Identification of *Cis*- Elements and *Trans*- Acting Factors that are Involved in Rat Placental Lactogen II (rPLII) Gene Expression in Placenta

by

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A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Physiology

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Identification of *Cis*- Elements and *Trans*- Acting Factors that are Involved in Rat Placental Lactogen II (rPLII) Gene Expression in Placenta

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Doctor of Philosophy

Dedicated to my father and mother,

Ali and Necla Öztürk

and

my lovely sister Nesrin

ABSTRACT

A large family of PRL related genes have been identified in rodents and ruminants, which are expressed only during pregnancy in the placenta and maternal decidua. The rodent family members demonstrate highly specific cell and temporal expression patterns, but little is known about the factors that regulate this expression. I have used the rat placental lactogen II (rPLII) gene as a model to further our understanding of the transcriptional regulation of this family and placental genes in general.

Previous work identified an enhancer element containing two DNAse I protected regions within a proximal 3 Kb 5' flanking fragment that appeared to contain sequences important for placental specific expression. I was able to show by electrophoretic mobility assays (EMSA) that the FP1 region bound members of the Elf subfamily of Ets transcription factors. When database searches were unsuccessful in identifying binding factors to the FP2 region I applied a proteomics approach using matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF MS) to identify nuclear factors from trophoblast giant cells that bound the enhancer element *in vitro*. Using mass matching and tandem MS sequencing my collaborators and I identified AP2γ, a known placental-specific transcription factor in rodents, as a factor that could bind the rPLII enhancer sequence. EMSA experiments confirmed this binding and showed that it was specific for the FP2 region of the element. Reporter expression studies

in transfected cells indicated that enhancing activity was increased by exogenous AP2γ and lost when the FP2 sequence was mutated. Chromatin immunoprecipitation experiments confirmed that AP2γ was associated with DNA at the enhancer site in the chromatin of rPLII expressing Rcho cells. My data strongly suggest that AP2γ binding to the rPLII enhancer is likely to be an important component of the developmental expression of this gene during pregnancy.

With other members of the laboratory I undertook studies in transgenic mice, using a large P1 genomic clone to try to identify further regulatory sequences outside the 3 Kb 5' fragment that might be involved in the more complete, high level rPLII expression. We showed that the clone contained the rPLII gene, with approximately 4 Kb of 5' flanking sequence and complete 3' flanking DNA, a previously unknown rPLII-related pseudogene, the rPLP-I gene and the rPLP-B gene; I characterized the pseudogene, the first to be described in this gene family. The latter two genes contained complete 5' flanking regions and complete, or in the case of rPLP-B, partial 3' flanking sequences. Three transgenic mice were analyzed for rPLII and rPLP-B mRNA expression. Placentas from two fetuses expressed low levels of rPLII as compared to mouse PLII, but levels of rPLP-B that were comparable to the endogenous mouse gene. Our results suggest that regulatory sequences within a few kilobases of the transcription start site, as opposed to more distal sequences, play an important role in the high level expression of at least some members of this gene family, although they do not rule out a role for a locus control region as has been identified in some other large gene families.

To identify further regulatory regions in the rPLII 5' flanking DNA I carried out DNAse I hypersensitive studies using chromatin from rat placenta, rPLII-expressing Rcho rat trophoblast cells and non-expressing C6 rat glioma cells; results with rat placental genomic DNA indicated hypersensitivity in the region of the enhancer element. My results suggest that the Rcho cells which have served as a model system for the rat placental giant cell in many studies may not be as informative in these hypersensitivity studies as placental tissue itself.

My studies are the first to identify a role for a known placental-specific transcription factor in the regulation of a member of the PRL gene family; however, since AP2γ is expressed in all trophoblast cell types it cannot be solely responsible for the complete rPLII developmental expression. Future studies will be aimed at identifying these other regulatory proteins.

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ABBREVIATIONS

11β-HSD-2	11-Beta-Hydroxysteroid Dehydrogenase Type 2
A_{260}	Absorbance at 260 nanometers
A_{280}	Absorbance at 280 nanometers
ANOVA	Analysis of variance
ANF	Atrial Naturetic Peptide Gene
ATP	Adenosine triphosphate
bHLH	Basic Helix-Loop-Helix
bp	Base pair
C/EBP	CCAAT enhancer binding protein
ChIP	Chromatin Immunoprecipitation
Ch	Chromosome
DTT	Dithioreitol
DPE	Downstream Promoter Element
d/tPRL	Decidual/Trophoblast Prolactin-related Protein
E	Embryonic Day
EDTA	Ethylenediaminetetraacedicacid
EMSA	Electrophoretic Mobility Shift Assay
EPC	Ectoplacental Cone
ExE	Extraembryonic Ectoderm
FBS	Fetal Bovine Serum
FP	Footprint

GH	Growth Hormone
Gcm 1/Gcm a	Glial Cell Missing 1
GlyT	Glycogen Trophoblast Cells
GH-V	Growth Hormone Variant
GTF	General Transcription Factor
HAT	Histone Acetyltransferase
HDACs	Histone Deacetylases
HSS	hypersensitive sites
hCG	Human Chorionic Gonadotropin
hPL	Human Placental Lactogen Hormone
IGF-1	Insulin-like Growth Factor 1
ICM	Inner Cellular Mass
INR	Initiator Element
LCR	Locus Control Region
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time of Flight
MS	Mass Spectrometry
NK	Natural Killer Cells
NMR	Nuclear Magnetic Resonance
PIC	Pre-Initiation Complex
PLI	Placental Lactogen I
PLII	Placental Lactogen II
PRL	Prolactin
PLF	Proliferin

PLFRP	Proliferin-related Protein
PLP-A	Prolactin-like Protein A
PLP-B	PRL-like protein B
PLP-C	PRL-like Protein C
PLP-Cv	PRL-like Protein Cv
PLP-D	PRL-like Protein D
PLP-E	PRL-like protein E
PLP-F	PRL-like protein F
PLP-H	PRL-like protein-H
PLP-I	PRL-like protein-I
PLP-J	PRL-like protein-J
PLP-K	PRL-like protein-K
PLP-P	PRL-like protein-P
TAFs	TATA binding protein–associated factors
TBP	TATA-binding protein
TE	Trophectoderm
TG	Trophoblast Giant Cells
SpT	Spongiotrophoblast
SynT	Syncytiotrophoblast

Miscellaneous

bp	Base pair
h	Human

m	Mouse
r	Rat
A	Adenine
G	Guanine
T	Thymine
C	Cytosine
DNA	Deoxyribonucleic Acid
cDNA	Complementary DNA
RT-PCR	Real Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
mRNA	Messenger RNA
dNTP	Deoxyribonucleotide Triphosphate
TK	Herpes Simplex I Thymidine Kinase
PVC	Packed Cell Volume
Arg	Arginine
Lys	Lysine
His	Histydine
Ser	Serine
Thr	Threonine
Asn	Asparagine
Arg	Arginine
Glu	Glutamine

CHAPTER 1

INTRODUCTION

In eutherian mammals, the establishment and maintenance of pregnancy requires many physiological changes to take place in the maternal environment. The placenta, the transient organ formed during pregnancy plays an essential role in regulating these changes. The placenta is derived from the progressive interaction of the mother's uterus and the implanting embryo; it is a critical vascular compartment that facilitates the flow of maternal nutrients and fetal wastes between the maternal and fetal circulations and it functions as a protective barrier between the maternal immune system and the developing fetus (Cross, *et al.*, 1994; Robinson *et al.*, 1995; Kingdom *et al.*, 2000; Cross, *et al.*, 2003; Huppertz and Peeters, 2005). The placenta is also an endocrine organ responsible for manufacturing hormones, growth factors and enzymes, which are essential for embryonic development and the adjustment of maternal physiology to the new demands of pregnancy (Gluckman, 1986; Gootwine, 2004; Murphy *et al.*, 2006). The placenta synthesizes various cytokines and growth factors, including interferons, angiogenic factors, leptin and members of the growth hormone/prolactin gene family, including placental lactogens. Expression of these placental factors is highly regulated according to specific spatiotemporal patterns that reflect the needs of the developing fetus and maternal physiological adaptations.

Although human and rodent placental morphology is different, there are similarities in the roles of the specific cell types in each species, and their structural arrangements within the organ (Huppertz *et al.*, 2006). In particular we now know that there are several key transcription factors involved in placental development that are highly conserved in humans, rodents and some other species (Rossant and Cross, 2001; Cross *et al.*, 2002; Malassinè *et al.*, 2003). This finding makes rodents an important model for studying placental development and gene expression in general. In particular, targeted mutagenesis studies in mice have identified many important transcription factors that are involved in placental development. Data from such studies can provide insights not only into the genetic control of placental development, but also into the spatial and temporal regulation of placental tissue specific target genes, which are important for the fetal growth and maternal adaptations.

1.1. Development of the Rodent Placenta

There are two major events that are essential for the progressive formation of placenta - implantation and the development of the placental compartment that brings fetal/maternal blood supplies into close proximity allowing exchanges of nutrients and wastes (Huppertz and Peeters, 2005). Both events involve cells of the trophoblast cell lineage, which are derived from the outer cells of the blastocyst.

1.1.1. Early development and attachment

In the mouse the development of the placenta is initiated with the blastocyst stage at embryonic day (E) 3.5. After fertilization and development to the 8-cell stage, the events of compaction and polarization take place. Up to this point, all cells have equal developmental potential. At the 8 cell stage, an apical-basal polarization of the blastomeres have an essential role. The E-cadherin based cell-cell adhesions create an asymmetry in each blastomere. During further cleavage, radially oriented separation of some of the blastomeres leads to segregation of two domains into different daughter blastomeres. This event generates two different cell populations (Sutherland *et al*, 1990). The outer, polarized blastomeres form the epithelial trophectoderm layer of the blastocyst. This layer encloses the inner, highly cohesive non-polarized blastomeres, called the inner cellular mass (ICM) as illustrated in **Figure 1.1**. The trophectoderm (TE) layer leads to the establishment of extraembryonic cell linages to form the yolk sac and placenta; the ICM progenitors are mainly involved in the development of embryo proper, but also contribute to the formation of the placenta through the allantois, which is derived from extraembryonic mesoderm.

The trophoectoderm contains progenitors of several trophoblast cell types. The position of these cells relative to the ICM creates two regions called, mural and polar TE (**Figure 1.1**). TE cells in these two regions have different fates in the post-implantation embryo.

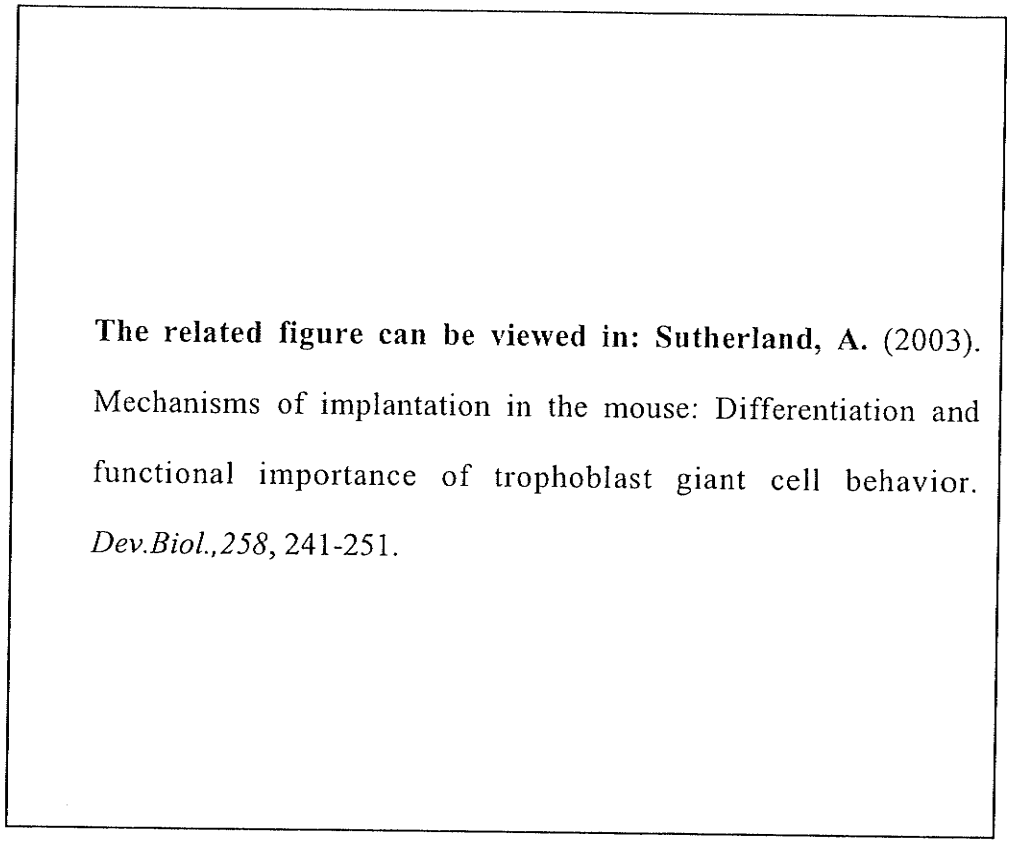


Figure 1.1: Schematic diagram in the early developmental localization of trophoblast progenitor cells.

