly higher levels of luciferase expression than the promoterless vector control ($p<0.05$), but their expression levels were significantly lower than that of the complete -3031 fragment ($P<0.05$). Most interestingly, the -3031 construct deleted for the *Eco*RI fragment produced even lower activity, showing no significant difference with the promoterless vector control. The activity of the deleted construct was lower than that of -1729 construct ($P<0.05$), suggesting there were repressor elements located between -3031 and -1729. The activity of the -1435 construct was higher

Figure 5

Figure 5. Deletion analysis of 3031 bp rPLII 5' flanking region.

A restriction enzyme map of this region is shown on the top left, and the bent arrow indicates the transcription start site. A series of deletion fragments were cloned into the luciferase reporter vector, pXP2, and tested for expression in Rcho cells by transient transfection. The 3' end of all constructs is nucleotide +64. The Rcho cells were transfected at day 14 after plating, and harvested at day 16. Luciferase activity was measured in light units/mg protein. Results are presented as relative luciferase activity, which is measured by mean $\pm$ SEM expressed relative to the pXP2 vector control, which was set as 1. All values have been corrected for plasmid uptake as described in Materials and Methods. Results are from two to four experiments where the number of separate transfections varied from 6 to 12. Asterisks indicate constructs that are significantly less active than the entire 3031 bp 5' flanking DNA, indicating the loss of enhancing sequences. Double asterisks indicate constructs that they also have significantly higher activity than the construct deleted for -2838 to -1729 (an *Eco*RI fragment), suggesting the presence of inhibitory sequences between -3031 to -2838 and -1729 to -1435.



Sac I
Eco RI
Eco RI
Bgl II
Pvu II
Eco RV
-3031
-2838
-1729
-1435
-765
-118
Luc
-3031
-1729
-3031
-2838
-1729
-1435
-765
-118
**
*
**
*
*
0
2
4
6
8
10
12
Relative Luciferase Activity

than that of -1729 ($P<0.05$), suggesting that further repressing elements may be located between -1729 to -1435. The -765 and -118 constructs showed no significant difference from the promoterless vector control, suggesting that the -1435 bp fragment contained the minimum information necessary to direct placental expression.

3.2 Functional analysis of the enhancer region

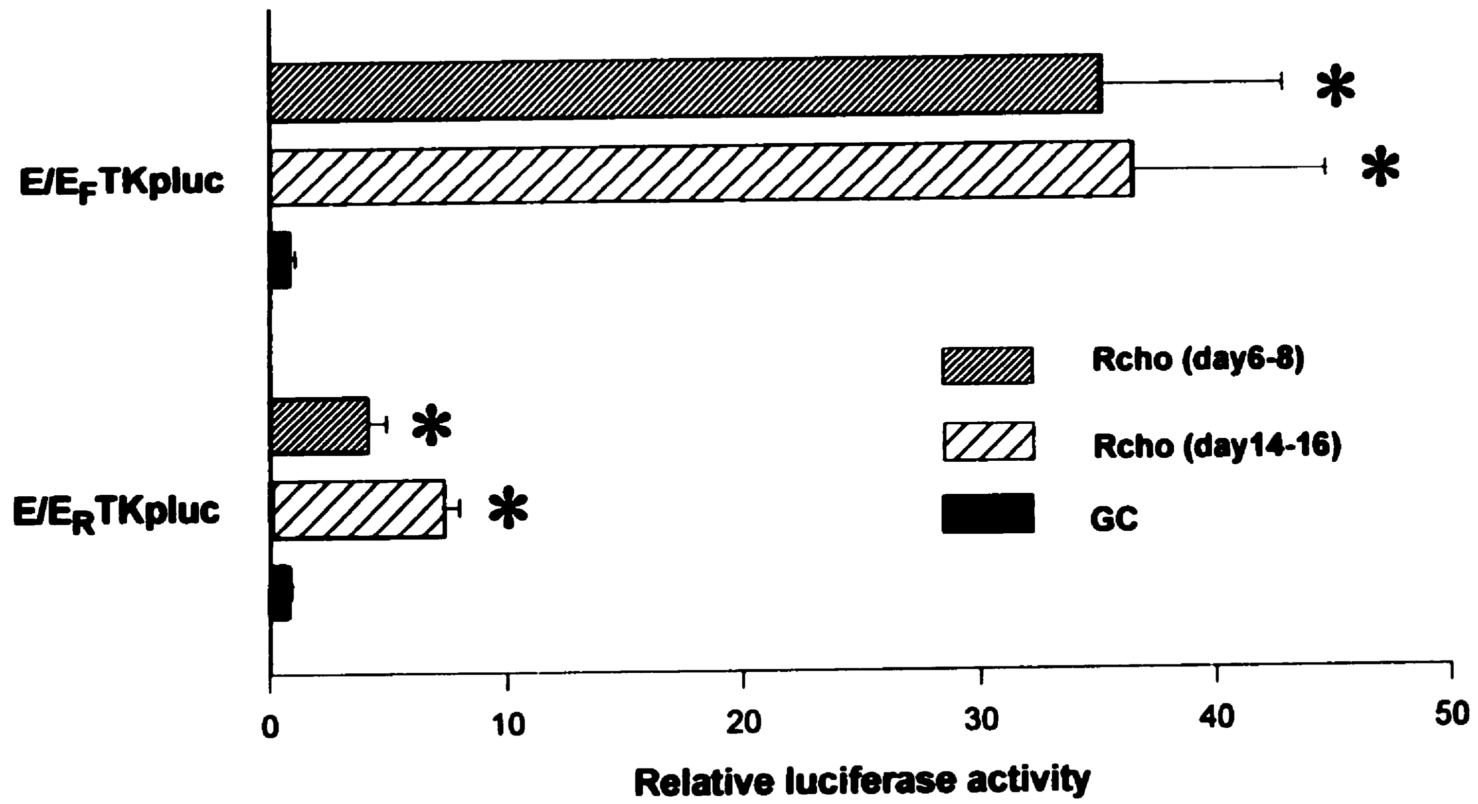
To test whether the sequences within the *Eco*RI fragment (-2838 to -1729) could function as a tissue specific enhancer, the *Eco*RI fragment was cloned in the forward and reverse orientations into a luciferase vector, pT81luc (TKpluc), which contains a minimal thymidine kinase promoter (Nordeen 1988). Previous experiments showed that rPLII expression in Rcho cultures was barely detectable before day 10, but was highly expressed after day 14 (Shah et al. 1998). The forward (E/E_FTKpluc) and reverse (E/E_RTKpluc) constructs were transfected into Rcho cells at day 6 and day 14 after plating, when we had previously shown rPLII expression in Rcho cells to be either very low or high. The pituitary GC cell line was used as a non-placental cell control.

Figure 6 shows that the -2838 to -1729 fragment was active with a minimal thymidine kinase promoter. Even though the level of activation of the forward and reverse orientation constructs was different, luciferase expression in both orientations was significantly higher then that of the pT81luc control alone ($P<0.05$). There were no significant differences in activity between Rcho cells transfected at day 6 or day 14 ($P>0.05$). Neither construct showed significant expression in non-placental GC cells. This fragment was effective with the heterologous TK promoter when placed in either orientation only in the Rcho cells, suggesting that it contained tissue-specific enhancer elements. The enhancing effects were

Figure 6

Figure 6. Sequences between -2838 to -1729 act as a placental cell-specific enhancer.

The *Eco*RI fragment containing sequences from -2838 to -1729 was cloned in the 5' to 3' (E/E_FTKpluc) and 3' to 5' (E/E_RTKpluc) orientations into a luciferase vector pTK81luc, which contains the minimal 81 bp *Herpes simplex* thymidine kinase promoter. Constructs were transfected into Rcho cells grown for either 6 or 14 days after plating and GC cells at 30-40% confluence. Cell lysates were harvested 48 hours after the beginning of the gene transfer. Results are from four separate transfections. Luciferase activity (mean $\pm$ SEM) is expressed relative to vector control pTK81luc, which was set as 1. Both orientations of this fragment produced significantly higher levels of luciferase activity than the controls in Rcho cells (*, $P<0.05$). There was no difference in activity between these Rcho cells cultured for 6 days or 14 days.


E/E$_F$TKpluc
E/E$_R$TKpluc
*
Rcho (day6-8)
Rcho (day14-16)
GC
0
10
20
30
40
50
Relative luciferase activity

detected in both early (when rPLII is low) and late Rcho cultures (when rPLII is high), suggesting this fragment alone may not be sufficient to define the normal temporal expression of rPLII as seen in placenta.

To study whether the -2838 to -1729 fragment could have a placental-specific enhancing effect in other species, E/E$_F$TKpluc was transiently transfected into the human choriocarcinoma cell lines JEG and JAR. JEG cells (Kohler and Bridson, 1971) grow as a monolayer of multinucleated cells; JAR cells (Pattillo et al. 1971) show a multi-layer growth with formation of cellular clusters or "piles". These cells have the ability to fuse and form syncytial placental bed giant cells. The transfection results shown in Figure 7 indicate that the rPLII enhancer also functioned in the human choriocarcinoma cell lines, JEG and JAR, although to different extent. This difference in enhancer activity may reflect differing concentrations of factors in these cell lines. These data suggest, however, that the sequences contained within -2838 to -1729 are important for the expression of rPLII in placenta, and that the *cis*-elements on this fragment are recognized across species.

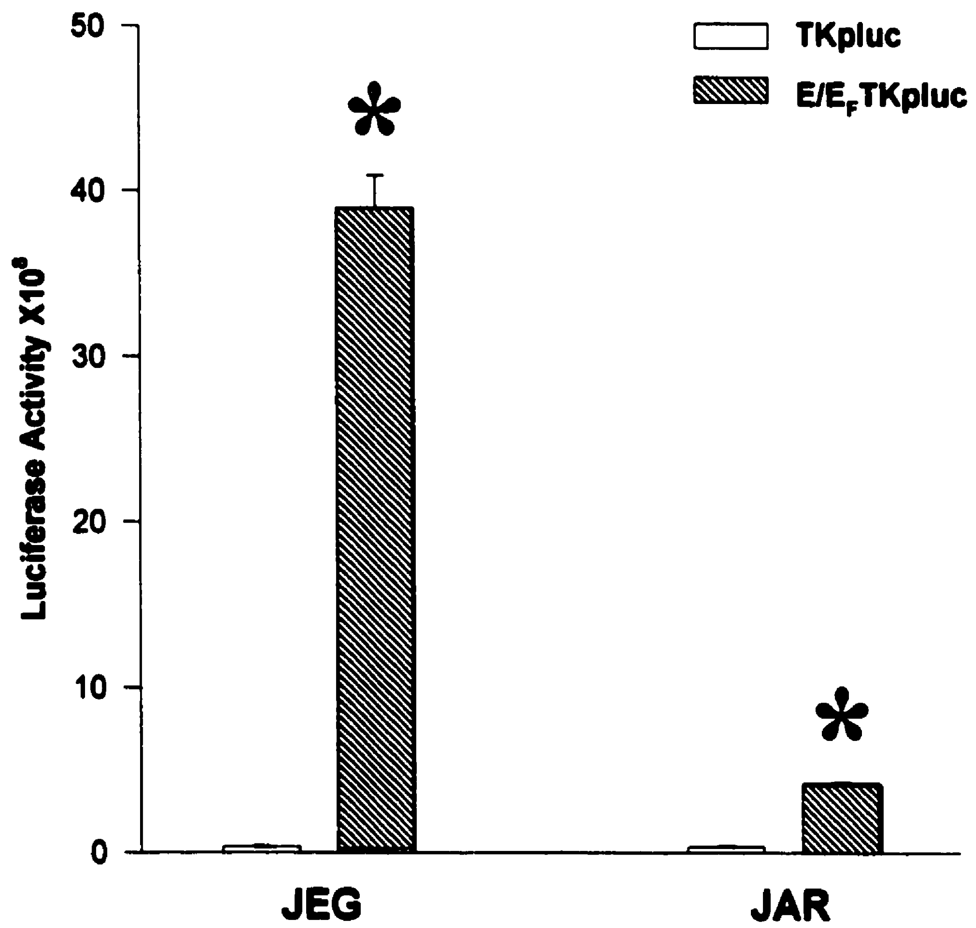
3.3 Identification of the enhancer sequences

In order to identify the functional elements in the enhancer region, a series of luciferase clones containing various regions of the *Eco*RI fragment were cloned into pT81luc. All fragments were cloned in a 5' to 3' orientation. Figure 8 shows deletion analysis of the enhancer region (-2838 to -1729). Only the constructs containing the 3' end of the *Eco*RI fragment (F3, F5, F7 and F9) showed enhancing activity, which had no significant difference from that of the entire *Eco*RI fragment ($P>0.05$). The more 5' fragments showed no significant enhancing activity as compared to the vector control.