The trophectoderm cells show transition to an invasive phenotype. The hatched blastocyst has a polarized, transporting, epithelial trophectoderm layer (TE), which exhibits no motility. The trophectoderm can be divided into two regions based on proximity to the inner cell mass (ICM), the polar TE (in green) and the mural TE (in yellow). As the blastocyst becomes competent to implant, the TE cells alter their polarity and begin to extend apical protrusions. During implantation in vivo, these protrusions aid in displacement and phagocytosis of the apoptotic uterine epithelial cells, and give rise to the laminar extensions that ultimately form the blood sinuses of the yolk sac placenta.

The mural TE cells, which are not in contact with the ICM, are the first to differentiate giving rise to the primary trophoblast giant cells. They initially adhere to antimesometrial cells of uterus and contact with maternal spiral arteries that supply blood to the implantation site at the beginning of development (Copp 1978; Copp 1979; Bevilacqua and Abrahamsohn 1988; Cross *et al.*, 2003). These cells are analogous to human extravillous cytotrophoblast cells. The process of the trophoblast cell differentiation continues to form polyploid trophoblast giant cells by endoreduplication, resulting in cells with multiple copies of the genome in very large nuclei. These cells participate in the formation of the early yolk sac placenta (**Figure 1.1**), which is also called the choriovitelline placenta. In contrast, the polar TE cells, which are adjacent to the ICM cells, are maintained as diploid cells in a proliferative and undifferentiated state by signals derived from the ICM. They give rise to the ectoplacental cone (EPC) and extraembryonic ectoderm (ExE). The EPC contains proliferative cells that provide a continuing supply of differentiating trophoblast cells, that serve to expand the area of the yolk sac placenta during the initial period of rapid growth but while the embryo is still very small (**Figure 1.2**) (Cross, 1998; Cross, 2000). The ExE develops into the chorion layer, which contains trophoblast stem cells; later, as the embryo becomes larger in size, the association of chorion layer with the allantois contributes to the labyrinth region of the definitive chorioallantoic placenta represented in **Figure 1.3** (Rossant and Croy, 1985; Cross, 2000).

During pregnancy, fetal blood vessels are derived from the allantois. The allantois originates from embryonic mesoderm at E8.0 and interacts with chorionic plate at E8.5.

This process is called the "chorioallantoic attachment". The chorion layer starts to form villi and fetal blood vessels begin to project from the allantois filling in the villous branches (Rossant and Cross, 2001; Cross, 2003). This highly vascularized region which contains both fetal and maternal blood vessels, is called the labyrinth; with its formation, the choriovitelline placenta is replaced by the chorioallantoic placenta which is second stage of the placental development. At this stage of development, the rodent placenta is made up of two functional zones - the labyrinth zone and the junctional zone.

The junctional zone is positioned between the maternal decidua and the labyrinth; trophoblast giant cells interface with and invade the maternal decidua while spongiotrophoblast and glycogen cells develop between the giant cells and the labyrinth. These latter cells are assembled like a sandwich in compact layers and correspond to the cytotrophoblasts of the human placenta. The labyrinth region is a complex vascular region with tree-like branches of fetal blood vessels and maternal blood sinuses, which are covered by a well-organized layer of trophoblast giant cells and syncytial trophoblast cells. It provides a permeable barrier between the maternal and fetal vascular structures. The villous structure with its many branches grows with the developing fetus. This extensively branched labyrinth region is related in function to the human chorionic villi.

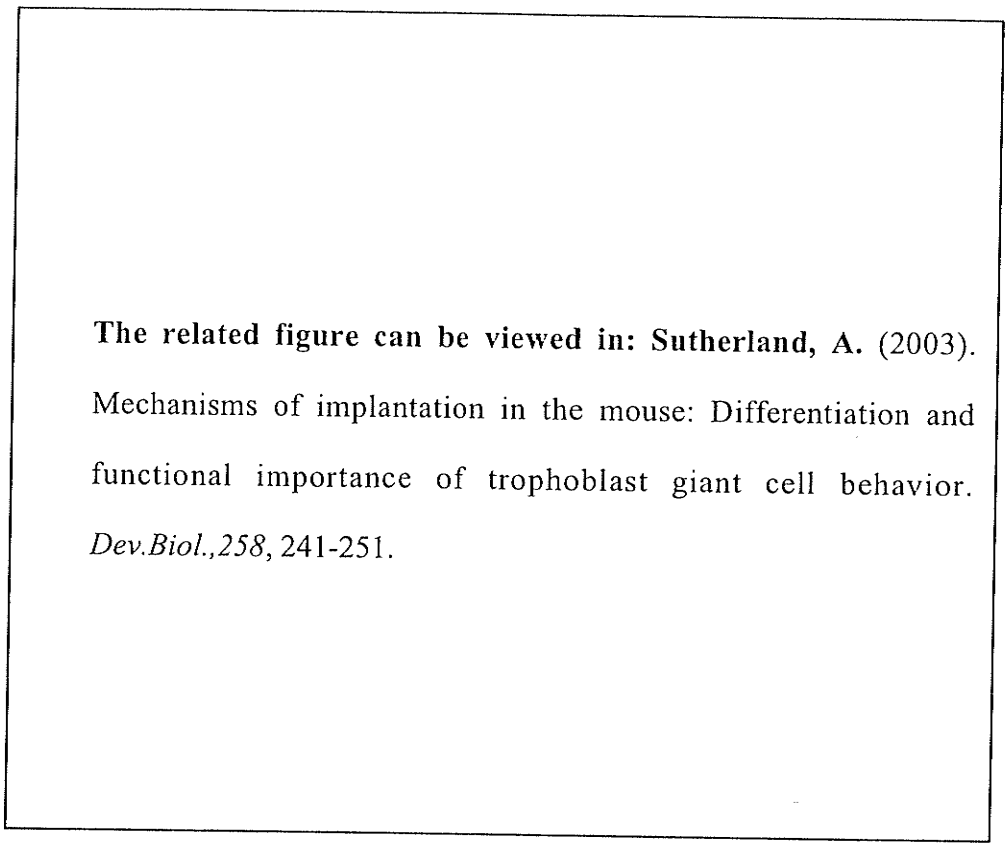


Figure 1.2: Schematic diagram of patterning of trophoblast giant cells in early placental development.

Primary giant cells, derived from outer cells of the blastocyst are found attached to Reichart's membrane, surrounding the developing fetus. Secondary giant cells develop at the outer periphery of the invading ectoplacental cone.

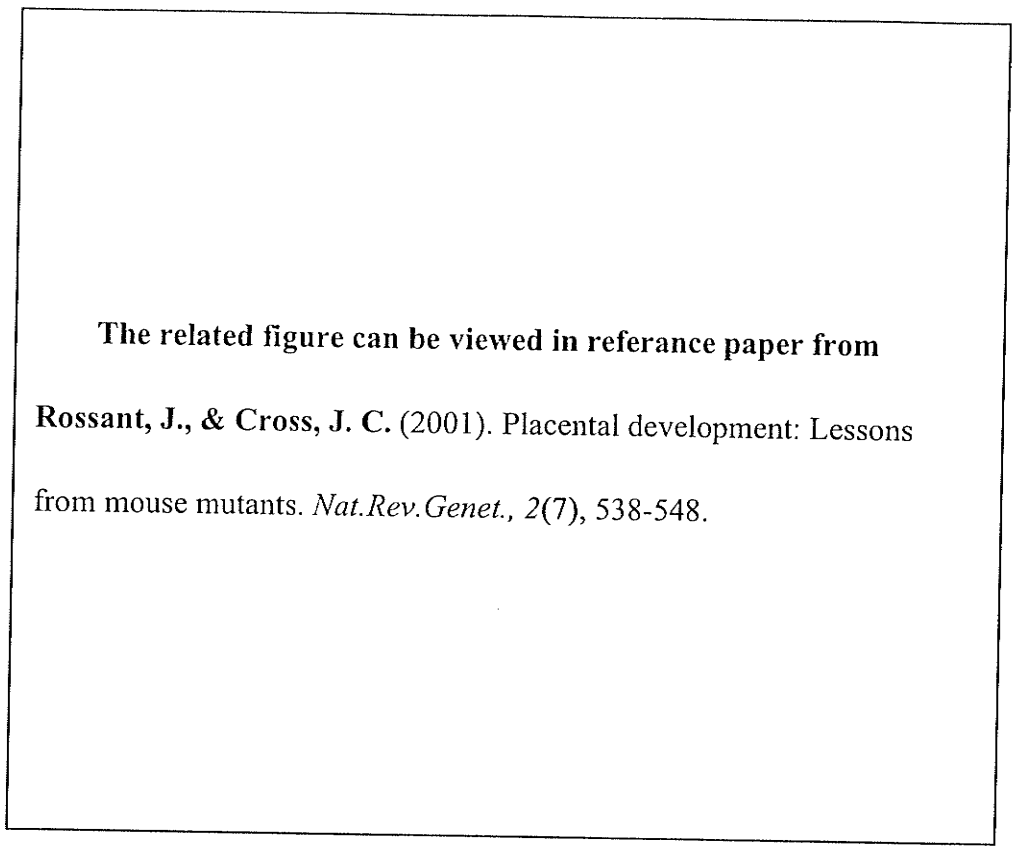


Figure 1.3: Developmental features of the chorioallantoic mouse placenta.

1.1.2. The development of the trophoblast cell lineages

There are four major differentiated cell types derived from trophectoderm in rodents; trophoblast giant cells (TG), spongiotrophoblast (SpT), syncytiotrophoblast (SynT), and glycogen trophoblast cells (GlyT) (Rossant and Cross, 2001; Cross *et al.*, 2003; Sahgal *et al.*, 2005; Sahgal *et al.*, 2006; Soares and Hunt, 2006). TG cells make up the interface between the decidua and placenta; a subtype of TG cells invades the maternal arteries in the decidua to supply blood to the implantation site at the beginning of development (Rossant & Cross, 2001; Cross et al., 2002; Adamson *et al*, 2002) (**Figure 1.3**). SpT cells are derived from trophoblast precursors in the ectoplacental cone; these cells arise at midgestation in association with the formation of the chorioallantoic placenta. SpT cells produce unique secreted proteins. In addition, they may be the precursors of the GlyT cells (Rossant, 1985; Cross *et al.*, 2001).

Following the chorio-allantoic fusion, chorionic trophoblast cells differentiate into two types of cells in the labyrinth - SynT cells and mononuclear trophoblast cells. The SynT cells make up the bilayer surrounding the fetal capillaries. SynT cells are multi-nucleated cells that form as a result of cell-cell fusion. The mononuclear trophoblasts line the maternal blood sinus. Together these cell layers function as the major transport surface for nutrient and gas changes between maternal and fetal circulation (Cross and Rossant, 2001; Cross *et al*, 2002). The GlyT cells have an unknown function; they appear only in late gestation, first within the SpT layer and later diffusely invade the interstitium of the decidualized uterus (Adamson *et al*, 2002).

1.1.3 A comparison between placental development in humans and rodents and endocrine function

Although there are differences between mouse and human placenta in morphogenesis and endocrine functions, the mouse placenta is now a widely accepted rodent model for the human placenta (Rossant and Cross, 2001). Both are the chorioallantoic type and though structurally different, analogous regions and cell types have been recognized and these species share several transcription factors that specify placental cell lineages during development. The gestation time for the mouse is 19 to 20 days (two days longer in rat) and for the human approximately 270 days. Some similarities and differences are outlined briefly below.

In both cases there are progressive interactions between the blastocyst and endometrium. During early pregnancy, the uterus has a key role in establishment of implantation and placental development (Fazleabas and Strakova, 2002). The process of decidualization, which is the transformation of stromal fibroblasts cells into differentiated decidual cells, is essential for implantation and survival of the embryo (Kim *et al.*, 1999). In rodents, trophoblast cells from mural trophoectoderm invade the uterine luminal epithelium of the antimesometrial decidua close to the basal lamina of the epithelium. This process initiates the decidual cells to penetrate progressively to basal lamina and lie under trophoblast cells. As implantation progresses, the basal lamina is displaced and left distal mural trophoblast cells close to the decidual cells, forming intercellular junctions

(Schlafke *et al.*, 1985; Blankenship and Given, 1992). One difference between humans and rodents, is that this penetration is deeper in humans (Ain *et al.*, 2006).

By 10 to 12 weeks of human pregnancy, there are two main functional villous structures – the floating villi, which are in contact with the maternal blood supply (Kaufmann *et al.*, 2003) and the anchoring villi, made up of proliferating cytotrophoblast cells which invade the decidua in an event called interstitial invasion (**Figure 1.4**). An accompanying event is the infiltration by cytotrophoblast cells into the lumen of maternal arteries and the replacement of vascular endothelial cells by trophoblast cells, which is called endovascular invasion (Malassiné *et al.*, 2003).

There are a number of endocrine differences between the mouse and human during pregnancy (**Figure 1.5**) (Albrecht and Pepe, 1990; Malassiné *et al.*, 2003; Malassiné and Cronier, 2002; Evian-Brion and Malassiné, 2003). Progesterone is required during gestation and has to be produced by *corpus luteum* throughout pregnancy. In the mouse, the *corpus luteum* is modulated by prolactin-like hormones. Mouse pituitary prolactin (mPRL) is the predominant hormone regulating *corpus luteum* function until midgestation, after which it is no long secreted (Freeman *et al.*, 2000; Soares and Linzer, 2001; Henkes *et al.*, 2003). Hypophysectomy studies carried out in pregnant mice and rats revealed that pituitary PRL is required early in pregnancy, but that secreted hormones from the placenta rather than pituitary can replace the functional role of PRL after midgestation (Pencharz and Long, 1931; Selye, 1933; Astwood and Greep, 1938; Linzer and Fisher, 1999).

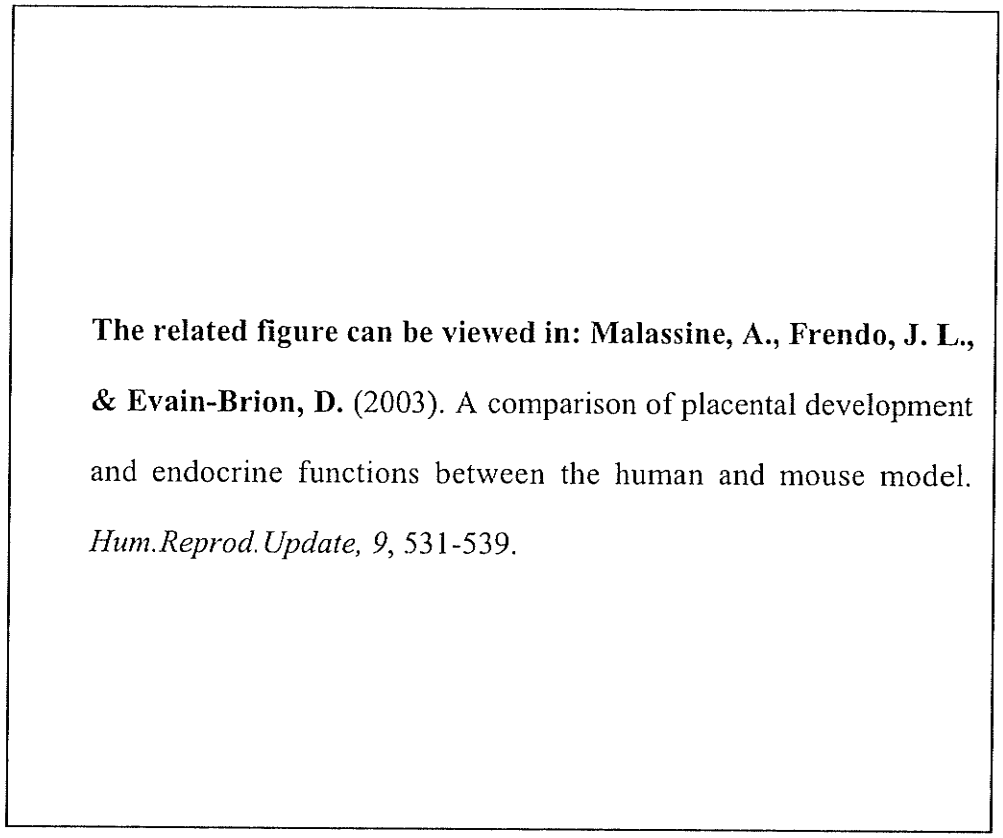


Figure 1.4: Trophoblast cellular layers of human placenta.

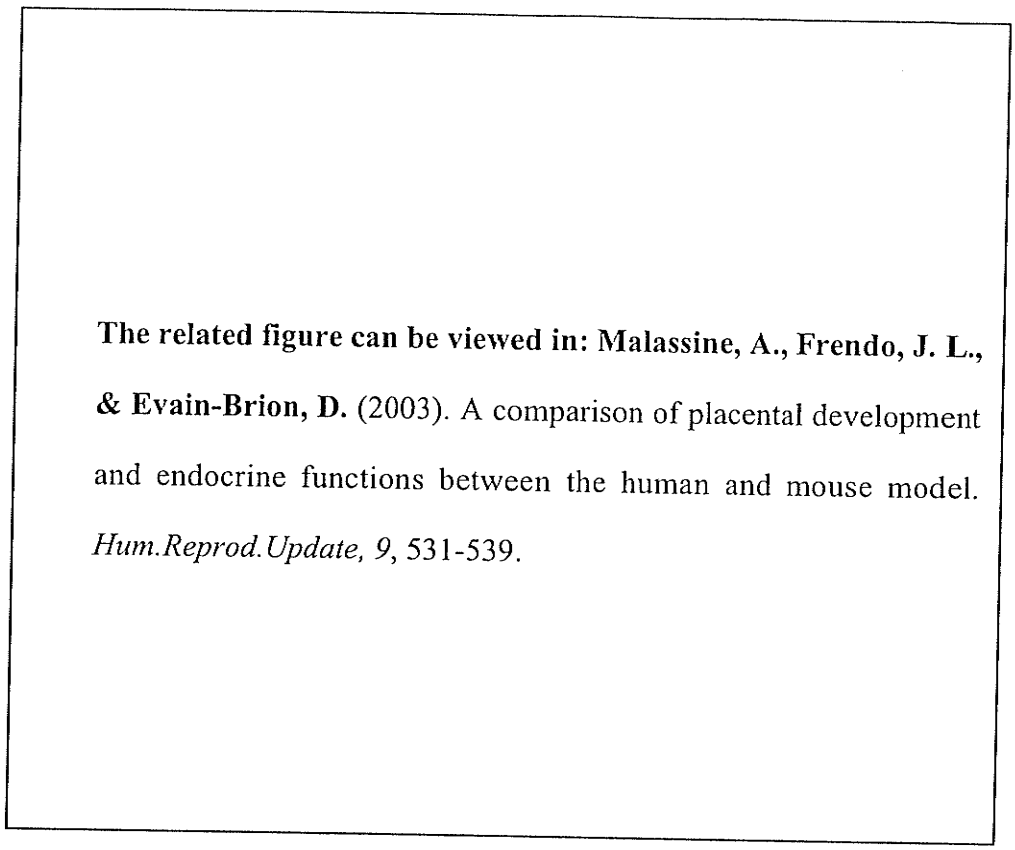


Figure 1.5: Expression of prolactin (PRL) - associated factors during gestation in rodents versus human.

In rodents and humans, a group of placental hormones expressed in placenta is classified as closely related to the pituitary homones prolactin (PRL) and growth homone (GH) according to their sequence similarity and in some cases shared receptors (Kelly *et al.*, 1991; Goffin and Kelly, 1997; Soares *et al.*, 1998; Harris *et al.*, 2004; Soares, 2004; Kossiakoff, 2004). In relation to the lactogenic effect and growth hormone-like activities during pregnancy, two factors demonstrating PRL-like activity were identified in mouse and rat (Kelly *et al.*, 1975 and 1976). They were shown to have different patterns of expression during gestation, but their activities were demonstrated not to be the same (Robertson and Friesen *et al.*, 1981; Soares *et al.*, 1982; Kishi *et al.*, 1988; Colosi *et al.*, 1988; Getler and Djiane, 2001). Later, they were defined as placental lactogens I and II (rPLI and –II), with target tissues such as the ovary and mammary gland. In mouse and rat, trophoblast giant cells of the placenta begin to produce placental lactogen I (PLI) from implantation until midgestation. In principle, PLI replaces pituitary function after implantation and placental development (Ogren and Talamantes, 1988; Soares and Linzer, 2001). Rat PLI hormone activity was detected from day 8 to 14, reaching its peak around E8 and then declining; in mice it remains at a low level until E10. Placental lactogen II is produced starting at midgestation and briefly overlaps with PLI expression. In the rat, PLII production starts around E10-12 and reaches a peak around day 14, remaining at a high level until parturition. Both PLI and PLII can bind the PRL receptor, but whether one or both of these proteins has a role in maintaining *corpus luteum* function has not been clarified; it is known that hypophysectomy after midpregnancy in mice does not cause termination of pregnancy (Strauss *et al.*, 1996).

The initiation and maintainance of pregnancy in humans do not require the involvement of the pituitary gland. In humans the first 8 weeks of gestation relies on the production of human chorionic gonadotropin (hCG) from the cytotrophoblast cells for the maintenance of the *corpus luteum* and progesterone production. This hormone also promotes the differentiation of trophoblast cells (Sirisuparp *et al.*, 2001; Cameo *et al.*, 2003; Cameo *et al.*, 2004). This hormone is essential for the early stages of pregnancy and serves as an early marker of pregnancy. After 8 weeks, syncytiotrophoblast cells produce progesterone and this production rescues pregnancy even if the ovary is not present. In contrast, rodents do not produce chorionic gonadotropin. The human placental syncytiotrophoblasts also produce chorionic somatomammotrophin (hCS) and a growth hormone variant (hGH-V) of the pituitary hormone (Pelmetshofer *et al.*, 1995; Martines-Rodriguez *et al.*, 1997; Hu *et al.*, 1999; Lewis *et al.*, 2000; Handwerger and Freemark, 2000; Silva *et al.*, 2002). Human CS, also sometimes called human placental lactogen hormone (hPL), is expressed by syncytiotrophoblast cells and is highly related in structure to hGH. It has been suggested this hormone has a role in fetal growth as well as in the adaptation of maternal metabolism to pregnancy. It has been suggested that hGH-V gradually replaces the pituitary hormone during pregnancy and has paracrine and autocrine effects on placental development (Lacroix *et al.*, 2002). Placental hGH-V modulates the levels of maternal insulin-like growth-factor 1 (IGF-1) and its decreased expression has been correlated with fetal growth retardation (Caufriez *et al.*, 1993 and 1994; Reece *et al.*, 1994; Fuglsang *et al.*, 2003).

The human syncytiotrophoblast cells also synthesize estrogen although placental estrogen synthesis is not required for the maintainance of the pregnancy, unlike in mice (Kwekel *et al.*, 2005). In addition, the 11-beta-hydroxysteroid dehydrogenase type 2 (11β-HSD-2), which plays a role in inactivating cortisol, is expressed in both mouse and human placenta. This enzyme is not expressed after midgestation in mice; it is expressed throughout the gestation in humans and appears to facilitate fetal growth, since mutations or low level expression of this enzyme have been associated with intrauterine growth retardation.

In rodents, a group of proteins related to pituitary prolactin (PRL), prolactin-related factors, are expressed mainly at the implantation site and in placental cells. These PRL family members are involved in the regulation of maternal and fetal adaptations during gestation. The pituitary, decidualized uterus and placenta show unique expression patterns of individual PRL family members, exhibiting cell type- and temporal–specific patterns in placental tissue during pregnancy.

1.2 Prolactin

Prolactin is a polypeptide hormone that is synthesized in the lactotroph cells of the pituitary gland. Across species more than 300 types of biological activities have been reported, including functions in reproduction, lactation, morphogenesis and immunity (Freeman *et al.*, 2000; Bole-Feysotet *et al.*, 1998). This protein is composed of a single amino acid chain with intramolecular disulfide bonds. As mentioned, PRL is essential in

rodents for the establishment of pregnancy and, until midgestation, for the maintainance of pregnancy (Freeman and Neill, 1972).

The PRL synthesized from the mammalian pituitary is involved in mammary gland development and growth. Prolactin is important for both maturation of the gland and during pregnancy when PRL contributes to the differentiation of the alveolar cells in the end buds of the mammary gland. PRL plays a key role in the synthesis of milk proteins after delivery (Horsemann *et al.*, 1999). Another PRL target is the ovary where it has a role in development and progesterone production during the luteal phase of pregnancy (McNeilly, 1997; Niswender *et al.*, 2000; Grosdemouge *et al.*, 2003; Bachelot and Binart, 2005).

1.2.1 The GH/PRL gene family

Human Family

The placenta produces a large number of hormones, some of which are normally expressed by other endocrine organs, and others which are unique to the placenta. Many of this latter group are members of the PRL/GH gene family. In humans, these hormones include the previously mentioned chorionic somatomammotropin (hCS) and growth hormone variant (GH-V), which are highly related to human pituitary growth hormone, both at the level of the protein structure and gene structure.

Rodent Family

In non-primates, particularly rats and mice, a large number of hormones have been identified that are related to PRL. These include PLI and PLII as well as a large number of other PRL-related hormones in mice and rats. These proteins show amino acid sequence similarity to the PRL protein and share very similar gene structures. Of these rodent proteins, only PLI and PLII bind to the PRL receptor. At mid-pregnancy in the rat, rising PLI concentrations are thought to result in the suppression of pituitary PRL secretion (Lee and Voogt, 1999). Certainly PRL levels fall at this point, and since hypophysectomy at this stage does not result in termination of the pregnancy it has been long felt that hormones produced by the placenta can take over the functions of PRL at this point (Selye, 1931; Astwood and Greep, 1938).