Figure 7

Figure 7. Sequences between -2838 to -1729 act as an enhancer in human placental JEG and JAR cells.

The E/FFTKpluc construct was transfected into human JEG and JAR cells. The data are from two separate experiments with sample numbers of 6 and 8. The activity of E/E_FTKpluc was compared to vector control pTK81luc (TKpluc). An asterisk indicates $P<0.05$. The results suggest that sequences within the *Eco*RI fragment function in human placental cells as well as Rcho cells.

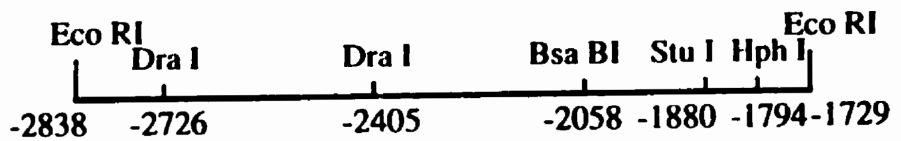


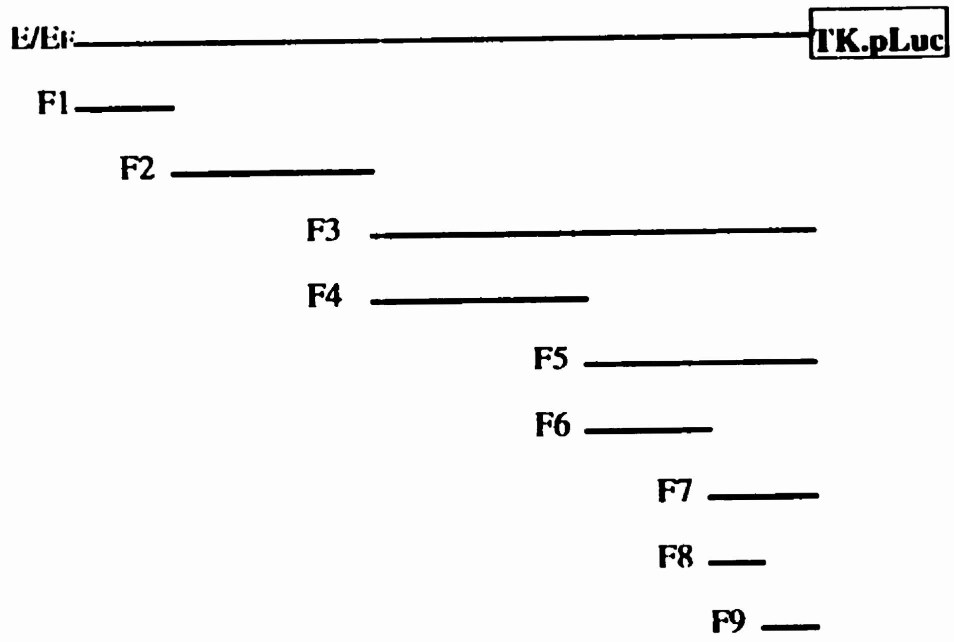
TKpluc
E/E$_F$TKpluc
Luciferase Activity X10^3
50
40
30
20
10
0
*
*
JEG
JAR

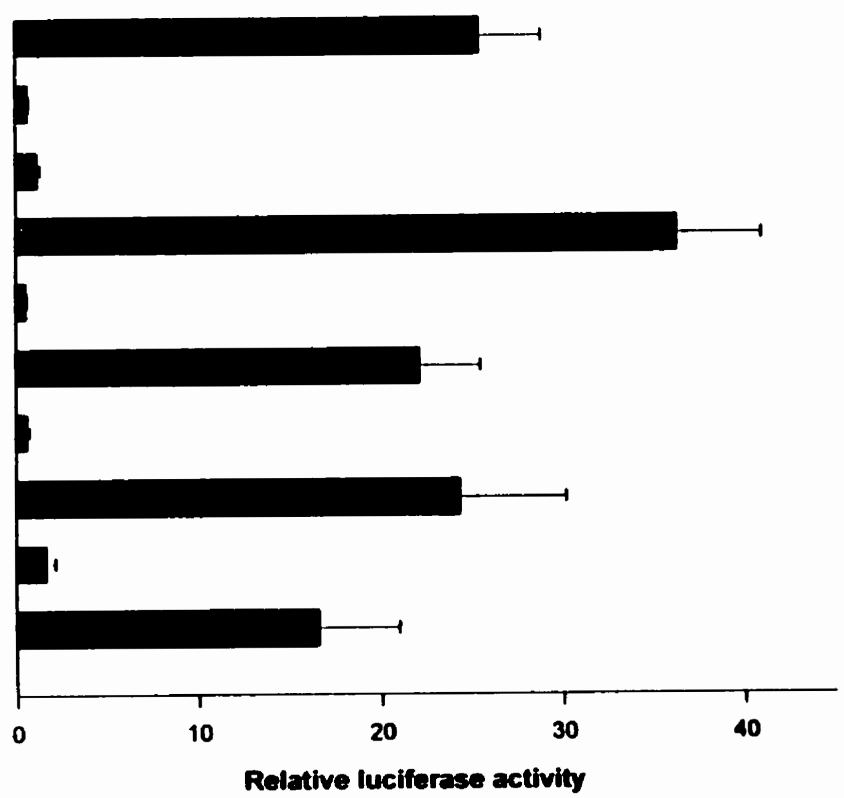
Figure 8

Figure. 8. Deletion analysis of the -2838 to -1729 fragment.

A restriction enzyme map of the enhancing -2838 to -1729 *Eco*RI fragment indicates the sites used for subcloning into the mimimal thymidine kinase promoter vector, pTK81luc. Transfections were carried out in Rcho cells grown for 6 days. Results are from at least six separate transfections. Luciferase activity (mean $\pm$ SEM) is shown relative to the vector control. All fragments that contain the most 3' sequence in the *Eco*RI fragment (F3, F5, F7, F9) have significantly higher activity ($P<0.05$) than the vector control, which was set as 1. There is no significant difference among the activities of these fragments. The more 5' fragments (F1, F2, F4, F6, F8) produce luciferase activities that are not significantly different from the vector control.


Eco RI
Dra I
Dra I
Bsa BI
Stu I
Hph I
Eco RI
-2838
-2726
-2405
-2058
-1880
-1794
-1729


E/E
TK.pLuc
F1
F2
F3
F4
F5
F6
F7
F8
F9


0
10
20
30
40
Relative luciferase activity

These more detailed deletion studies indicated that enhancing DNA binding sites were located in the F9 fragment, a 65 bp fragment between -1794 to -1729 bp.

4. PROTEIN-DNA INTERACTION ANALYSES

4.1 DNAse I protection studies

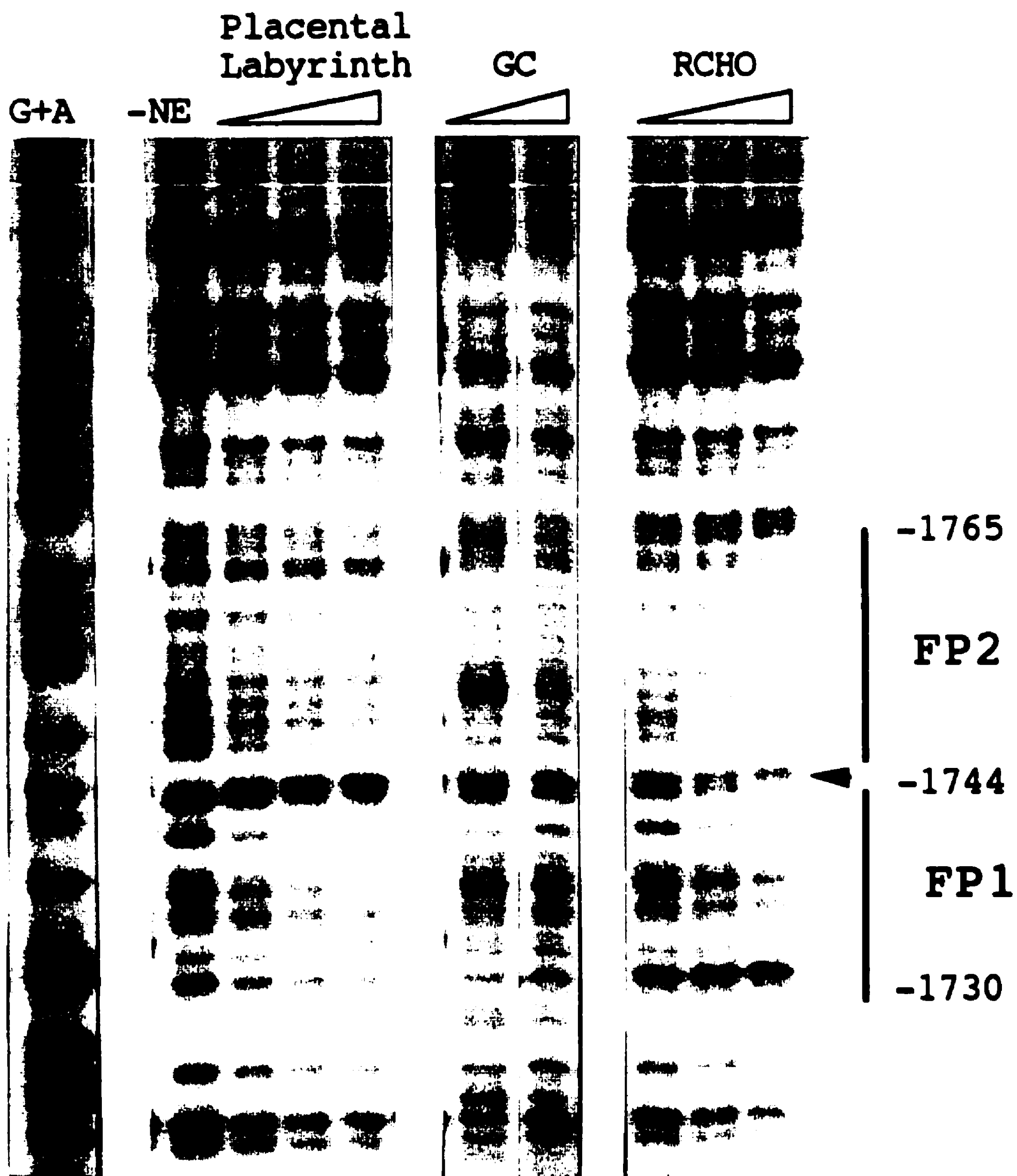
To identify the *cis*-acting elements between -1794 to -1729, DNAse I protection assays were employed. Since the labyrinth region of placenta is the major source of rPLII late in pregnancy (Duckworth et al. 1990, 1993), placental nuclear extracts were isolated from the labyrinth region of day 16 to day 18 rat placenta. Rcho nuclear protein extracts, were made from cultures collected at day 6 after plating when most cells have differentiated to giant cells; remaining small undifferentiated cells were pre-removed from the culture as described in Materials and Methods. The non-placental control pituitary GC cells were harvested at near confluence. To identify the *cis*-acting element between -1794 to -1729 (F9), the -2058 to -1729 (F5) fragment, a more suitable size for a DNAse I protection probe, was end-labelled.

Figure 9 is a representative autoradiogram of the DNAse I protection assays. Two adjacent regions (FP1 and FP2) were protected by rat placental labyrinth and Rcho nuclear extracts. With increasing amounts of nuclear proteins the protection became clearer. FP1 is more protected with placental labyrinth nuclear extracts than Rcho extracts, while FP2 is more protected with Rcho nuclear extracts, suggesting that the relative amounts of binding factors responsible for FP1 and FP2 was different in placental labyrinth and Rcho cells. The protection pattern of GC nuclear extracts was distinct from that of the two protected regions seen with the placental cells. This suggests the binding of markedly different protein

Figure 9

Figure. 9. DNAse I protection analysis of the rPLII enhancer region.

The antisense strand of the F5 fragment (-2058 to -1729) was end-labeled, incubated with increasing amounts of placental labyrinth (20 - 80 μg), Rcho (10 – 40 μg) and GC (20, 40 μg) cell nuclear extracts, and partially digested with DNAse I. G+A indicates a Maxam and Gilbert sequencing reaction that was used as a sequence marker. -NE indicates no protein was added. Two adjacent protected regions, FP1 and FP2, are seen with the placental and Rcho extracts. A different pattern is seen with the GC extract. A hypersensitive site, which is visible in FP1 in placental and Rcho extracts, is marked by an arrow. This is a characteristic shift in sensitivity from the adjacent nucleotide as compared with the -NE lane. The sequences beyond -1729 are vector polylinker sequences.



Placental
Labyrinth
GC
RCHO
G+A
-NE
-1765
FP2
-1744
FP1
-1730

complexes in these two tissues.

The FP1 sequence matched a consensus Ets binding site, while the FP2 sequence was related to a consensus AP-1 binding site. A characteristic shift in DNAse I sensitivity from one nucleotide to the adjacent nucleotide (marked by arrow) was observed in FP1. This hypersensitive site is similar to a reported feature of Ets binding to DNA with murine Ets1 (Gunther et al. 1990) and *Drosophila* E74A (Urness andThummel 1990).

Features of the -1882 to 1724 region (F7) containing the DNAse I protected regions are shown in Figure 10. An *Hph*I site is at the 5' end of F9, which contains the enhancing elements as shown by transfection studies. The underlined sequences indicate the protected areas identified as putative Ets and AP-1 binding sites. There are two putative GATA binding sites on the larger fragment; only one of these is on the enhancing fragment. GATA factors have been demonstrated to be important in mPLI gene expression (Ng et al. 1993, 1994), but no protection was detected at the putative GATA binding sites in the rPLII enhancer.

4.2 Site-directed mutagenesis studies

To determine whether the putative Ets and AP-1 DNA binding sequences were functionally important for the rPLII enhancer activity, primers were designed with mutations at the core nucleotide of the consensus Ets and AP-1 binding sites and used in site-directed PCR mutagenesis. Even though no protection was detected for GATA binding sites in DNAse I protection assays, a GATA-site mutated primer was also generated to exclude a possible role for GATA binding in the rPLII enhancer. The mutated sequences are indicated by * in Figure 10. The -1880 to -1724 (F7) fragment cloned in pBspKS was

Figure 10

Figure 10. Features of the placental specific enhancer region 151 bp F7 fragment

The approximate regions protected placental and Rcho nuclear extracts are underlined and designated as F1 and F2. F1 contains a putative Ets site and F2 a putative AP-1 binding site, as shown in bold. The protected region is shown as a double-stranded sequence since the footprints were best observed on the antisense strand. The arrow marks a new hypersensitive site. The *Hph*I restriction enzyme site further divides this fragment into an inactive 5' (F8) and an active 3' (F9) fragment. Putative GATA sites are also marked in bold. Other putative AP-1 sites are shown in lower case. Asterisks mark the nucleotides that were altered by site-directed mutagenesis in the constructs discussed in Figure 11.

-1882
AGGCCTAagagtcaAAATTTTCTCtgcatcaCTGCAAACACTACATACTTTTCCC

GATA
** ***
TTCTGAACTAACTACACTGTAGGtgagtaa**GATA** TGTA**GATA**TGTGTTTTACGCA
HphI

AP-1 Ets
**** *** -1724
TTTCCAGGGTTAT**TTGCTCA**AGGGTAAAC**AGGAA**GTAGGGCTTGAATTC
AAAGGTCCCAATAAACGAGTTCCCATTTGTCCTTCATCCCGAACTTAAG

F2 F1

used as a DNA template. The mutated primers were paired with M13 reverse primer in PCR reactions. These newly generated Ets, AP-1 or GATA mutated enhancer fragments were further cloned into pT81luc for transfection studies.

Transfection studies (Figure 11) in Rcho cells indicated that the Ets mutant had only 30% of the native enhancer activity. The AP-1 mutant had 60% of native enhancer activity. The double mutation of Ets and AP1 abolished the enhancing activity, suggesting that Ets and AP-1 binding factors are important for the expression of rPLII. The Ets and AP-1 sites appear to function independently and each contributes a portion of the activity of the enhancer, which suggests that these sites function in an additive rather than a synergistic fashion. While GATA appears to be important in mPLI placental expression, the GATA mutations did not decrease activity of the fragment containing rPLII enhancer, which was consistent with DNAse I protection studies. Even though PLI and PLII are related, these studies suggest that the regulation of these two genes could be quite different.

To test whether the mutations affect the enhancing effect in other species, the mutants were also tested in the human placental JEG cell line (Figure 12). The single Ets mutation decreased the activity significantly, suggesting that the Ets protein that binds to this site may also be present in human placental cells. Interestingly, the AP-1 and GATA mutations significantly increased the activity of the enhancer in the JEG cell line.

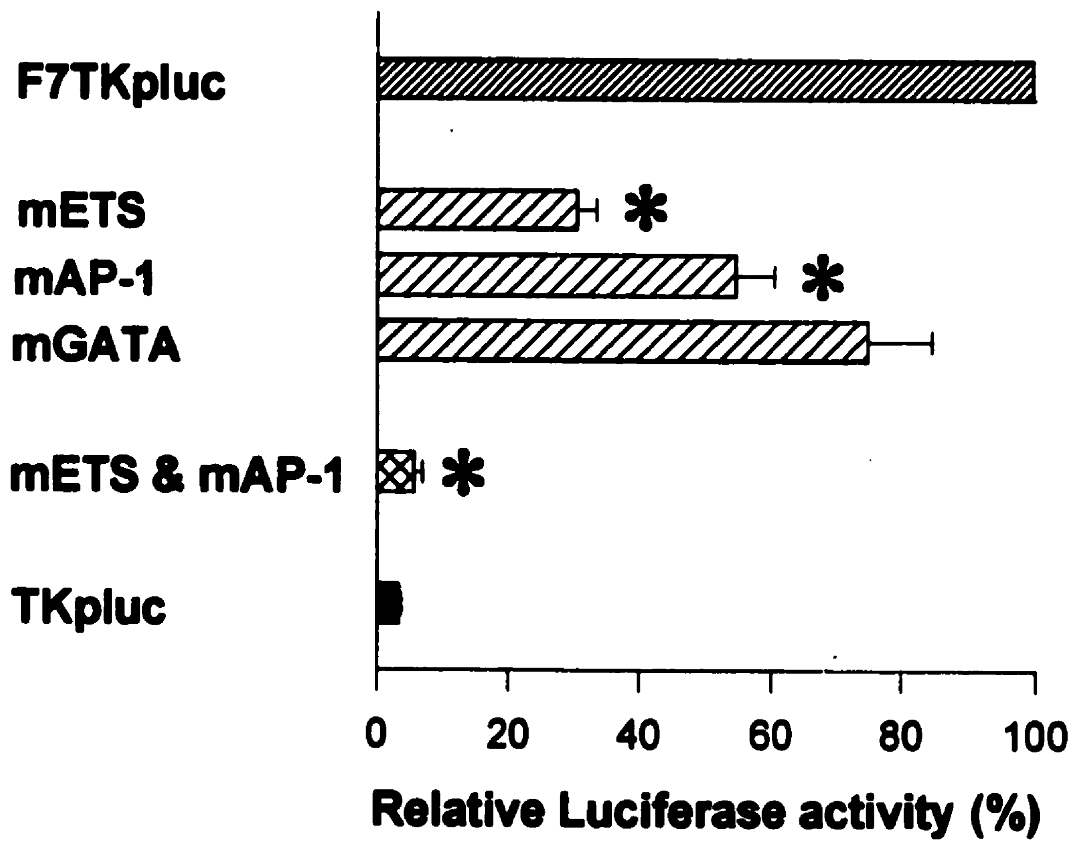
4.3 Expression of Ets transcription factors

Ets1 and Ets2 have both been implicated in the placental expression of matrix metalloprotease genes, collagenase (MMP1) and stomelysin (MMP3) in human (Wasylyk et al. 1991, Butticè et al. 1996, Basuyaux et al. 1997). MMP1 and MMP3 genes are expressed

Figure 11

Figure 11. Expression of the mutated rPLII enhancer constructs in Rcho cells.

The consensus Ets and Ap-1 binding sites in the protected region were individually or doubly mutated in the context of the 151 bp *Stu*/*Eco*RI fragment (F7) as described in Materials and Methods. Two consensus GATA sites were also mutated. The nucleotides that were altered are indicated in Figure 10. The mutated and wild type F7/Tkpluc clones were transiently transfected into day 6 Rcho cells. Results were from two experiments with at least 12 separate transfections and are expressed as a percentage of the luciferase activity (mean $\pm$ SEM) seen with the wild type fragment. The individual Ets (mEts) and AP-1 (mAP-1) mutations had significantly (* $P<0.001$) lower activity than the wild type fragment. Combined mEts/mAP-1 mutations reduced luciferase levels to the level of the vector control. There was no significant loss of activity when GATA sites are mutated.