The PRL gene family in both rats and mice consists of at least 24 members. (Weimers *et al.*, 2003; Öztürk *et al.*, 2003; Soares, *et al.*, 2004; Alam *et al.*, 2006). Although all the genes are structurally related to the PRL proteins, they have been classified into two general groups based on their ability to bind the PRL receptor. As previously mentioned, only PLI and PLII have been shown to bind to the PRL receptor; all other members do not to bind the PRL receptor and in some cases have been shown to utilize other signaling pathways (Boyle-Feysot *et al.*, 1998). The major target tissues for classical PRL-receptor mediated signaling are the *corpus luteum* and the mammary gland, although PRL receptors are found in many tissues including the brain, pancreas and immune systems (Freeman *et al.*, 2000; Soares *et al.*, 2004). Receptors for the

non-classical members of the PRL family have not yet been identified in most cases; one protein expressed in the mouse, proliferin (PLF), has been shown to bind to the mannose-6-phosphate/IGF II receptor 2 (Lee and Nathans, 1988; Volpert *et al.*, 1996).

Prolactin-like Protein A (PLP-A) is a non-classical member of the rodent PRL family that uses an unknown receptor (Muller *et al.*, 1999). Studies show that it targets the uterine natural killer cells (NK), which reside in the decidua close to the uterine blood supply. To determine the function of mPLP-A, Ain *et al.* (2004), created a null mutation in mice. There appeared to be no effect of the mutation on the outcome of a normal pregnancy, but under conditions of hypoxia trophoblast cells did not invade the uterus normally, causing a significant effect on placental vascularization and early fetal death, perhaps due to lower nutrient and gas exchange between the placenta and fetus. This study suggests that PLP-A has an essential role for the development of normal hemochorial placentation under hypoxia, or perhaps more likely, other stress-induced adaptations. In humans, placental GH members have been suggested to have a role in adaptation to metabolic stress (Bole-Feysot *et al.*, 1998).

1.2.2. Spatial expression patterns of the rodent PRL family

The expression of PRL family members is developmentally regulated according to specific temporal and cell-type specific patterns. Cell-specific expression of family members is seen in the lactotroph cells of the anterior pituitary (PRL), in cells of the trophoblast lineage in placenta, and in cells of the decidualized uterus.

Trophoblast Giant Cells: A number of the PRL family members are expressed in trophoblast giant cells. Placental lactogen I (PL-I), placental lactogen II (PL-II) and proliferin (PLF), are exclusively expressed in trophoblast giant cells (Yamaguchi *et al.*, 1994). Both PL-1 and PLF are expressed at early postimplantation in the giant cells at the uterine face of the invading ectoplacental cone (Carney *et al.*, 1993). While PLI expression is terminated at midgestation, PLII expression begins at this time, slightly overlapping in expression with PLI, and continues until term. PLF expression continues at reduced levels throughout the rest of the gestational period (Faria *et al.*, 1990, Duckworth *et al*, 1990). Several other non-classical members are also expressed at midpregnancy in basal zone giant cells, although at a lower level than the PL's as judged by *in situ* hybridization. These include PRL-like protein-A (PLP-A), PRL-like protein-C (PLP-C), PRL-like protein-D (PLP-D), PRL-like protein-Cv (PLP-Cv), PRL-like protein-E (PLP-E), PRL-like protein-F (PLP-F) and PRL-like protein-H (PLP-H). Depending on whether it is the rat or the mouse, the majority of these genes are more highly expressed in spongiotrophoblast cells (Muller *et al.*, 1998; Dai *et al.*, 1998; Ishibashi and Imai, 1999; Saghal *et al.*, 2000; Toft and Linzer, 2000; Weimers *et al.*, 2003; Ain *et al.*, 2003; Ain *et al.*, 2005).

Spongiotrophoblast cells: As listed above, these cells express many PRL family members (Soares, 1996). The PRL-like protein-B (PLP-B) is not expressed in the trophoblast giant cell, but is highly expressed in spongiotrophoblasts from midpregnancy to term in rat (Duckworth *et al.*, 1990). While rat PLP-F is also expressed in giant cells, the mouse PRL-like protein-F (PLP-F) expression is restricted to spongiotrophoblast

cells, (Sahgal *et al.*, 2000). Both rat and mouse PLP-I are expressed within the junctional zone of the chorioallantoic placenta in spongiotrophoblasts (Ishibashi and Imai, 1999; Weimers *et al.*, 2003). In differentiated Rcho cells, its expression was not detectible by RT-PCR analysis (Dr. M. L. Duckworth). PLP-I *in situ* hybridizations demonstrated that rat PLP-I is expressed in spongiotrophoblast cells located in junctional zone of chorioallantoic placenta (Alam *et al.*, 2006).

Rat PLP-P expression was recently identified and shown to be expressed in the chorioallantoic placental tissue; it is expressed in both the junctional zone and in invasive trophoblast cells (Alam *et al.*, 2006). Expression was increased during the late stage of pregnancy. Its expression was also determined in Rcho-1 trophoblast cells and the level of mRNA transcript was relatively higher in proliferating cells rather than differentiated cells. rPLP-P expression was not identified in other tissues. The rat PLP-P does not have a mouse ortholog.

Labyrinthine trophoblast cells: There are cells related to the trophoblast giant cell type in the labyrinth region of chorioallantoic placenta, which appear to have a distinct phenotype from the basal zone giant cells. Some PRL family members, including PL-II, PRL-like protein-K (PLP-K) and proliferin-related protein (PLF-RP or PRP) are produced in these cells during the last week of pregnancy (Champell, 1989; Duckworth *et al.*, 1990, Dai *et al.*, 2000).

Decidual Cells: Four members of the PRL family are expressed in the maternal antimesometrial decidua. These include PLP-B, decidulal/trophoblast prolactin-related protein (d/tPRL), PRL-like protein-J (PLP-J) and PRL itself (Duckworth *et al.*, 1988; Croze *et al,* 1990; Roby *et al*, 1993; Gu *et al.*, 1994; Lin *et al.*, 1997; Choick *et al*, 1997; Orwig *et al.*, 1997; Orwig *et al.*, 1999). PLP-J is the only one of these that is restricted in expression to the decidual cells (Toft and Linzer, 1999). Decidual expression of these proteins is lost at midpregnancy as the antimesometrial decidua regresses; PLP-B and d/tPRP expression begins in the placenta at about this time.

The tempral of expression patterna and cell types are summarized in **Table 1.1.**

Table 1.1. Expression patterns of the rat prolactin family of proteins

Gene Name	Cell Type	Copartment	Time of Expression
PRL	Lactotroph cells Antimesometrial Decidual cells	Pituitary Decidua	Postnatal
PL-Iα	Trophoblast Giant Cells	Junctional Zone	Day 7-12
PL-Iβ	Trophoblast Giant Cells	Junctional Zone	Day 7-12
PLP-J	Decidual cells	Decidua	Day 7-10
PL-II	Trophoblast Giant Cells	Junctional and Labyrinth Zone	Day 11-term
PLP-I	Spongiotrophoblasts	Junctional Zone	Day 18-term
PLP-B	Spongiotrophoblasts	Junctional Zone	Day 13-term
	Antimesometrial Decidual cells	Decidua	Day 7-13
dPRP	Trophoblast Giant Cells Spongiotrophoblasts	Junctional Zone	Day 13-16
	Antimesometrial Decidual cells	Decidua	Day 7-13
PLP-K	Trophoblast Giant Cells	Junctional Zone	Day 16-term
PLP-D	Trophoblast Giant Cells Spongiotrophoblasts	Junctional Zone	Day 14-term

Table 1.1. Expression patterns of the rat prolactin family of proteins

Gene Name	Cell Type	Copartment	Time of Expression
PLP-Cv	Trophoblast Giant Cells Spongiotrophoblasts	Junctional Zone	Day 14-term
PLP-C	Trophoblast Giant Cells Spongiotrophoblasts	Junctional Zone	Day 14-term
PLP-H	Trophoblast Giant Cells Spongiotrophoblasts	Junctional Zone	Day 14-term
PL Iv	Trophoblast Giant Cells Spongiotrophoblasts	Junctional Zone	Day 14-term
PLP-Cβ	Trophoblast Giant Cells Spongiotrophoblasts	Junctional and Labyrinth Zone	Day 13-term
PLP-N	Trophoblast Giant Cells	Junctional Zone	-
PLP-Fβ	Trophoblast Giant Cells	Junctional Zone	Day 13-term
PLP-Fα	Trophoblast Giant Cells	Junctional Zone	Day 13-term
PLP-RP	Trophoblast Giant Cells Spongiotrophoblasts	Junctional and Labyrinth Zone	Day 13-19

Table 1.1. Expression patterns of the rat prolactin family of proteins

Gene Name	Cell Type	Copartment	Time of Expression
PLP-M	Trophoblast Giant Cells Spongiotrophoblasts	Junctional and Labyrinth Zone	Day 14-term
	Decidua	Metrial Glands	Day 14-term
PLF	Trophoblast Giant Cells	Junctional and Labyrinth Zone	Day 11-term
	Antimesometrial Decidual cells	Decidua	Day 8-term
PLP-A	Trophoblast Giant Cells Spongiotrophoblasts	Junctional Zone	Day 14-term
	Decidua	Metrial Glands	Day 14-term
PLP-L	Spongiotrophoblasts	Junctional Zone	Day 14-term
	Decidua	Metrial Glands	Day 16-term
PLP-P	Trophoblast Giant Cells Spongiotrophoblast	Junctional Zone	Day 15-term
	Decidua	Metrial Glands	Day 15-term

1.2.3 Genes identified to be involved in placental development

The use of "knock-out" mouse technology provided the identification of more than 100 genes that are important in placental development; a list is shown in **Table 1.2** (Cross, 2005). These genes can be grouped into transcription factors, receptors and signaling molecules, growth factors, and adhesion molecules among others. Below, a number of factors that are involved in controlling key events in placental development and determination of placental cell lineages are given, grouped according to cell-type phenotypic defects (Cross, 2005).

Table 1.2. Genes involved in defects of mouse placental development

Trophoblast stem cells

Activin, Cdx2, Eomes, Err2, Fgf4, Fgfr2, Dp1, Erk2, mTOR, Nipp1, Nodal, Talin

Trophoblast giant cells

CyclinE1/E2, Hand1, I-mfa, Eed, Epcr, Fbw7, K18/19. Kip2, Lifr, Mfn2, Socs3

Spongiotrophoblast

Mash2, Egfr, Arnt, Nodal

Chorioallantoic attachment

Bmp5/7, Cdx2 Cyclin F, Cyr61, Dnmt, Edd, Err2, Fgfr2, Itga4, Lim1, Lpp3, Mrj, Nodal, Smad1, T, Tbx4, Vcam1, Zfp36L

Syncytiotrophoblast and labyrinth

α7 adrenergic receptors, Ap2γ, Arnt, Chm, Cited2, Met, Ctbp2, Cx26, Cx31, Cx43, Dlx3, Egfr, Erk2, Erk5, Esx1, Fzd5, Gab1, Hey1/2, Hsp90b, Hgf, Igf2, Il10, Itgav, Itgb8, JunB, K8/18, Kip2, Lbp1a, Lifr, Mek1, Mekk3, Nex1, Nodal, Nte, Cul7, p38MAPK, Kip2, Pdgfb, PKBa, Plk2, Pparγ, RAP250, Rb,Rxra, Sos1, Tfeb, Vhl, Wnt2

Glycogen trophoblast differentiation

Igf2, Kip2

1.2.4 Basic helix-loop-helix transcription factors

A number of basic helix-loop-helix (bHLH) transcription factors regulate the formation of specific trophoblast cell lineages, particularly early in fetal/placental development (Cross, 2000). Members of this transcription factor family are evolutionarily conserved and tissue specific factors (Hollenberg *et al.*, 1995; Firulli, 2003). They contain a basic protein domain, which facilitates DNA-binding to a CANNTG element termed an E-box, and a HLH region, which is responsible for dimerization with other transcription factors (Massari and Murre, 2000).

One of the members of this family, Hand 1 is the key factor for determination of trophoblast giant cell differentiation; secondary giant cells emerging from the leading edge of the ectoplacental cone express Hand-1 (Cross *et al.*, 1995; Morikawa and Cserjesi, 2004). Hand-1 is expressed in extra-embryonic membranes and is essential for the differentiation of dividing trophoblast cells to giant cells. In mice, Hand-1 homozygous null mice showed dramatically reduced number of giant cells and subsequent placental defects. Homozygous Hand-1 mutants arrest at E7.5, displaying a smaller ectoplacental cone (Riley *et al.*, 1998; Scott *et al.*, 2000). Interestingly, Hand 1 does not appear to be expressed in developing human placenta, although it has been detected in human placental cell lines (Knofler, 2002; Liu *et. al.*, 2004). It has been suggested that Hand-1 may have a role in regulating PLI expression (Anthony *et al.*, 2001; Anthony *et al.*, 2003), but no direct interaction has been demonstrated with a PLI regulatory sequence in either mouse or rat. Hand-1 has also been shown to have role in

the developing mouse heart (Thattaliyath *et al.*, 2002; Furilli, 2003; Togi *et al.*, 2004; McFadden *et al.*, 2005).