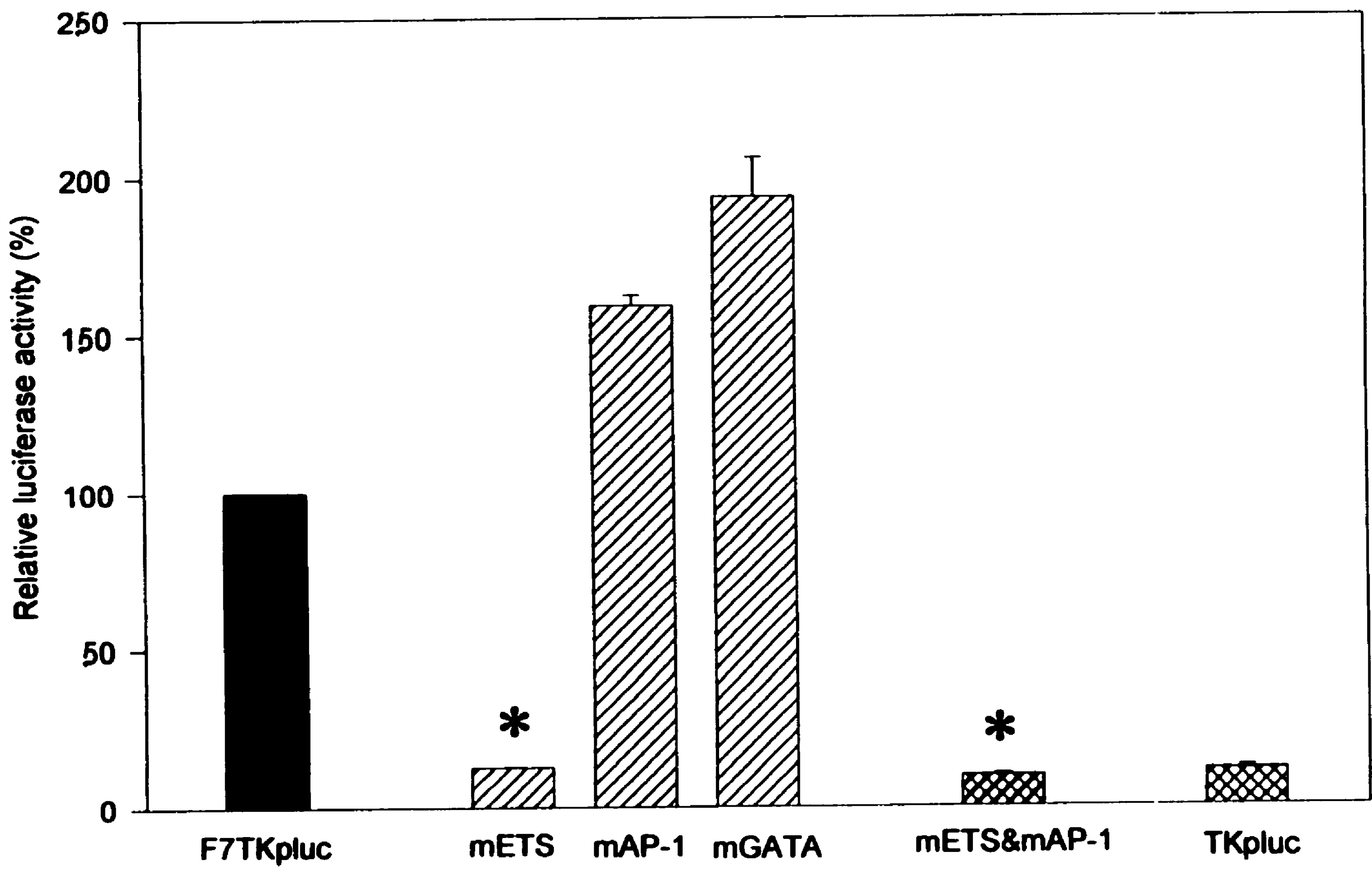


F7TKpluc
mETS
mAP-1
mGATA
mETS & mAP-1
TKpluc
0
20
40
60
80
100
Relative Luciferase activity (%)

Figure 12

Figure 12. Expression of the mutated rPLII enhancer constructs in JEG cells.

The constructs in Figure 11 were transiently transfected into JEG cells. Of the individual mutations, only mEts showed significantly decreased in activity ($P<0.05$). The combined mutations, mEts/mAP-1, also showed a significant decrease in activity, but the level was comparable with the Ets mutation alone. The results were from two experiments with a total of 4 to 6 transfections.


Relative luciferase activity (%)
250
200
150
100
50
0
*
*
F7TKpluc
mETS
mAP-1
mGATA
mETS&mAP-1
TKpluc

during growth and development in a variety of cell types, but in particular by invading trophoblast cells of the placenta (Vettraino et al. 1996). It has been reported that Etsl is expressed in human trophoblast cells (Luton et al. 1997), and recently it has been reported that a targeted mutation of Ets 2 in mice caused early embyonic death due to defective trophoblast function (Yamamoto et al. 1998).

Since Ets1 and Ets2 were already known to be important for some placental gene expression, human Ets1 and Ets2 cDNA probes were hybridized to rat placenta and Rcho RNA blots to determine whether these genes were also expressed in the rat placenta. Hybridization to hEts2 cDNA was detected in both placenta and Rcho cells (Figure 13). No hybridization was detected with hEts1 cDNA. Recently we have found that when an Ets1 Ets domain probe is used to screen a rat placental cDNA library, most selected clones were Ets2, suggesting that Ets2 is abundantly expressed in placenta.

4.4 Overexpression studies of Ets and AP-1 binding factors

To test further the enhancing role of Ets2 and AP-1 binding factors (Jun/Fos), overexpression studies of these proteins were carried out. Human Ets2 and rat c-Jun/c-Fos cDNA expression plasmids were co-transfected with the 65 bp rPLII enhancer reporter clone, F9Tkpluc, into Rcho cells. Results shown in Figure 14 indicated that overexpression of either Ets2 or c-Jun/c-Fos increased reporter gene expression two fold. When Ets2 and c-Jun/c-Fos were cotransfected together, the activity was approximately four fold. This result again suggests an additive effect of Ets 2 and c-Jun/ c-Fos consistent with the mutagenesis data, which showed each mutation decreasing activity only partially.

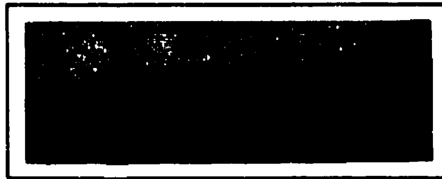
Figure 13

Figure 13. Ets2 expression in placenta and Rcho cells.

Total RNA was isolated from rat placenta at different days of pregnancy and Rcho cells at different days after plating. Thirty μg of each RNA was electrophoresed on a formaldehyde agarose gel and blotted onto Nitroplus membranes. The blots were hybridized to either hEts1 or hEts2 cDNA probe using stringent conditions. The Ets2 expression is shown. No Ets1 hybridization was detected after two weeks exposure in either placenta or Rcho cells. Loading was essentially equal as estimated from the 18S and 28S ribosomal RNAs on the ethidium bromide stained gels. The expression of Ets2 was low in day 11 placenta compared to later days of pregnancy. The level of Ets2 expression in Rcho cells was essentially unchanged with length of time in culture.

11 12 14 16 18 21 Day

Rat placenta



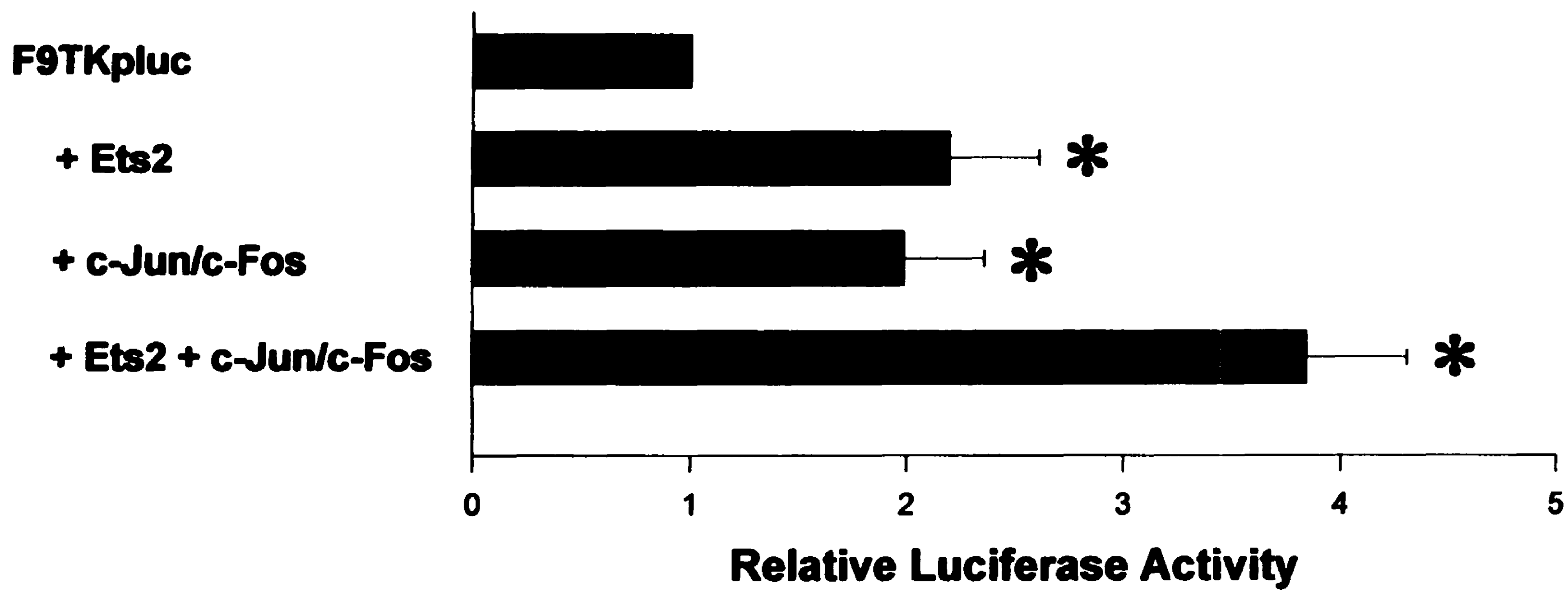
4 6 8 10 12 14 16 18 22 26 Day

Rcho cells

Figure 14

Figure 14. Overexpression of Ets2 and Jun/Fos in Rcho cells.

The human Ets2, rat c-Jun, and rat c-Fos cDNA were cloned into the expression vector, pcDNA3. Five micrograms of each cDNA were co-transfected with 10 μg of the luciferase expression clone, F9/Tkpluc, into day 6 Rcho cells. The total amount of transfected plasmid was kept constant at 25 μg in all cases by addition of pcDNA3 vector DNA. Results are from three experiments, including 10 separate transfections. Luciferase activities are expressed relative to activity in cells co-transfected with F9/TKpluc and the vector pcDNA3, which was set at 1. Co-transfection of either Ets2 or c-Jun/c-Fos increased luciferase activity approximately 2 fold. When the three clones were co-transfected, luciferase levels were increased by approximately 4 fold. The asterisk indicates that the activity was significantly higher than the F9TKpluc expression clone transfected with vector pcDNA3 alone ($P<0.05$).


F9TKpluc
+ Ets2
+ c-Jun/c-Fos
+ Ets2 + c-Jun/c-Fos
*
*
*
0
1
2
3
4
5
Relative Luciferase Activity

4.5 Electrophoretic mobility shift assays

The overexpression study suggested that Ets2 and Jun/Fos could regulate the activity of the rPLII 65 bp enhancer, but it could not determine whether the effects were direct or indirect. Electrophorectic mobility shift assays (EMSA) were used to investigate whether Ets and AP-1 family proteins could directly interact with the enhancer fragment. Further, EMSA studies were used to investigate which Ets family member was directly involved in the enhancing effect.

A labelled 65 bp enhancer fragment (-1794 to -1729), which contains the putative Ets and AP1 sites, was used as a probe. The probe was incubated with *in vitro* transcribed and translated Ets1, Ets2, c-Jun and c-Fos proteins. A specific retarded complex was formed with a mixture of recombinant c-Jun/c-Fos proteins (Figure 15A), which could be competed with a consensus AP-1 binding oligonucleotide. A Jun specific antisera also competed the band, while an unrelated oligonucleotide and a 65 bp enhancer fragment containing mutations of Ets and AP-1 sites did not. There was no shift of the fragment with recombinant c-Jun alone, suggesting that only Jun/Fos heterodimers were bound. There was also no specific shift with Ets2 or the related Ets1. When either Ets protein was incubated with c-Jun/c-Fos, only the AP-1 complex was formed, no further complex was seen (data not shown). Other shifted bands were seen in all reactions and appear to be non-specific, since they were present in control reactions which contained only reticulocyte lysate.

The EMSA studies were also carried out using placental, Rcho and GC nuclear extracts with the 65 bp enhancer fragment (Figure 15B). Several specific complexes were formed with placental extracts. One of these (complex 2) was completed by both the 65 bp fragment itself and a consensus AP-1, but was not completed by the oligonucleotide

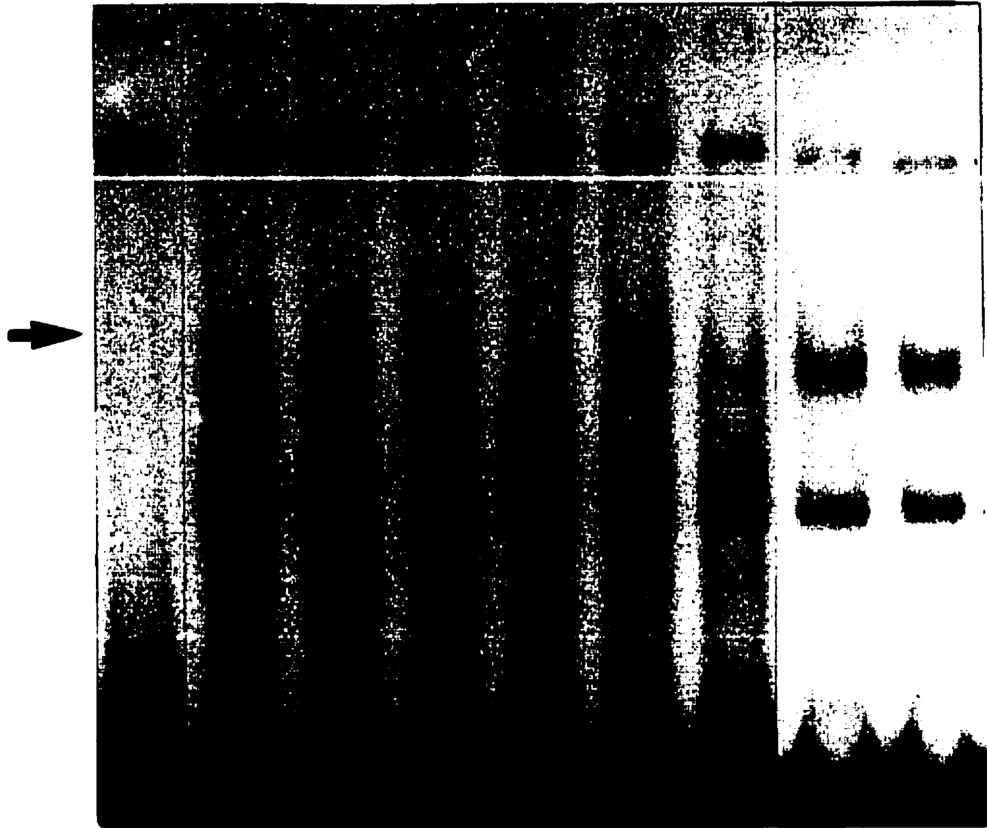
Figure 15

Figure 15. Electrophoretic mobility shift assays of the enhancer fragment.

F9 fragment was end-labeled, and incubated with *in vitro* synthesized proteins or nuclear extracts. The binding conditions are described in Materials and Methods.

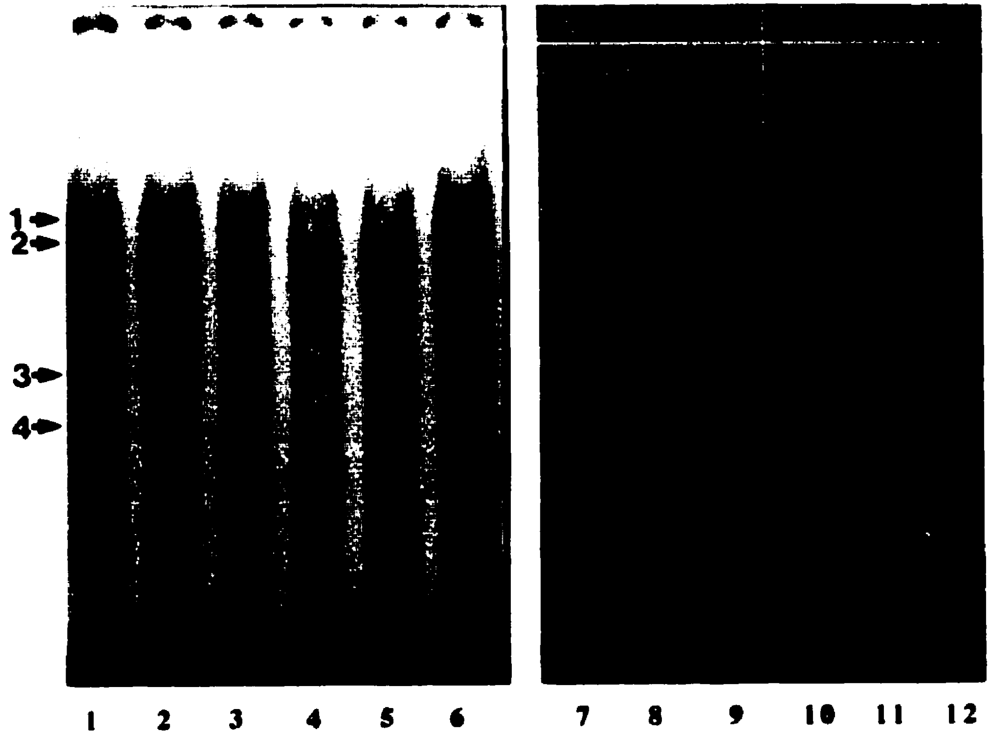
A. F9 fragment binds *in vitro* synthesized AP-1, but not Ets1 or Ets2 proteins. Lane 1 shows the free probe; lane 2 shows the complexes formed when *in vitro* synthesized rat c-Jun/c-Fos dimers bind to the labeled 65 bp F9 fragment. Addition of a consensus AP-1 binding oligonucleotide at 100-fold molar excess (lane 3) or c-Jun antisera (lane 5) specifically competes the upper band, marked by an arrow. Neither an unrelated oligonucleotide (lane 4) nor an oligonucleotide containing a mutated AP-1 site (lane 6) competes the specific complex at 500-fold molar excess. *In vitro* synthesized c-Jun protein alone does not produce this specific complex (lane 7). No specific complexes were formed in the presence of *in vitro* synthesized human Ets1 (lane 8) or Ets2 (lane 9).

A



	1	2	3	4	5	6	7	8	9
<u>Protein</u>									
c-Fos	-	+	+	+	+	+	-	-	-
c-Jun	-	+	+	+	+	+	+	-	-
Ets1	-	-	-	-	-	-	-	+	-
Ets2	-	-	-	-	-	-	-	-	+
<u>Oligo</u>									
AP-1	-	-	+	-	-	-	-	-	-
Non-Specific	-	-	-	+	-	-	-	-	-
mAP-1/mEts	-	-	-	-	-	+	-	-	-
<u>Antiserum</u>									
Anti-Jun	-	-	-	-	+	-	-	-	-

B. F9 fragment forms different complexes with placental and GC nuclear extracts. Lanes 1-6 show complexes formed with rat placental nuclear extract. A number of complexes are produced (lane 1); those complexes that are competed with 200-fold molar excess of the probe (lane 4) are marked with arrows and numbers. None of the complexes appear to be competed by a 500-fold excess of an Ets2 binding oligonucleotide (lane 2), but complex 2 is competed by a 150-fold excess of a consensus AP-1 oligonucleotide (lane 3). An oligonucleotide containing mutations in the AP-1 and Ets sites, at 500-fold excess, does not compete for complexes 2, 3 or 4, but does compete complex 1 (lane 5). An unrelated oligonucleotide at 500-fold excess does not compete for any complexes (lane 6). Complexes formed with GC nuclear extracts are shown in lanes 7-12. Neither an Ets2 nor an AP-1 consensus binding site competes at 500-fold excess for any of the complexes (lane 8 and 9). All complexes are partially competed by unlabeled probe at 400-fold excess (lane 10), as well as by 500-fold excess of the oligonucleotide containing mutations in the AP-1 and Ets binding sites (lane 11). There is no competition by an unrelated oligonucleotide at 500-fold excess (lane 12).



Competitor	Placental extract						GC extract					
Ets2	-	+	-	-	-	-	-	+	-	-	-	-
AP-1	-	-	+	-	-	-	-	-	+	-	-	-
Wild type	-	-	-	+	-	-	-	-	-	+	-	-
mAP-1/mEts	-	-	-	-	+	-	-	-	-	-	+	-
Non-specific	-	-	-	-	-	+	-	-	-	-	-	+

containing the double Ets and AP-1 mutations or an unrelated oligonucleotide. These data suggest that this complex is formed when AP-1 factors bind. Complex 3 was specifically competed by the probe fragment itself, but not by the Ets and AP-1 mutated oligonucleotide or a non-specific oligonucleotide. This complex potentially could be an Ets binding complex. Complex 3 was not completed by Ets2 oligonucleotide, however, suggesting that the binding Ets factor might be an Ets member other than an Ets2.

The binding pattern with GC extracts was quite different from that of placental extracts. Neither Ets2 nor AP-1 consensus oligonucleotide was able to compete, while the 65 bp fragment and the mutant fragment both showed the same competition pattern, suggesting that the competed complexes were not due to either Ets or AP-1 binding (Sun and Duckworth. 1999).

5. FURTHER INVESTIGATION OF ETS FACTORS THAT BIND THE PLACENTAL SPECIFIC ENHANCER

5.1 Other Ets family members

The sequence of FP1 is related but not identical to an Ets2 or Ets1 binding site. Although overexpression of Ets2 in Rcho cultures increased the rPLII enhancer activity, EMSA did not detect a specific shift with either Ets2 or Ets1. Even though all Ets family proteins contain a highly conserved DNA binding domain that recognizes a core consensus sequence, A/CGGAA/T, there is a degree of variation among the binding specificities of different Ets proteins. The specificity of binding in this family is thought to involve sequences outside the core region (Wasylyk et al. 1993, 1998). According to the sequence

similarity in the Ets binding domain, the Ets family has been divided into different subgroups (Lauded et al. 1999).

In an attempt to identify the Ets family member that specifically binds the rPLII enhancer Ets site, several other Ets factors were tested. Ets1 and Ets2 belong to the ETS subgroup, Elk-1 belongs to the TCP subgroup, and ESX and Elf-R belong to the Elf-1 subgroup (Lauded et al. 1999). Of these, ESX and ElfR have been shown to be highly expressed in human placenta (Chang et al. 1997, Aryee et al. 1998). The binding sequence of ESX (CTTGAGGAAGTATAAG, Chang et al. 1997) is very close to the Ets binding site (AAACAGGAAGTAGGGC) on the rPLII enhancer. In RNA analysis using an ESX cDNA probe lacking the Ets domain, we have shown that ESX-related mRNA is expressed in Rcho cells and to a lesser extent in rat placenta (data not shown).