Development and survival of spongiotrophoblast cells is dependent on another bHLH transcription factor, Mash-2 (Guillemot *et al.*, 1994). This factor is required for the maintainance of proliferating precursor cells that develop into both giant cells and spongiotrophoblasts. The expression of both Hand-1 and Mash-2 has been demonstrated in the ectoplacental cone but in non-overlapping trophoblast cell types (Riley *et al.*, 1998), suggesting that Hand-1 suppresses Mash-2 expression during early giant cell differentiation. When Mash-2 is overexpressed, it prevents differentiation of giant cells *in vitro*. Mash-2 knock-out mice are developmentally arrested at E10.5 as a result of a lack of spongiotrophoblasts in the junctional zone. Mash-2 function can be modulated by other bHLH proteins such as HEB and ITF-2, which are also expressed in the ectoplacental cone in mice (Scott *et al.*, 2000). The human homologue of Mash-2, Hash-2 is expressed in human cytotrophoblast cells (Janatpour *et al.*, 1999; Westerman *et al.*, 2001).

Members of the bHLH proteins can form homo- and hetero-dimers through their HLH domain. For example, Hand-1 and Mash-2 can form heterodimers with other bHLH factors (Johnson *et al.*, 1992; Voronova and Lee, 1994). It has also been proposed that both I-mfa and Hand-1 may heterodimerize with Mash-2 and eliminate its function (Scott *et al.*, 2000). There are other placental HLH proteins, Id1 and Id2, that do not contain DNA-binding domains. Id proteins are downregulated in giant cells and it has been

proposed that these factors may be function to suppress giant cell differentiation by forming heterodimers with Hand-1 (Jen *et al.*, 1997). Interestingly, Id2 is expressed in the first trimester human placenta, suggesting that it may have a role in deciding between the proliferation versus differentiation pathways for trophoblast cells (Janatpour *et al.*, 2000).

Hand-2 is expressed in at high levels in the deciduum and at lower levels in extra-embryonic membranes. Although Hand-1 and Hand-2 are structurally related, Hand-2 expression was enhanced in Hand-1 null mutant mice (Morikawa and Cserjesi, 2004). Interestingly, Hand-2 is also recruited to promoter element region via protein-protein interactions with GATA4 (Dai *et al.*, 2002). In heart development Hand-2 can also interact with a homeodomain factor Nkx 2.5 in combination with E12, enhancing transcriptional activity of the atrial naturetic peptide gene (ANF) promoter (Thattaliyath *et al.*, 2002).

Stra13 is a retinoic acid-inducible member of the bHLH transcription factor family. Stra13 expression is induced during trophoblast cell differentiation and facilitates arrest of cell proliferation and induction of trophoblast giant cell differentiation (Yan *et al.*, 2001). It has been reported that overexpression of Stra13 promotes terminal differentiation of trophoblast stem cells in the chorion (Hughes *et al.*, 2004).

1.2.5 Involvement of other transcription factors

Glial cell missing 1 (Gcm 1/Gcm a), is a transcription factor with a *GCM* domain, which is a zinc-containing DNA-binding domain, (Hashemolhosseini and Wegner, 2004). Gcm a/1 binds directly to an octameric GC-rich DNA-binding site (Lin *et al.*, 2005). Gcm 1 is expressed in trophoblast stem cells of the chorionic plate as early as E8; expression is restricted to villous branch points following chorioallantoic attachment (Cross *et al.*, 2001). Gcm1 is expressed during villous branching at the tip of elongating branches. This location is where trophoblast cells elongate and fuse to form the syncytiotrophoblast cell layer of the labyrinth. Targeted deletion of the Gmc1 gene demonstrated that the chorioallantoic attachment proceeded but chorioallantoic branching did not start and fetal vessels remained in the allantois (Anson-Cartwright *et al.*, 2000).

The zinger-finger transcription factors GATA2 and GATA3 have been implicated in the developmental regulation of a number of genes in a variety of cells (Ma *et al.*, 1997). Their expression has been localized to trophoblast giant cells in the early mice placenta (Ng *et al.*, 1994). Targeted mutation of either GATA2 or GATA3 leads to reduced expression of both PRL family members, PLI and PLF, in the invading ectoplacental cone (Ma *et al.*, 1997). Disruption of both GATA genes caused repression of mPLI expression and arrest at midgestation of placental development. Loss of GATA2 also affects the expression of PLP-A. In the mouse, this gene is usually expressed in secondary trophoblast giant cells of the basal zone, providing a specific marker to differentiate these cells from primary giant cells. In the GATA2 mutant mouse PLP-A is

expressed in both primary and secondary trophoblast giant cells (Ma and Linzer, 2000). GATA factors may therefore function as both positive and negative regulators of trophoblast giant cell expression and have a role in establishing or maintaining the distinct functions of secondary and primary trophoblast giant cells.

In rodents, AP2γ is involved in placental development; its expression is restricted to the trophoblast cell lineages. AP2γ homozygous knock-out mice do not survive beyond E8.5, showing failure to establish the early choriovitelline placenta (Werling and Schorle, 2002; Auman *et al.*, 2002). In these transgenics, extraembryonic tissue is malformed and the number of trophoblast giant cells is dramatically reduced compared to normal pregnancies. Members of the AP2 family of transcription factors, in particular AP2α and AP2γ have been implicated in the regulation of human and mouse genes specifically expressed in differentiated trophoblast cells (Morasso *et al.*, 1999, Shi and Kellems, 1998, LiCalsi and Mellon, 2000, Knofler and Husslein, 2000, Richardson *et al*, 2001, Roberson *et al.*, 2001; Jin *et al*, 2004, Knofler *et al.*, 2004).

Several CREB-binding protein/p300-interacting transactivator with ED-rich tail (*Cited*) proteins have been shown to interact with AP2 transcription factors. *Cited* proteins do not interact with DNA, but function as co-activators through interactions with both p300/CREB-binding proteins and AP2 factors. Co-activators facilitate interactions with the general transcription machinery and function as histone acetyltransferases (HAT), allowing chromatin remodeling or acetylating transcription factors to modulate their activity (Kouzarides, 2000). *Cited* 1, 2 and 4 have been shown to interact with

members of the AP2 family (Braganca *et al.*, 2003); *Cited*1 is expressed in trophectoderm-derived cells and a mutation in this gene results in a disorganized spongiotrophoblast layer and a reduced labyrinth area (Rodriguez *et al.*, 2004). Maternal blood vessels are also enlarged in these mutants, resulting in less area for nutrient exchange; most null embryos die shortly after birth. *Cited* 2 is widely expressed in the placenta and extraembryonic tissues; *Cited* 2 null mice have significantly smaller placentas at E12.5 and E14.5 and die *in utero* (Withington *et al.*, 2006). The effects of these null mutations on placental development may be due to the interactions of the *Cited* proteins with AP2.

1.3. Gene Regulation

Eukaryotic cellular organisms possess a common genetic material called "genomic DNA", that encodes information for the production of more than 6000 to 10000 proteins (Lamond and Earnshaw, 1998). During the last several decades, studies on the structure and function of DNA in the nucleus has provided information on various aspects of the control of gene expression; a complete understanding of the regulated expression of the many genes in eukaryotic organisms throughout development has yet to be obtained. We now understand something of the general protein machinery that is involved in the regulation of transcription by RNA polymerase II from basic DNA gene promoter sequences and we are extending our knowledge to a better understanding of the roles of short and/or long distance regulatory DNA elements such as enhancers, insulators, locus control regions and their associated protein complexes. Most recently we

are beginning to understand the role of the dynamic DNA/protein structure of genomic DNA – chromatin in gene regulation. In this section, I will present some of our current knowledge in these areas.

1.3.1 General features of DNA recognition by transcription factors

A DNA strand is composed of a deoxyribose sugar-phosphate backbone convalently linked to two types of purines bases, adenine (A) and guanine (G) and two types of pyrimidine bases, thymine (T) and cytosine (C), which protrude from the backbone and give characteristic diversity to the structure. Genomic DNA is made up of two long polynucleotide chains (or strands) in a double helix structure. Each base forms hydrogen bonds with its complementary base on the opposite nucleic acid chain, creating the double-stranded DNA (**Figure 1.3.1a**). The hydrophobic nature of the bases favors double helix formation. There are about 10 base pairs in a complete turn of 360°. Base pairing is between A and T or G and C; the GC pairing with three hydrogen bonds is somewhat stronger than the AT pair with only two hydrogen bonds (**Figure 1.3.1b**). The bases can pair only if the two strands of the double helix are antiparallel – the polarity of the one strand must be the opposite of the other. The coiling of the two strands around each other creates a major (wide) groove and a minor (narrow) groove in the double helix DNA structure. It is the specific sequence of the base pairs that encodes the genetic information. The major groove is the primary region where DNA binding proteins interact (Dickerson and Chiu, 1998).

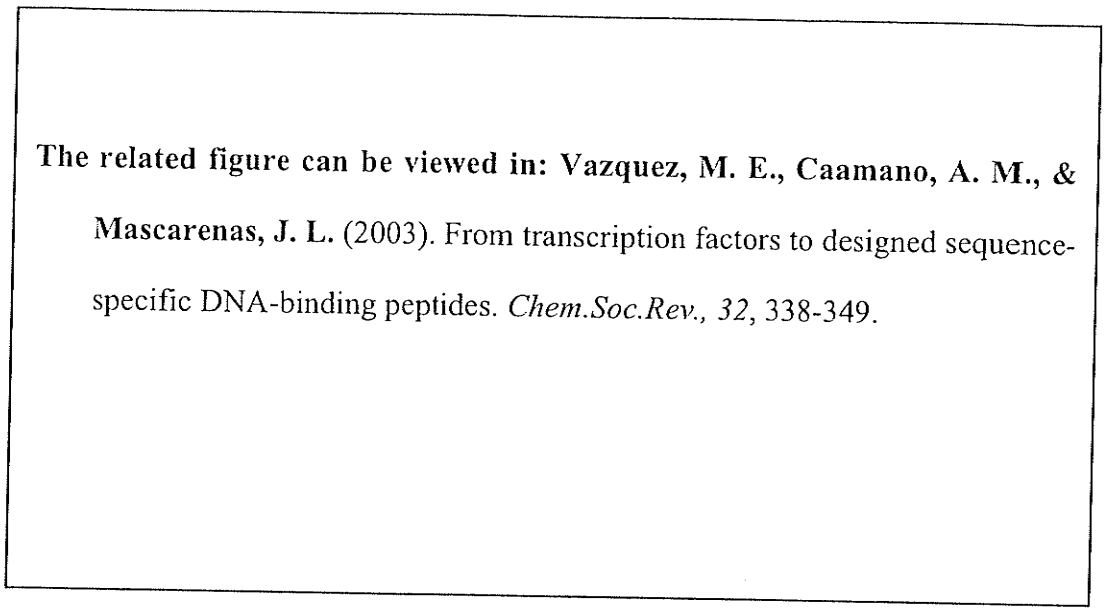


Figure 1.3.1a: **B form of double stranded DNA.**

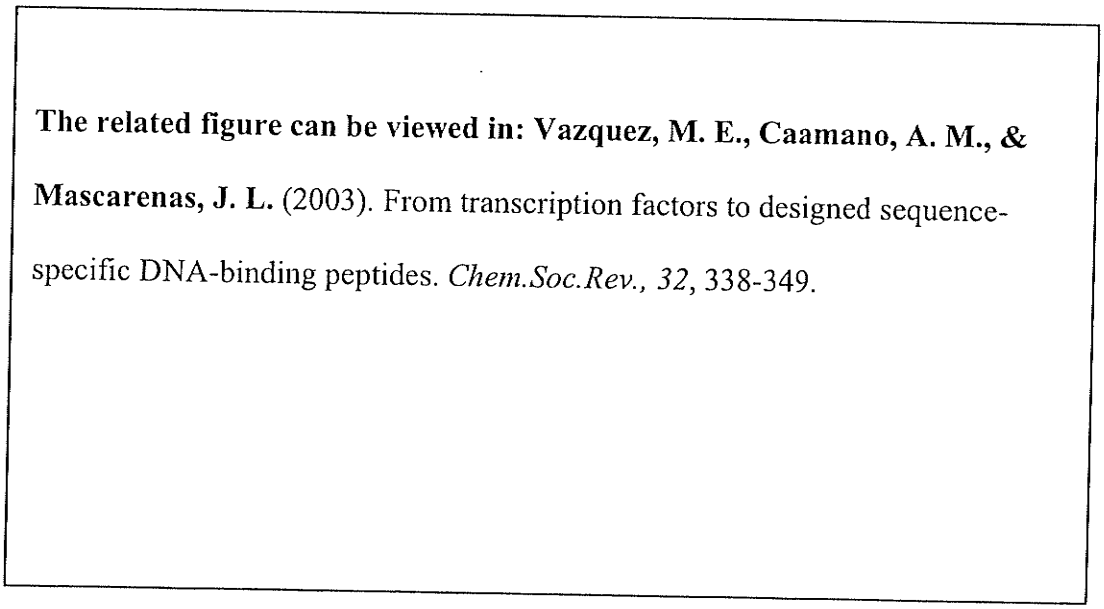


Figure 1.3.1b:Schematic diagram of base pairing by hydrogen bonding of the exposed functional groups between A and T, and G and C bases are represented.

The proteins that regulate genes must recognize specific DNA sequences contained within the strands. Most DNA binding proteins interact with DNA through exposed groups of the bases usually in the major groove (**Figure 1.3.2**). The specific sequence of in the DNA represents a distinctive pattern of hydrogen bond donors, acceptors and hydrophobic interactions. A specific domain of the protein, the DNA-binding motif, recognizes a particular DNA sequence and interacts with the functional groups of bases of double helix DNA through these bonds. The principle of this interaction is called the "DNA recognition code" (Choo and Klug, 1997; Suzuki *et al.*, 1995; Hendry *et al.*, 1984). For example, a common observation of protein interactions with DNA was through Arg and Lys amino acids, which can bind to A and T bases by hydrogen bonds. However, as seen in X-ray structure analysis the same amino acids can also bind to G. Some studies suggest that there is no such universal recognition code but that there are many amino acid-base interactions, which are modulated by the combination of many different types of interactions. It might seem likely, however, that there are some constraints on binding since specific DNA binding sequences are common among families of transcription factors with their similar DNA binding domains (Nadassy *et al.*, 1999; Jones *et al.*, 2001; Luscombe and Thornton, 2002).

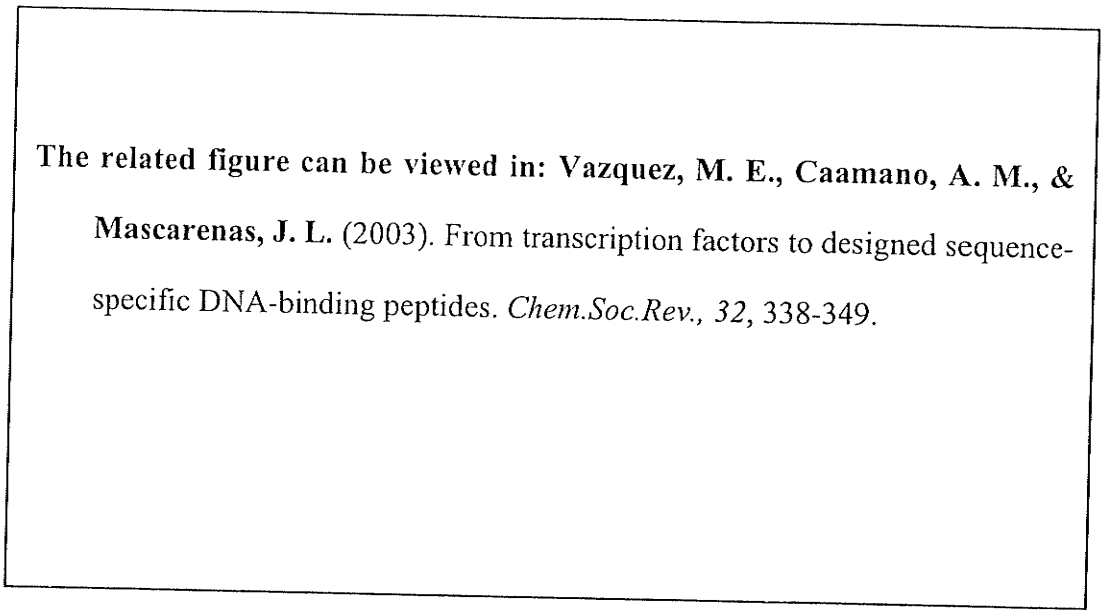


Figure 1.3.2: Hydrogen bonding interactions between bases and amino acid side chains of Arg, Lys, Asn, and Gln.

A number of major types of interactions between proteins and nucleic acids have been identified. These include:

A. Salt bridges and hydrogen bonds established between the DNA phosphodiester backbone and amino acid residues of basic amino acid protein domains (Arg, Lys, His). These chemical bonds help to orient the DNA-binding motif and stabilize DNA-protein complexes. These interactions are also involved in specificity for recognition process.

B. Hydrogen bonds between the sugars or bases in the DNA and polar side chains of amino acids. Based on the analysis with X-ray and nuclear magnetic resonance (NMR) structures, hydrogen bonds involved in amino acid-nucleotide interactions are

classified in various ways (Vazquez *et al.*, 2003): 1. Single hydrogen bond interactions between an amino acid and base. 2. Bidentate interactions with two or more hydrogen bonds between an amino acid a nucleotide or nucleotide pair. 3. Complex interactions.

The majority of hydrogen bonds take part in bidentate and complex interactions. Changes in local DNA structure are often dependent on the physical environment of the nucleic acid. On the protein side, polar and charged residues play a central role. Arg, Lys, Ser and Thr are the most common amino acids that participate in this type of hydrogen bonding. In particular, bidentate hydrogen bonds form between a single protein side-chain and a base pair, providing a means of increasing the bond energy per amino acid-base pair while conferring a higher degree of specificity for a given sequence (**Figure 1.3.2**).

C. Non-polar interactions. It has been suggested that a hydrophobic amino acid side chain within a protein domain may provide a selectivity for recognition of the DNA sequence (Luscombe and Thornton, 2002; Luscombe *et al.*, 2001).

D. Water-mediated hydrogen bonds. This type of interaction is established between polar and charged amino acids such as Lys, Asn, Arg, Gln and Glu, and the DNA backbone (Feng *et al.*, 1994; Suzuki *et al.*, 1994).

At the cellular level, sequence specific protein-DNA interactions are key factors in the processes of transcription and replication. In general, the sequence specific DNA

binding proteins utilize many types of bonds to recognize and interact with their consensus binding sites (Marmorstein and Harrison, 1994; Marmorstein and Fitzgerald, 2003). Many of these proteins are grouped into families based on the amino acid sequence and structural homologies within the DNA-binding domain. In eukaryotes, sequence specific DNA binding domain containing proteins are grouped into helix-turn-helix, zinc-finger, helix-loop-helix, leucine zipper, steroid receptors and homeo domain-containing classes of transcription regulatory proteins.

The secondary and tertiary structure of given protein domains, reflect the architecture of the protein-DNA complex interaction, showing a highly complementary shape recognition at interacting surfaces, which reflects chemical bond formation. Filter-binding, gel-shift and DNAse I protection analyses are standard procedures to assess DNA-protein interactions. However, a number of detailed approaches from thermodynamic and kinetic analysis to chemical and stereochemical analysis have been developed to uncover the detailed mechanisms of determining the specificity and stability interactions between proteins and DNA (Rhodes, *et al.*, 1996; Oda and Nakamura, 2000). Recently, the tools of modern structural biology, such as X-ray and NMR, have provided three-dimensional structural information and have improved our understanding of the principles involved in recognition between regulatory protein domains and DNA/RNA (Tateno *et al.*, 1997; Yang and Van Duyne, 2004). It is this type of result that has established that certain amino acids have preferences for interactions with certain bases, such as the interaction of arginine with N7 and O6 of guanine and of asparagine and glutamine with the N7 and N6 of adenine **(Figure 1.3.2)** (Mandel-Gutfreund and Marlit,

1998; Luscombe *et al.*, 2001).

1.3.2 The structure of chromatin

To understand transcription factor protein-DNA recognition and functionality in genomic DNA, it is first necessary to understand the higher forms of DNA packing that occur in the nucleus and how accessibility to that DNA is regulated. In the nucleus the minimum basic structure of DNA is organized into a "beads on a string" structure in which the DNA is wrapped around a core of histone proteins. This structure is called a nucleosome. Each protein octamer is formed from two histone H2A-H2B dimers and a histone H3 - H4 tetramer (Kronberg and Thomas, 1974; Arents *et al.*, 1991; Luger *et al.*, 1997; Hansen, 2002). These proteins contain domains called histone-fold motifs, which facilitate interactions among the histone subunits as well as the DNA. Individual nucleosomes are separated by linker DNA of varying lengths. Including the DNA wrapped around the core histone octamer and the linker DNA each nucleosome is associated with approximately 160 nucleotides. This is the basic unit of chromatin. In the nucleus nucleosomes are more usually packed on top of one another. Both specific intra- and inter-nucleosomal interactions among the nucleosome core proteins and the association of histone H1 and its variants to the linker DNA increase the compaction of the chromatin to a 30 nm fiber and enhances further contacts with other fibers (Thoma *et al.*, 1979; Woodcock and Horowitz, 1995; Iarovaia *et al.*, 2004). Total compaction of the DNA in chromatin is from 1000 to 10,000 times, depending on the stage of the cell cycle and the chromatin region. Chromatin is further organized in the nucleus by attachments to

the nuclear matrix via loop structures containing from 5000 to 100000 bp of packed DNA. This elaborate organization restricts the accessibility of regulatory proteins involved in gene expression (Beato and Eisfeld, 1997).

1.3.3. Activation of eukaryotic genes and transcriptional regulation

Regulatory mechanisms involving both ubiquitously expressed and cell-specific protein factors play essential roles during the development and differentiation of multi-cellular organisms. Modulation of the precise expression patterns genes is driven by the interaction between *cis*-acting DNA elements and *trans*-acting transcription factors as well as factors associated with chromatin modification. Below I will address our current knowledge of these interactions.

Eukaryotic genes can be in one of two chromatin states – transcriptionally inactive or transcriptionally active (or poised for action). The inactive state is called heterochromatin and is the result of a high degree of chromatin condensation, which limits accessibility of DNA binding protein factors. The state of chromatin that is transcriptionally active or poised for activity is called euchromatin, which is in a more open chromatin state (30 nm) and therefore more accessible to the basal and activated transcription machinery. Even in this more open state, whether gene transcription occurs or not is dependent on the presence of stable transcription initiation complexes. At a minimum, transcription requires core promoter DNA elements on which to assemble the RNA polymerase II protein initiation complex. As shown in **Figure 1.3.3**, these basic

DNA elements include a TATA box sequence, a BRE/TFIIB initiator element, an initiator element (INR) and a downstream promoter element (DPE). A few genes are known that contain a TATA-less promoter, in which case the INR may bind the TFIID protein of the initiator complex more strongly (Weis and Reinberg, 1992; Smale, 1997; Butler and Kadonaga, 2002; Smale and Kadonaga, 2003). Beyond these basic DNA sequences found within the promoter region, transcription is affected by more distant DNA elements, called enhancer or repressor sequences that bind proteins factors which can either stabilize or destabilize this transcription initiation complex, affecting whether transcription will take place (Gross and Garrard, 1987; Levine and Tjian, 2003).

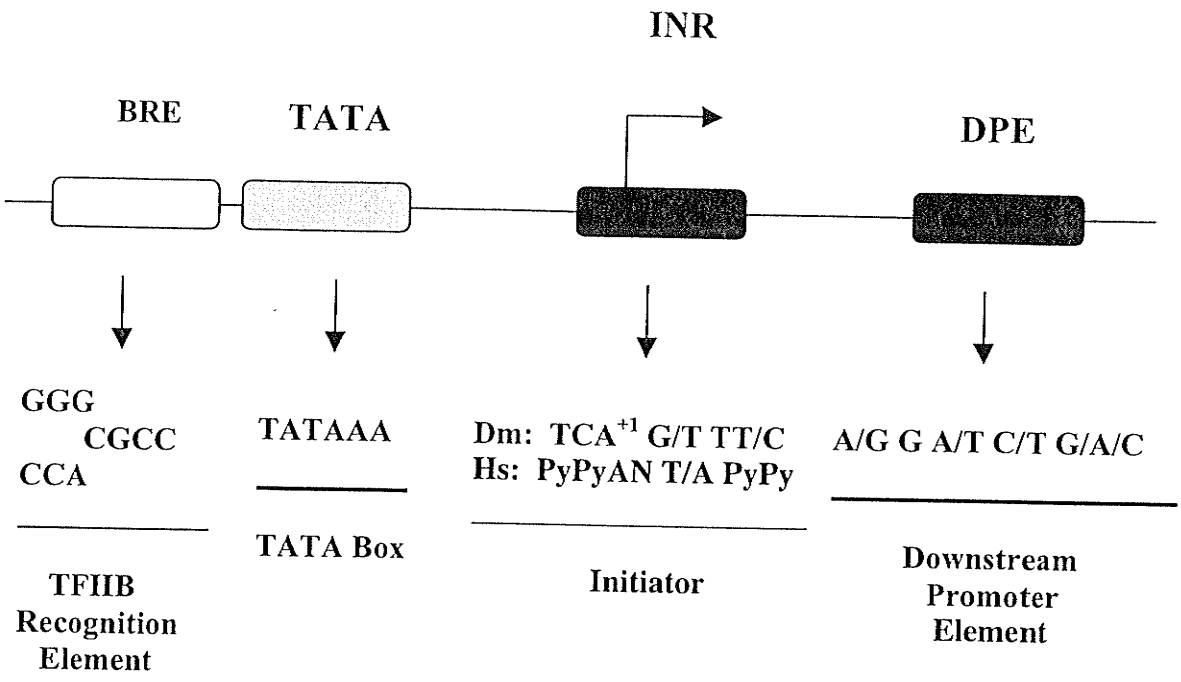


Figure 1.3.3: Scheme of the common core promoter elements is shown. Some promoters may contain at least two or three of these elements. TCA^{+1}, may have an additional A; Py, pyrimidine; N, any nucleotide.