5.2 Transfection studies of the new Ets factors

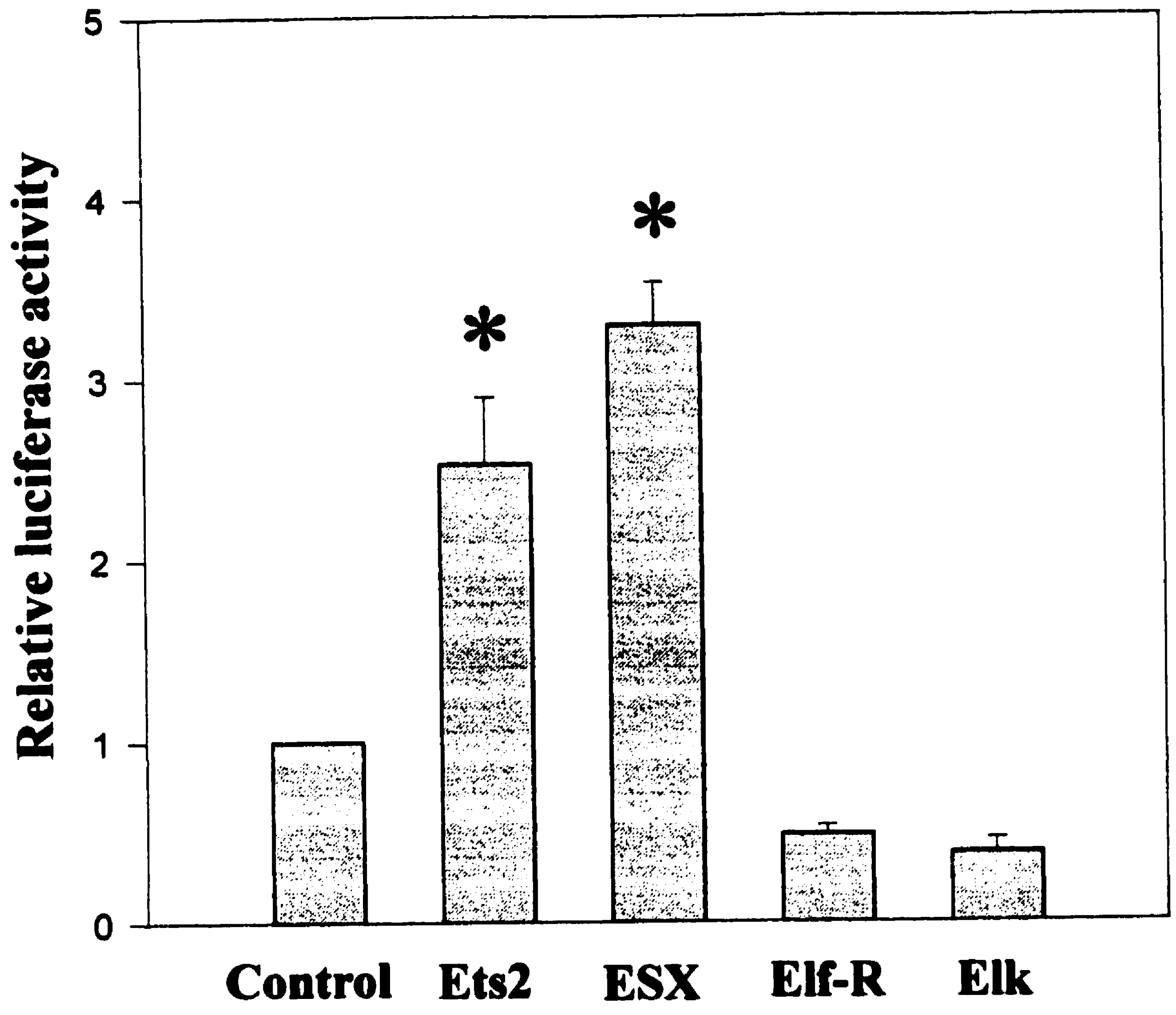
Expression plasmids of Ets2, ESX, Elf-R, Elk-1 were co-transfected with F9TKpluc into Rcho cells. Control cells were co-transfected with expression vector alone. Only Ets2 and ESX showed a significant increase ($P<0.05$) in the activity of the rPLII enhancer as measured by an increase in luciferase expression (Figure 16).

To determine whether the enhancing effect was due to direct or indirect Ets binding, Ets2 or ESX overexpression plasmids were co-transfected with luciferase constructs driven by a TK promoter and an enhancer fragment containing the mutated Ets site as shown in Figure 11. The Ets2 clone increased luciferase activity of this construct ($P<0.05$), but ESX had no effect (Figure 17). This result supported our EMSA data that Ets2 doesn't directly bind to the Ets site on the rPLII enhancer. ESX was not able to increase luciferase

Figure 16

Figure 16. Overexpression of other Ets family members.

Day 6 Rcho cells 1 were co-transfected with 10μg of the luciferase F9/TKpluc clone and 5μg of expression clones for human Ets2, ESX, Elf-R or Elk-1. Control cultures received 5μg of pcDNA3 vector. Luciferase levels obtained with control transfections were set at 1. Ets2 and ESX transfected cultures showed significantly increased activity (*, $P<0.05$) over the control; Elf-R and Elk-1 transfected cultures showed no significant differences. The results are from two separate experiments with a total of 6 to 8 transfections.

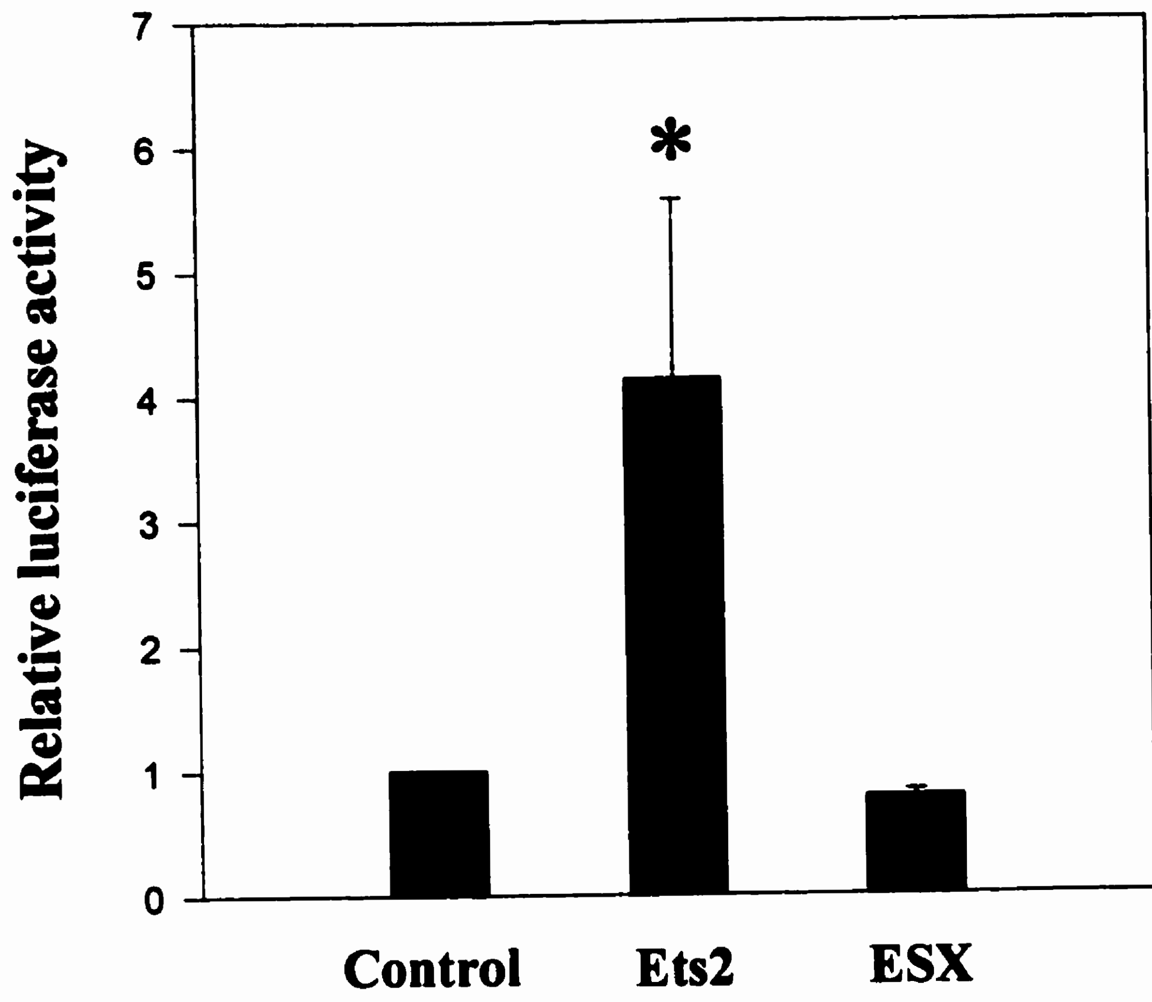


Relative luciferase activity
5
4
3
2
1
0
*
*
Control
Ets2
ESX
Elf-R
Elk

Figure 17

Figure 17. Effect of overexpression of Ets2 or ESX on the Ets mutated rPLII enhancer.

The mEtsTKp.luc clone, described in Figures 10 and 11, and either human Ets2 or ESX expression clones, were co-transfected into day 6 Rcho cells. The control was co-transfected with expression vector pcDNA3. Luciferase expression levels for the control were set at 1. The total amount of transfected plasmid was kept constant at 15 μg. The activity of Ets2 was significantly higher than the control (*. $P<0.05$), while the activity of ESX showed no significant difference. Results are from two separate experiments with a total of 6 to 8 transfections.



Relative luciferase activity
7
6
5
4
3
2
1
0
*
Control
Ets2
ESX

expression with the mutated enhancer, suggesting that ESX may directly bind to the Ets site of rPLII enhancer.

5.3 Electrophoretic mobility shift assays using *in vitro* translated ESX.

To further determine whether ESX could bind to the rPLII enhancer Ets site directly, human ESX was synthesized *in vitro* and tested in an electrophoretic mobility shift assay. The 65 bp rPLII enhancer fragment was used as a probe. *In vitro* synthesized c-Jun/c-Fos and hEts2 served as positive and negative controls. ESX, but not Ets2, showed a distinct binding complex (Figure 18), suggesting that ESX may directly bind the rPLII enhancer Ets site. The complete competition experiments will further confirm the affinity and specificity of this complex.

Figure 18

Figure 18. Electrophoretic mobility shift assay of ESX.

The -1794 to -1729 F9 fragment was end-labeled and incubated with *in vitro* synthesized rat Jun/Fos, hEts2 or ESX proteins. From previous results (Figure 15A) the c-Jun/c-Fos lane served as a positive control; the Ets2 lane was a negative control. A distinct shifted complex different from that of Jun/Fos was seen with ESX.

Jun/Fos

ESX

Free
Probe
Jun/Fos
Ets2
ESX

DISCUSSION

Placental lactogen I and II genes are expressed in a temporal and cellular specific fashion in placenta, which makes them good candidates to study placental specific gene regulation. There is a switch in expression between these two proteins at mid-pregnancy (Duckworth et al. 1993); the factors that control this switch are as yet unknown. To better understand this intriguing biological switch, my project has focused on the regulation of rPLII gene expression. To identify the *cis*- and *trans*- factors involved in the full developmental expression of rPLII, I have functionally and structurally characterized a part of the 5' flanking region of the rPLII gene both *in vitro* and *in vivo*.

Our promoter deletion study indicated that the proximal 765 bp of the rPLII 5' flanking region appears to be insufficient to direct placental expression. Sequences between -3031 and +64 were able, however, to confer expression in both rat and human placental cell lines, but not in non-placental cell lines, suggesting that the 3031 bp rPLII 5' flanking DNA may contain elements that are able to direct placental-specific expression across species.