During transcription by a eukaryotic RNA polymerase II, protein complexes are assembled and disassembled at promoter in various stages to form the pre-initiation complex (PIC), and complexes required for initiation of transcription, promoter clearance, mRNA elongation and RNA chain termination. The initiation stage requires the catalytic enzyme RNA polymerase II and general transcription factors (GTFs), which include TFII-A, TFII-B, TFII-D, TFII-E, TFII-F and TFII-H. These are also referred to as TATA binding protein–associated factor (TAFs). Other protein cofactors such as mediator, CRSP and TRAP that facilitate multi-domain protein-protein interactions, and various chromatin modification or remodeling complexes (SWI/SNIF, NURF, ACF) also

have a role in initiation (Wang *et al.*, 2001; Baek *et al.*, 2002; Taatjes and Tjian., 2004). The factors involved in the initiation of transcription appear to be conserved across species and in general use for a variety of promoters; (Roeder, 1996; Novina and Roy, 1996; Pugh, 2000; Lemon and Tjian, 2000; Woychik and Hampsey, 2002; Muller and Tora, 2004).

RNA polymerase II must recognize and bind the core promoter sequence for initiation of transcription to occur. The first step appears to be the binding of TFIID, which is composed of TATA-binding protein (TBP) and a number of TBP-associated factors (TAFs) to the TATA region; RNA polymerase recognizes and binds this complex. The TAFs such as TFIIA, -B, -E, -F and -H have a role in establishing specific interactions between co-activators (also termed transcriptional activators), and TFIID, which bind to proximal promoter elements. This role of the TAF complex can be seen with two models for the classical activation of gene transcription. One is the activation of transcription on a naked DNA element *in vitro* (**Figure 1.3.4a**); the other one is the activation of transcription *in vivo* within the context of chromatin (**Figure 1.3.4b**). There are two basic functional *cis*-regulatory elements – the core promoter and an enhancer/activator element. The specific interaction of activator protein with the enhancer element triggers the recruitment of the basal transcription machinery to the core promoter. While the *in vitro* TFIID and RNA polymerase II complex interacts with the enhancer protein and produces an active promoter initiation complex, *in vivo* an activator protein bound to the (proximal/distal) enhancer element also promotes the recruitment of ATP-dependent chromatin-remodelling and histone modifying factors to the core

promoter. The chromatin-remodelling factors facilitate the incorporation of an additional interactive protein complex, in particular *mediator*, between the activator protein and basal transcription machinery bringing the two elements closer. This interaction stabilizes the initiation complex making it more likely that productive transcription can occur.

In recent years it has been suggested that general transcription factor (GTF)-associated complexes bound to different promoters may show some diversity and that this represents an additional level of transcriptional regulation (Muller and Tora, 2004). RNA polymerase II was shown, by chromatin-immunoprecipitation and footprinting analysis *in vivo*, to be recruited to distal enhancer elements in the prostate-specific antigen (PSA) and the Pdβ1 genes (Spicuglia *et al.*, 2002; Louie *et al.*, 2003). The recruitment of GTF-associated factors to distal regulatory elements has also been identified (Verrijzer and Tjian, 1996; Orphanidies and Reinberg, 2002; Szutorisz *et al.*, 2005). It has been suggested that this distal binding may play an essential role in the timing of gene activation in different cell types during development (Rastegar *et al.*, 2004; Fisher, 2002). A well-studied model of transcriptional regulation during development has been the human and mouse β-globin gene loci. *In vivo* and *in vitro* comparative analysis of a DNAse I hypersensitive site localized in an enhancer element of the mouse β-globin locus control region (LCR) showed that GTFs and RNA polymerase II occupy the enhancer element during the mouse erythroid cell differentiation (Gribnau *et al.*, 2000; Plant *et al.*, 2001; Vieira *et al.*, 2004).

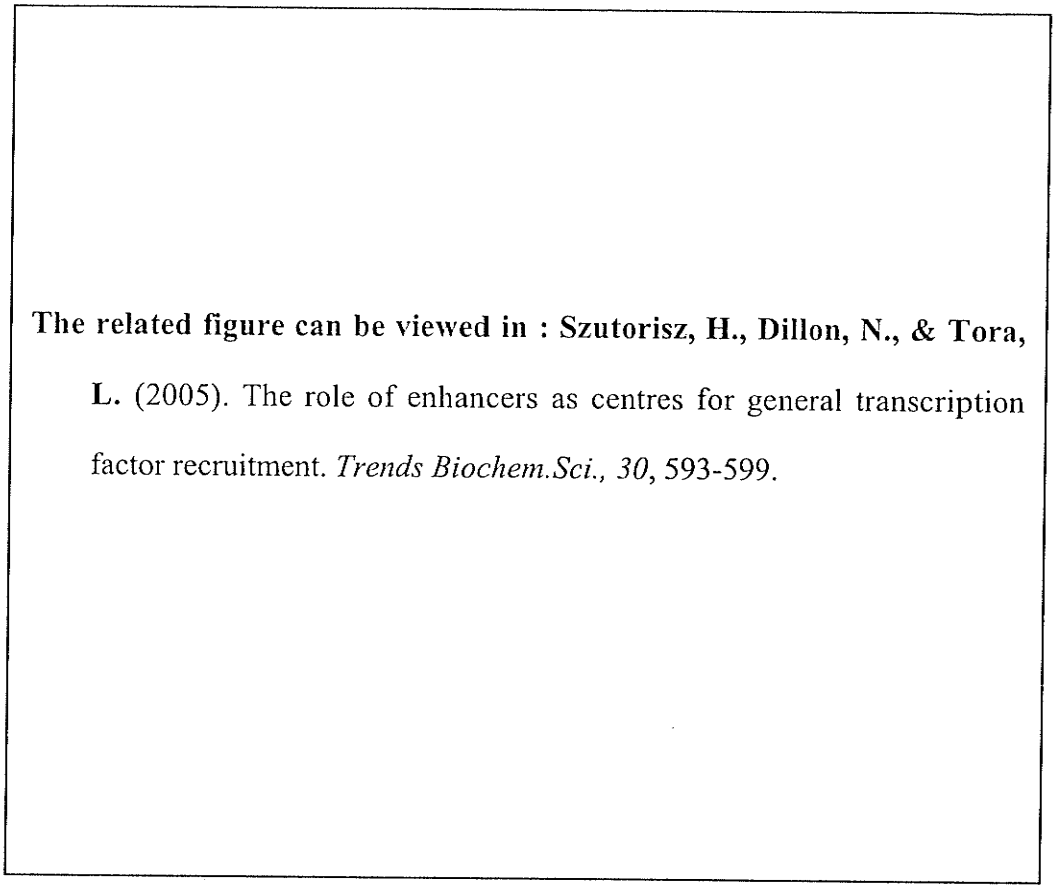


Figure 1.3.4: Classical models for the activation mechanisms of transcriptional activation.

Access to gene regulatory elements in genomic DNA is another point of transcriptional control. The highly conserved N-terminal tails of te core histone proteins play a crucial role in regulating chromatin structure. Each tail can undergo a number of protein modifications, including acetylation of lysines, methylation of lysines and phosphorylation of serines (Jenuwein and Allis, 2001). These modifications can have a profound effect on the state and stability of the chromatin fibers. Histone acetylation, which is carried out by enzymes called histone acetyl transferases (HATs), serves to

destabilize the higher order chromatin structures and exposes regions of the histone proteins that can interact with other proteins to cause further decompaction. Deacetylation, carried out by histone deacetylases (HDACs), results in compaction of the chromatin. Methylation of H3 lysine residues may result in repression of transcription and the formation of the more compacted heterochromatin; methylation of the H3 Lys4 residue, however, is associated with the transcriptional activity (Eberharter and Becker, 2002; Lachner and Jenuwein, 2002). These enzymes are usually part of multisubunit proteins that are brought to particular regions of DNA through interactions with other proteins, in particular the sequence-specific DNA binding proteins, as shown in Figure 1.3.4b. It has been suggested that the combination of modifications on the histone tails may represent a histone code that conveys a particular meaning to a region of DNA, which would then attract specific proteins to carry out the appropriate function (Strahl and Allis, 2000; Turner, 2002; Fischle *et al.*, 2003). Gene expression is also controlled by the dynamic cooperative functions of histone protein modifications and DNA methylation, which are key epigenetic mechanisms for the control of gene expression in embryonic development and cell differentiation (Im *et al.*, 2002; Katan Khaykovich and Struhl, 2002; Grewal and Moazed, 2003).

ATP-dependent chromatin remodeling complexes are also involved in loosening the interactions of the DNA and histones so that nucleosomes are more able to move in relation to the DNA and provide access for other proteins. These complexes are made up of multiple subunits and the complexes may differ somewhat in composition and therefore properties. Some complexes are thought to form to allow access to DNA, others

to restrict access (Pollard and Peterson, 1998; Vignali *et al.*, 2000; Lusser and Kadonaga, 2003).

Regions of decreased nucleosome density, and therefore regions which are accessible to regulatory proteins and are either transcriptionally active or poised for gene activation, can be visualized experimentally by DNAse I hypersensitivity in the context of chromatin (Felsenfeld, 1996; Felsenfeld and Groudine, 2003). These regions are usually small (200-300 bp) and show a 10 to 100 times sensitivity to digestion by DNAse I as compared to the surrounding chromatin (Elgin, 1984; Gross and Garrard, 1988; Fraser and Grosveld, 1998). Hypersensitivity is caused by the binding of specific protein factor complexes to DNA binding sites resulting in displacement of nucleosomes. These hypersensitive sites (HSS) appear to occur prior to the actual initiation of a transcribed gene. Hypersensitivity studies commonly support the conclusion that formation of tissue or cell type-specific HSS sites in a gene are associated with expression of that gene in those cells.

1.3.4. Functional gene expression domains and models

Apart from the site for efficient RNA polymerase binding, normally a TATA sequence or TBP site, it is the gene specific combination of DNA *cis*-regulatory elements and their protein binding factors that regulate tissue-specific, cell-type specific and developmental-specific gene expression. These DNA elements are grouped according to

their functions into promoters, enhancers, repressors, insulators and locus control regions to name the most well-studied.

A promoter is generally located within a hundred base pair region with transcription initiation site usually 25 to 42 bp upstream of the transcription start site. However, an enhancer is a more flexible element and can be positioned at much greater distances either 5' or 3' of the defined transcription start site. In eukaryotes, enhancers can be localized as much as 100 Kb from the promoter site of a target gene and may even be positioned within intron or exon regions (Merika and Thanos, 2001; Sipos and Gyurkovics, 2005). A defining feature of an enhancer DNA sequence is that its enhancing effect on a promoter is both position and orientation independent. Individual enhancer elements may contain multiple DNA factor binding sites, which lead to interacting protein complexes within a region and/or cooperative interactions with complexes formed on other regulatory elements, either other enhancers or the promoter. As mentioned earlier, enhancers facilitate the recruitment of transcriptional coactivators and RNA polymerase II to the promoter, stabilizing the transcription complex. Enhancer binding initiates the release of nucleosomal repression from a distance, resulting in formation of DNAse I hypersensitive sites and hyperacetylation of histones in nucleosomes located at the promoter region, which is the marker for chromatin opening and transcriptional activation of a target gene (Schubeler *et al.*, 2000; Forsberg *et al.*, 2002; Hatzis and Talianidis, 2002; Kim and Dean, 2003). There are hypothetical models that attempt to explain how enhancer-dependent gene activation at a distance functions through promoter elements (**Figure 1.3.5**). Recruitment of RNA polymerase II and

chromatin remodelling complexes to the enhancer elements either promote enhancer sliding to the promoter along the localized chromatin segment ("tracking") or a distally located enhancer element can interact with the target promoter directly through associated factors by "looping" or indirectly through multiple protein complex interactions by "linking" (Blackwood and Kadonaga, 1998; Bulger and Groudine, 1999; Bondarenko *et al.*, 2003; Li *et al.*, 2006).