Deletion analysis of this 3031 bp 5' flanking fragment demonstrates that a fragment from -2838 to -1729 functions as an enhancer in both rat and human placental cells. Even though there is no related PLII gene in human, the fact that rPLII regulatory elements function in human placenta suggests that the same transcription factors are present and that studies of these factors may bring insights into regulation of human placental gene expression.

The rat trophoblast Rcho cell line has been shown to be a good model system to study PLI and PLII expression (Duckworth. et al. 1993). In early experiments, we observed that a significant level of rPLII expression in Rcho cell cultures did not appear until 14 days

after plating (Sun et al. 1998), which suggested that the Rcho cell line may be used to investigate the switch in expression between rPLI and rPLII. In placenta, rPLI is expressed in placental giant cells at day 7 and declines by day 12, while rPLII is expressed in these same cells from day 11 to term. To determine whether the fragment we had identified at -2838 to -1729 could have a specific role in the temporal expression of rPLII, we tested this fragment with a heterologous promoter at day 8 and day 16 after plating for the ability to enhance luciferase reporter gene expression. A comparable level of expression was detected at both days suggesting that the enhancing sequences were not directly involved in the temporal regulation of rPLII. However, when we redid the rPLII time course study in a cell passage number similar to that used for transfection experiments, we observed that the Rcho cells differentiated earlier than the previous cultures. As well, significant levels of rPLII mRNA appeared earlier after plating (data not shown), indicating that the Rcho cell line may have changed during passaging. These data suggest that one must be cautious when using the Rcho cell model to study the temporal expression of placental lactogen genes. The role of the rPLII enhancer fragment in the control of temporal expression of rPLII is still unclear and will require further investigation. It seems that a better way to study temporal expression of rPLII would be to develop transgenic mouse lines from reporter constructs under the control of different regulatory fragments and monitoring the luciferase expression at different stages of placenta development.

Detailed deletion analysis of the enhancing fragment, -2838/-1729, indicates that the enhancing activity is located in a 65 bp fragment from -1793 to -1729. DNAse I protection studies of the 65 bp enhancer identified two adjacent regions, FP1 and FP2, that are protected by placental and Rcho nuclear extracts. These regions correspond to putative Ets

(F1) and AP-1 (F2) binding sites. At comparable protein concentrations, FP1 appeared to be protected more by the placental nuclear extracts, while FP2 was protected more by Rcho extracts. This difference could be explained by the fact that the placental extracts are from a mixed cell population while the Rcho cells are pure trophoblast giant cells. Alternatively, since the placenta expresses much higher levels of the rPLII mRNA than do the Rcho cells, the footprints may reflect a real difference in the amounts of active transcription factors present in these two sources.

Since we were interested in whether the protein-DNA interactions of the enhancer region are placental specific, nuclear extract from pituitary GC cells were also tested in the DNAse I protection assay. The binding pattern to GC nuclear extract at the putative Ets and AP-1 sites is markedly different from that seen with placental and Rcho extracts. There is no clear protection of FP1; in particular, a new hypersensitive site, which develops with both Rcho and placental nuclear extracts, is missing with GC extract. A hypersensitive site at this location is characteristic of an Ets factor binding to DNA (Gunther et al, 1990; Urness and Thummel, 1990) and supports FP1 being an Ets binding site.

The importance of the AP-1 and Ets binding sites to the activity of this rPLII enhancer is demonstrated by the PCR site directed mutagenesis studies. Mutation of core nucleotides in the Ets site alone decreased the activity by 70%; changes to nucleotides in the AP-1 site alone decreased activity by 30%. The double mutation totally abolished the activity of this enhancer, demonstrating that both sites are crucial for the activity. The effect of these two sites appears to be additive rather than synergistic, suggesting that binding of the Ets and AP-1 factors is independent rather than co-operative.

The AP-1 transcription factors, Jun and Fos, binding as Jun-Fos heterodimers or Jun-Jun homodimers are involved in a wide range of regulatory processes linked to cellular proliferation and differentiation (Curran and Franza 1988, Johnson et al. 1993). AP-1 transcription factors have been implicated in the placental expression of several placental genes including mouse and rat PLI (Shida et al, 1993)

Ets transcription factors regulate a diverse array of biological functions including cell proliferation, differentiation and oncogenic transformation (Wasylyk et al. 1993, 1998), but it was a novel observation that an Ets factor is involved in the placental expression of a prolactin family gene. Ets transcription factors consist of a large gene family (Wasylyk et al. 1993, 1998). Ets1 and Ets2 have both been implicated in the placental expression of matrix metalloproteinase genes (MMP) including collagenase (MMP1) and stomelysin (MMP3) which degrade connective tissue and basement membranes (Wasylyk et al. 1991, Butticè et al. 1996, Basuyaux et al. 1997). MMP1 and MMP3 are expressed during growth and development in a variety of cell types, but in particular by invading trophoblast cells of the placenta (Vettraino et al. 1996). A recent report has shown that targeted deletion of the conserved DNA binding domain of murine Ets2 results in placental defects and embryonic death before day 8.5 of embryonic development. The primary defect appears to be mediated through deficient expression of the MMP9 gene (gelatinase B) (Yamamoto et al. 1998) which leads to insufficient invasion of the placenta. Interestingly, it was also observed that mPLI was increased in the absence of Ets2. Unfortunately pregnancy is terminated before expression of PLII in these animals.

Since both Ets1 and Ets2 had been shown to regulate the expression of genes in trophoblast cells we tested for expression of these Ets members in rat placenta and Rcho

cells. In RNA blot analysis Ets2 but not Ets1 mRNA was detected in both placenta and Rcho cells. Taken together with the role of Ets2 in other placental gene regulation these findings suggested that Ets2 would be a reasonable candidate for binding to the rPLII enhancer element.

It is known Ets proteins bind to DNA as monomers, but protein-protein interactions appear to be important for their function. One of the most common interactions reported is with the Jun/Fos (AP-1) family of transcription factors (Wasylyk et al. 1993, 1998). Co-transfection of either c-Jun/c-Fos or Ets2 expression clones with 65 bp enhancer/luciferase construct in Rcho cells resulted in increased reporter gene expression in both cases (2 folds of control). To test whether Ets2 could interact with AP-1 factors on the rPLII enhancer, c-Jun/c-Fos and Ets2 were cotransfected together with the enhancer/reporter construct. Luciferase activity was twice as high as the activity expressed when Ets2 or Jun/Fos were transfected individually. This additive effect is consistent with our mutagenesis data. Expression of MMP1 and MMP3 gene require the binding of Ets and AP-1 factors but the interactions appear to be different. The effects of Ets and AP-1 appear to be co-operative in the MMP1 gene (Butticè et al. 1996, Basuyaux et al. 1997) but independent in MMP3 gene (Wasylyk et al. 1991). Even though the AP-1 and Ets binding sites are adjacent on the rPLII enhancer, similar to those sites in the collagenase MMP1 gene, both our overexpression and mutagenesis data suggest that Ets and AP-1 factors interact with the rPLII enhancer independently rather than co-operatively. To test whether Ets and AP-1 factors are the only factors required for the activation of this rPLII enhancer, overexpression of Ets and AP-1 factors could be carried out in other cell lines, in which the enhancer is inactive, such as GC and Hela.

Although we demonstrated that Ets2 and AP-1 factors increased the luciferase activity of the rPLII enhancer in over-expression studies with transcription factor clones, these effects could be through either direct or indirect actions on the enhancer/reporter construct. For instance, it is known that Ets2 can affect the expression of the c-Jun gene itself (Coffer et al. 1994, Aperlo et al. 1996). This meant that our over-expression study was inconclusive for establishing direct protein-DNA binding. To characterize direct protein-DNA interactions on the enhancer, electrophoretic mobility shift assays (EMSA) were employed. EMSA studies using *in vitro* transcribed and translated rat c-Jun and c-Fos proteins confirmed that the 65 bp F9 enhancer sequences bind Jun/Fos heterodimers but not Jun-Jun homodimers; a consensus AP-1 oligonucleotide was able to compete the binding complex efficiently, but a non-specific oligonucleotide was not. These data show that Jun and Fos factors directly interact with the AP-1 site on the 65 bp placental specific enhancer. In EMSA neither *in vitro* synthesized Ets1 nor Ets2 protein with or without c-Jun/c-Fos proteins was able to bind the 65 bp enhancer fragment, even though both recombinant Ets proteins were shown to shift a consensus Ets2 binding site (Stacey et al. 1995). This consensus Ets2 binding site oligonucleotide does not compete any of the complexes formed between placental nuclear extract and the enhancer fragment. The data suggest that Ets2 is not the specific Ets family member that directly binds to rPLII enhancer.

Overexpression of Ets2, however, does increase the rPLII enhancer activity. This effect could be produced through protein-protein interactions with another Ets protein, such as for the stromelysin gene where Ets2 and the Ets protein, ERG, have been shown to interact (Basuyaux et al. 1997). It also has been reported that Ets2 binds to and activates the Jun-B promoter (Coffer et al. 1994, Aperlo et al. 1996). It is therefore possible that Ets2

may increase the enhancer activity by increasing the expression of AP-1 factors, which then directly bind and activate the enhancer. A similar observation has been made for the stromelysin gene, where the effects of Ets1 and AP-1 binding appear to be additive but Ets1 does not bind directly to the Ets binding site. It has also been speculated that the Ets1 may act through increasing c-Jun and c-Fos synthesis (Wasylyk et al. 1991). An indirect effect of Ets2 on the rPLII enhancer would appear to be further supported by the transfection data in which Ets2 overexpression produces higher luciferase activity with the mutant Ets site enhancer (Figure 17) than the native sequence (Figures 14 and 16).

Rat d/tPRP is expressed in antimesometrical decidua, spongiotrophoblasts, and giant cells in the basal zone (Roby et al. 1993, Gu et al. 1994). Very recently, it has been reported that a proximal 93 bp rat d/tPRP promoter is sufficient to direct reporter gene expression in primary decidual cells and Rcho cells (Orwig and Soares, 1999). This sequence contains sites for Ets and AP-1 factors. Mutation of the AP-1 regulatory element reduced d/tPRP promoter activity in both primary decidual and Rcho cells. Mutation of the Ets site also reduced promoter activity in both cell types. The effect of the AP-1 site mutation appeared, however, to be more significant in decidual cells than Rcho cells, while the effect of the Ets site mutation was more significant in Rcho cells where there was a complete loss in activity (Orwig and Soares 1999). In EMSA, the patterns of nuclear proteins binding on the d/tPRP Ets site oligonucleotide were clearly different between the Rcho cell line where d/tPRP promoter is active and the HRP-1 trophoblast stem cell line where d/tPRP promoter is inactive. This suggests that the protein-DNA interactions with the Ets regulatory element are cell type specific and that the Ets factor(s) may be different in these two trophoblast cell types. Two complexes (I and II) were detected in EMSA of the d/tPRP Ets element with

Rcho nuclear extracts. The intensity of complex I decreased during trophoblast differentiation; in contrast, the intensity of complex II increased as the trophoblast cells differentiated, which suggests that the protein-DNA complex formed at the Ets binding site changes during trophoblast giant cell formation (Orwig and Soares 1999). Since Ets2 has been implicated in the regulation of several trophoblast expressed genes (Butticè et al. 1996, Basuyaux et al. 1997, Yamamoto et al. 1998, Ezashi et al. 1998), Ets2 was also tested. Similar to our rPLII studies, Ets 2 antibody was not able to supershift the complexes in EMSA, suggesting Ets2 does not directly interact with the proximal d/tPRL promoter (Orwig and Soares 1999). The Ets family member that binds the d/tPRP promoters remains to be determined.

There are more than 35 members in the Ets family of transcription factors (Wasylyk et al. 1998). Even though all the proteins contain the Ets binding domain (EBD), which recognizes a core consensus sequence A/CGGAA/T, many identified Ets sites are selective for different Ets family members. The specificity of these proteins appears to lie in the sequences that surround this core. The Ets family has been divided into different subgroups according to sequence similarities within the EBD and the position of the EBD within the protein (Wasylyk et al. 1993, 1998). To identify the specific Ets transcription factor which binds directly to the rPLII enhancer, several other Ets proteins (ESX, Elf-R and Elk-1) which belong to different Ets subfamilies have been tested. In our transfection studies in Rcho cultures, ESX functionally activates the enhancer and this activation is lost if the rPLII enhancer Ets site is mutated, suggesting that ESX may bind directly to the enhancer.

ESX is a member of the Elf-1 subgroup based on the similarity of the Ets binding domains. In addtiion to the EBD, ESX contains a pointed domain and a serine-rich box

homologous to the transactivating domain of the High Mobility Group Protein, SOX4. Although the pointed domain is similar to that of Ets1, it lacks a consensus proline in the only known functional portion of this domain, a MAP kinase substrate site. No known target genes of ESX have yet been identified. ESX expression appears to be restricted to epithelial cells and has been shown to be highly expressed in human placenta (Chang et al, 1997).

We have demonstrated the presence of ESX mRNA in Rcho cells. The Ets binding site in rPLII enhancer is more closely related to the binding site of ESX (Chang et al. 1997) then to the reported Ets2 consensus-binding site (Stacey et al. 1995). Our preliminary EMSA experiments suggest that ESX could be a candidate protein that directly interacts with the Ets site on the enhancer. More complete competition and antibody supershift EMSA experiments will be needed to confirm the specificity and affinity of this binding. It will also be important to show that ESX is present in the nuclear extracts from rat placenta and Rcho cells.

Several alternative approaches can be used to further identify the placental Ets factor that binds to the rPLII enhancer.

1) Design degenerate PCR primers complementary to the highly conserved amino acid residues within the Ets binding domain and use these primers to amplify late placental or Rcho cDNA (Giovane et al. 1994). Clone and sequence PCR products to identify known or novel Ets proteins. These *in vitro* translated factors would then be tested by EMSA for binding to the rPLII enhancer Ets sequence.

2) Screen a placental expression library (Vinson et al. 1988) using a labelled rPLII Ets binding site oligonucleotide to identify clones that bind this sequence specifically.

3) Screen a late pregnancy placental cDNA library with an Ets binding domain cDNA probe. Clones would be sequenced for identity then tested by EMSA for ability to bind the rPLII enhancer.

4) Use the yeast one-hybrid system to identify factors that bind directly to the rPLII enhancer Ets binding site (Li and Herskowitz 1993). This method would be the most direct means for looking for Ets factors that interacted with the rPLII enhancer site at a relatively high affinity.

Previously it was reported that in the mouse a 2.7 Kb 5' flanking DNA fragment of mPLII is able to direct SV40 large T antigen to the placenta in transgenic mice (Shida et al. 1992). This is a similar result to our transgenic data in which 3031 bp of rPLII 5' flanking DNA was able to target luciferase expression to placenta. Most recently Lin and Linzer (1998) have reported more detailed studies of the above mouse regulatory fragment. By transient transgenic analysis they localized the giant cell-specific regulatory region of the mouse placental lactogen II gene to sequences between -2019 and -1340 upstream of the transcriptional start site. Their transfection experiments in Rcho cells identified two positive regulatory elements in the -1471 to -1340 region (Lin and Linzer 1998). Sequence at the 5' end of the mPLII enhancer fragment, from -1471 to -1340, is very similar to the 3' end of our reported 65 bp enhancer fragment (-1794/-1729). Lin and Linzer generated a set of scanning mutations throughout this 132 bp sequence. The 10 bp sequence (TAAACTGTAAGT) at the most 5' end of the mPLII activating fragment was shown to be essential for mPLII expression. Even though this sequence overlaps and is highly related to our FP1 region it does not contain an Ets binding site. Lin and Linzer concluded that the 10 bp sequence represented a binding site for an unknown transcription factor. The mPLII

enhancer sequence did not extend as far 5' as our identified rPLII enhancer and did not include an AP-1 binding site. The identification of enhancing activity within highly related but not identical sequences in the rat and mouse PLII genes is an intriguing problem that remains to be resolved.

We have several lines of evidence to support a functional Ets binding site on the rPLII enhancer. First, mutation of the core sequence of the putative Ets binding site significantly decreases the enhancer activity. Second, in DNAse I protection assays, there is a distinctive feature of FP1 in the presence of placental and Rcho nuclear extracts; a new hypersensitive site appears on the noncoding strand at the nucleotide immediately 5' of the consensus Ets binding sequence. This specific shift in DNAse I sensitivity has been reported to be a characteristic feature of murine Ets1 and Drosophila E74A binding to the Ets binding site (Gunther et al. 1990, Urness and Thummel 1990). Finally, in EMSA studies, a specific placental complex is competed by the native sequence, but not by an oligonucleotide containing a mutation of the GGA core of the Ets binding site, suggesting that this complex is formed by an Ets factor binding. These data support Ets factor binding to the rPLII enhancer sequence. The differences between the mouse and rat data suggest that in spite of a high overall degree of relatedness between the PLII genes in rat and mouse, the regulation of these genes may not be identical in the two species.

In addition to Ets and AP-1 binding sites, there are two putative GATA sites in or near the rPLII enhancing fragment. GATA-2 and GATA-3 are expressed in trophoblast giant cells and have been reported to regulate transcription of the mouse PL I gene promoter in Rcho cells (Ng et al. 1993, 1994). Neither rPLII GATA site appeared to be protected by placental or Rcho nuclear extracts in the DNAse I protection assays. To test further whether

the rPLII GATA sites were functional we mutated core sequences in both GATA sites in our enhancer/luciferase construct. In transfection assays in Rcho cells these mutations had a slight but not significant effect ($P>0.05$) on reporter gene expression, suggesting that these GATA sites are not functional in the rPLII enhancer.

Using site-directed mutagenesis, Lin and Linzer (1998) investigated the role of other GATA sites in the mPLII enhancer sequence. These sites are also present in the rat 5' flanking sequence, but are more 3' than the rPLII enhancer element. Mutation of these sites also had no effect on the function of the mPLII enhancer. These observations are quite different from the mPLI gene where GATA binding has been shown to be crucial for expression (Ng et al. 1993,1994). These findings suggest that the same placental giant cell type makes use of different combinations of transcription factors for the regulation of the related PLI and PLII genes.

Our transient transgenic mouse studies showed that the 3031 bp rPLII 5' flanking fragment is sufficient to target luciferase expression to placenta, suggesting that this fragment includes sequences that are important for placental specific expression *in vivo*. The variation in luciferase levels seen with the different Fo transgenic placenta may relate to insertion sites. The luciferase activity of the transgenic placentas did not correlate with transgene copy number. Ectopic expression was also some within some fetuses. These findings suggest that other regulatory elements, either in proximal flanking regions or even a more distant locus control region (LCR), may also required for complete developmental expression of rPLII. The regulation of human growth hormone and placental lactogens have been shown to be controlled by a LCR (Jones et al. 1995, Su et al. 2000). All eighteen genes of the rat PRL family so far identified are located on chromosome 17 (Duckworth et

al. 1993, Soares et al. 1998), suggesting a very large gene locus. There is, however, no evidence for a role of an LCR in the expression of the rPRL gene itself in transgenic mice (Crenshaw et al. 1989). The role of an LCR in the expression of placental members of the PRL gene family still remains to be explored.

It is increasingly apparent that the regulation of gene expression in higher eukaryotes is greatly influenced by the local chromosomal environment. The modification and remodeling of chromatin, which changes its the conformation, controls the activation and inactivation of genes. Active chromatin is often uncondensed, is located near the center of the interphase nucleus, and is replicated early in the cell cycle. Active chromatin contains sites that are two orders of magnitude more sensitive to DNAseI digestion than bulk chromatin, reflecting its more open structure (Reviewed in Higgs 1998).

DNAse I hypersensitivity analysis (Gaasenbeek et al. 1999) could be used to identify other transcriptionally active DNA sequences associated with the complete developmental expression of the rPLII gene. A comparison of DNAse I hypersensitive sites in placenta, Rcho and non-placental (eg. GC cells) cell nuclei may identify further regions that are important in both cell-specific and temporal expression of the rPLII gene.

To help define the role of the rPLII enhancer in placental-specific expression *in vivo*, F0 transgenic mice have now been generated that carry luciferase transgenes regulated by either a 3031 bp rPLII 5' flanking with mutated Ets-AP1 sequences, or the 65 bp enhancer with a minimal TK promoter. Although somewhat difficult to interpret, the Ets/AP1 site-mutated 3031 bp rPLII 5' flanking DNA doesn't appear to lose the ability to target expression to placenta. Unlike with the native 5' flanking DNA, however, there is ectopic expression in essentially all of the corresponding fetuses. The 65 bp enhancer construct also

does not target luciferase expression only to placenta (data not shown). These *in vivo* data suggest that the 65 bp rPLII enhancer alone does not define the placental specificity of the rPLII gene. It is likely that there are further tissue-specific regulatory elements located either on the 3031 bp 5' flanking DNA or elsewhere within the rPLII gene.

The full developmental expression of a gene contains three properties: where the gene is expressed, how much is expressed and when it is expressed. The functional studies of the rPLII enhancer region in different placental cell lines from different species and in transgenic mice indicate that this enhancer is important in specifying placental expression (where). This enhancer appears to be very important for the level of rPLII expression - without it the expression is minimal (how much). Once we have identified the specific Ets transcription factor that binds the enhancer, a comparison of its mRNA expression patterns in early and late placenta with that of endogenous rPLII expression may be helpful in answering whether this factor has a role in determining the timing of rPLII expression (when).

It has been known that transcription factors involved in the expression of genes of highly differentiated cells often consist of ubiquitously expressed factors acting in cooperation with other factors expressed in restricted cell types (Hayashi and Scott 1990, Herr and Cleary 1995). It is unlikely that one transcription factor alone accounts for the full developmental placental-specific expression of rPLII. It is very likely that multiple placental-specific transcription factors, ubiquitous transcription factors and other protein cofactors together determine the precise development pattern of rPLII expression.

To answer the biological question of what factors control the switch in expression between rPLI and rPLII, we have to understand the transcriptional regulators of each of

these genes. The mechanisms that control the switch in expression between PLI and PLII could function either by increasing PLI activators and PLII repressors or by decreasing PLI repressors and PLII activators before mid-pregnancy; and the opposite would follow after mid-pregnancy. AP-1 and GATA factors are involved in PLI regulation. AP-1 factors (Jun/Fos) are ubiquitous (Curran and Franza 1988, Johnson et al. 1993). GATA-2/3 are expressed in various, although restricted cell types, and their expression in trophoblast giant cells continues throughout pregnancy (Shida et.al. 1993, Ng et al. 1993 and1994). It seems unlikely that these factors are directly involved in the temporal expression of the PLI gene. The identification of the Ets factor that binds to the rPLII enhancer will provide us with a further tool to explore the factors involved in the switch in expression between PLI and PLII during pregnancy. The molecular mechanisms of rPLII regulation will ultimately lead us to the better understanding of this switch and increase our knowledge of placental gene regulation in trophoblast cells.

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