The transcription of genes is modulated at several levels – where it is expressed, when it is expressed, how much is expressed. The characterization of DNA elements involved in eukaryotic gene expression uses *in vitro* techniques such as footprinting, electrophoretic mobility gel shift assays and transient transfections and assays. Individual elements are often analyzed *in vitro*, whereas the functionality of these elements *in vivo* may require further factors and cooperative interactions in the context of native chromatin. DNAse I hypersensitive studies help to identify multiple regions that may contain regulatory sequences in chromatin, sequence analysis is useful in identifying known factor binding sites within these sequences, and chromatin immunoprecipitation studies can address whether specific factors can bind the genomic DNA in the context of chromatin. Transgenic mice are often used for understanding the *in vivo* function of these DNA elements.

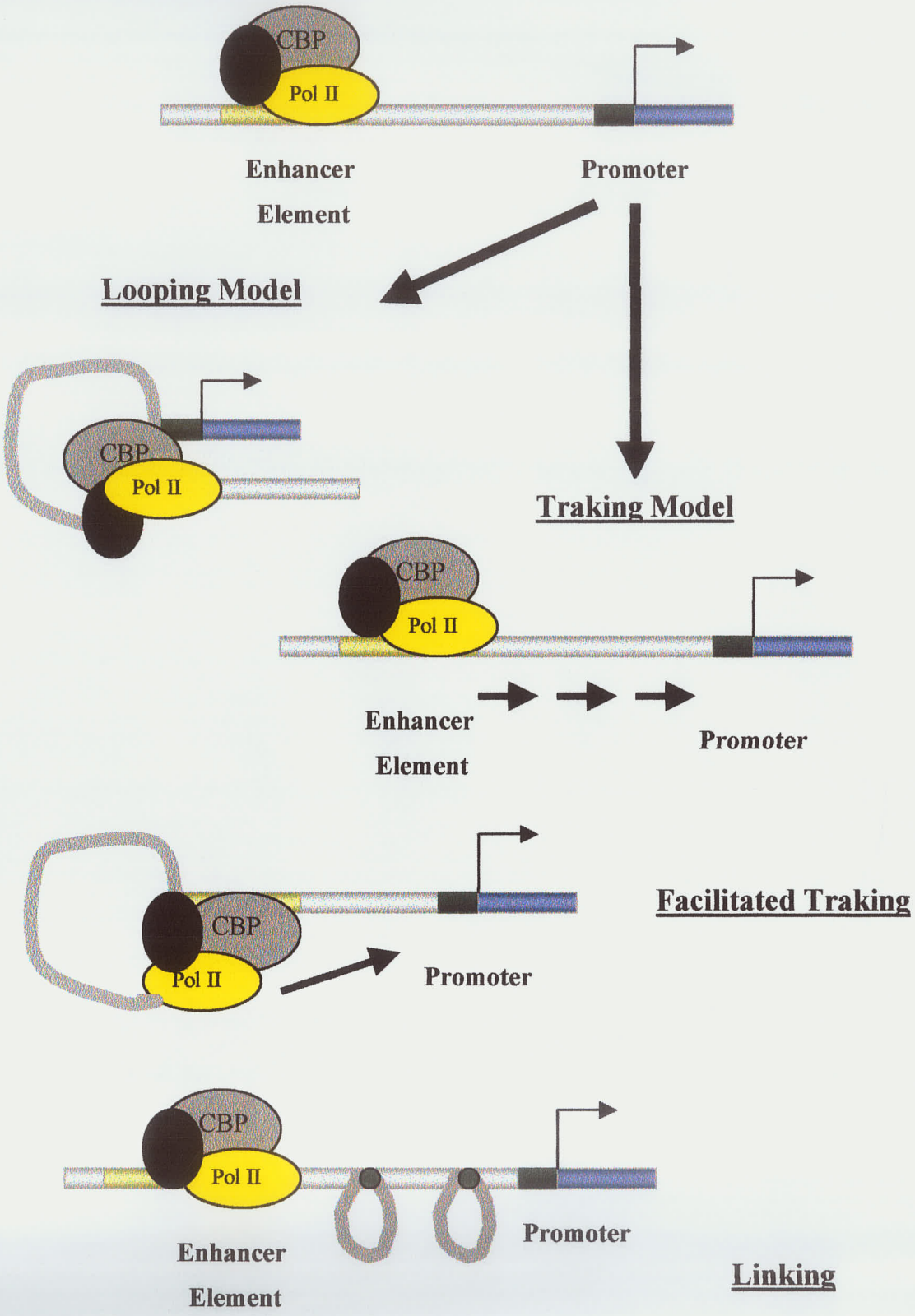


Figure 1.3.5: Models of gene activation by enhancer elements. A number of factors associated with the enhancer elements are defined: Pol II (RNA polymerase II), CBP (cell

specific binding protein), P (promoter). The transcription activator is indicated by an yellow oval feature. Association of these factors with the promoter element is facilitated by tracking, looping or linking mechanism driven by distant enhancer element resulting in high level gene activation as shown by the blue arrow.

Understanding the regulation of spatiotemporal expression of individual genes within a given gene locus, which can contain several linked genes, is particularly challenging. A single DNA element within a eukaryotic gene locus, which contains either a single or multiple genes, may not be sufficient to regulate expression *in vivo*. Studies of these loci in the context of chromatin have provided the evidence of functional transcriptional determinants such as the locus control region (LCR) and insulators or boundary elements. An LCR has been defined as a DNA region that when it binds appropriate DNA-binding factors can organize clusters of genes into an active "block" of chromatin. DNA within this region becomes more available for binding further transcription factors at other sites within the more open region. Insulator sequences are involved in creating independent chromatin domains between genes (Capelson and Corces, 2004). Both functional elements play important roles in marking chromatin loop formation and nuclear localization of functional domains.

As shown in **Figure 1.3.6** (Dillon and Sabattini, 2000), two models of conserved functional domains have been proposed; these have been referred to as the strong and weak domain models. According to the strong domain hypothesis, transcriptionally active genes are located within an open chromatin structure containing both enhancer and LCR regions located on a loop of more open chromatin that is flanked by inactive regions,

made up of more compacted chromatin. The edges of the compact regions mark the border between active and inactive domains of chromatin structure and represent insulator elements (**Figure 1.3.6a**). To try to explain the observation that independently regulated loci can partially or completely overlap, the weak domain model predicts the existence of various binding sites for activator factors in different locations along the DNA and that cooperation among these regions regulate the chromatin domain structure without insulators (**Figure 1.3.6b**). Sequences that interfered with gene specific enhancer-promoter interactions would be selected against if they occurred within a domain but not if they were found at the edges or between domains.

The related figure can be viewed in: Dillon, N., & Sabbattini, P. (2000). Functional gene expression domains: Defining the functional unit of eukaryotic gene regulation. *Bioessays, 22*, 657-665.

Figure 1.3.6: Two hypothetical types of functional gene expression domains.

An LCR has been functionally defined as an activating sequence or region that is able to confer position independent, copy number dependent expression of a linked gene in transgenic mice, although some exceptions suggest that this definition may be too simple (Feng, 2005). LCRs are composed of multiple dominant and positively acting transcription factor binding sequences that sustain DNAse I hypersensitivity and acetylation state; no LCR-specific factors have been identified. Specificity appears to depend on the precise arrangement of DNA sequences and the interaction of the factors that bind to them. LCRs often act at long-range to the promoters of the genes they influence (Wijgerde *et. al.*, 1995; Dillon *et. al.*, 1997; Carter *et al.*, 2002; Dillon and Sabattini, 2000) and can be localized either 5' or 3' of the target gene(s). The binding sites may be clustered or more widely dispersed and can even be localized within a neighboring gene that is independently regulated.

The most studied gene loci model systems have been the human and mouse β-globin gene locus and the human growth hormone gene locus. The β-globin genes are arranged in the order in which they are expressed during erythroid cell lineage (Fraser and Grosveld, 1998; Li *et al.*, 1999). The transcriptional activation of β-globin genes shows a temporal expression pattern during erythroid cell development – yolk sac, liver, bone marrow. The genes within the human growth hormone locus are expressed in a spatiotemporal fashion within two different tissues – the pituitary and the placenta. Despite the fact that specificity of hypersensitive sites in LCR regions, it has also been demonstrated that deletion of LCR-HSS in the mouse β-globin locus did not affect its expression in transgenic mice, opposing the idea of conserved domain structures in

mammals. In contrast, as shown in the mouse β-globin gene, specific hypersensitive sites within an LCR region can also be associated with transcriptional repression (Feng *et al.*, 2005). In the growth hormone gene locus LCR selectivity and functionality require histone modifications at the chromatin level (Ho *e. al.*, 2002; Kimura *et al.*, 2004); this has not been demonstrated in the β-globin locus (Dean, 2006).

Other regulatory DNA domains, called insulators, are suggested to play a functional role in establishing boundaries between the neighboring genes, thereby preventing the spread of repressive histone modifications between transcriptionally active and inactive genes (Cai and Shen, 2001; West *et al.*, 2002; Capelson and Corces, 2004). Insulators may also provide selectivity to enhancer elements (Dillon *et al.*, 1997).

1.4. Research Objectives

The rodent PRL family members show high level, tissue-specific, cell type-specific and temporal-specific expression patterns during pregnancy (Dustin *et al.*, 2003; Soares *et al.*, 2004). With the availability of genome sequence data it has now been demonstrated that there are 26 PRL family members in the mouse and rat; these genes are organized in a single gene locus in each species (Weimers, 2003; Alam *et al*, 2006). Regulation of the pituitary-associated PRL gene expression has been well studied, but there is only limited information on the molecular mechanisms that regulate the majority of the members of the rodent PRL gene family, that are expressed in placenta and decidual cells during pregnancy. The general question addressed by my studies is how

expression of the individual genes in the same locus is modulated during development in a temporal and cell specific fashion. The rat placental lactogen II gene (rPLII) was among the first of these pregnancy-specific genes, and the first PRL-related placental lactogen, to be identified (Duckworth *et al*, 1986). It is highly expressed only in trophoblast giant cells of the placenta from midpregnancy to term, and in my studies has served as a model gene to examine the regulatory factors, which define placental cell type specific gene regulation within the PRL locus.

Previous studies from our laboratory identified a 3 Kb 5' flanking fragment immediately proximal to the rPLII transcription start site, which consistently targeted reporter gene expression to the placentas of transgenic mice, suggesting that it contained key placental-specific regulatory sites (Shah *et al.*, 1998). An element within this region enhanced luciferase reporter gene expression from a minimal thymidine kinase promoter in Rcho cells, but not in rat pituitary GC cells (Sun and Duckworth, 1999). DNAse I protection assays defined two regions within this enhancer element, designated as FP1 and FP2. Changes to nucleotides within each of these regions reduced enhancing activity in Rcho cells; mutations in both regions caused a complete loss of activity (Sun and Duckworth, 1999). These previous studies concluded that the factor that bound FP2 was AP1 and that a member of the Ets family of transcription factors was a likely candidate for the FP1 binding factor.

The transgenic mouse studies had indicated that although the 3 Kb fragment contained sequences that were important for rPLII expression it was insufficient to

reproduce the complete rPLII expression pattern. My working hypotheses were that this enhancer region and its binding factors were important for rPLII placental-specific expression and that there would be other regions and factors that would be involved in the high level, temporal expression of the endogenous gene.

The specific aims of this thesis are:

1. To identify and characterize the regulatory [illegible] associated with the previously identified rPLII enhancer element in [illegible] *in vitro* and *in vivo*.
2. To identify further rPLII *cis*-regulatory elements using a larger P1 genomic clone in transgenic mouse studies.
3. To identify additional regulatory elements that are essential for the expression of rPLII in placental cells, using DNAse I hypersensitivity assays.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials:

Experimental materials as listed were purchased from the following sources:

Amersham-Pharmacia Biotech: Sephadex G50 fine columns, GFX™ PCR DNA and gel purification kits.

BD Biosciences/Clontech: Nucleobond BAC maxi kit, BAC 100 columns.

BIO RAD Laboratories: Bradford's reagent.

Boehringer-Mannheim: Calf intestinal phosphatase.

Dynal A.S., U.S.A.: Dynal M-280 magnetic beads, Dynal MPC-S magnetic separation apparatus.

Fisher Scientific: General laboratory chemicals, Nitro-Pure and Nitroplus blotting membranes.

Intersciences Inc.: Kodak XAR and MS X-ray films.

Invitrogen: RPMI-1640, DMEM, and HAMS F-10 media, fetal calf serum, penicillin, streptomycin, lipofectamine PLUS, calcium/magnesium-free phosphate buffered saline (PBS-CMF), trypsin-EDTA, glutamine, GlutaMAX™, platinum Taq PCR kit, mouse

Moloney murine leukemia virus reverse transcriptase (MMuLV RT), random primer labeling kit, low melting temperature agarose (LMA), Trizol reagent.

New England Biolabs: Most restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, β-agarase, various DNA size markers.

Perkin Elmer: $^{32}\gamma$P-ATP, $^{32}\alpha$P-dCTP, ^{35}S-L-methionine.

Promega: TNT SP6 and T7 coupled rabbit reticulocyte *in vitro* transcription/translation kits, dual luciferase reporter assay kits.

Qiagen: Mini and maxiprep plasmid isolation kits, Qiaquick spin columns, HotStar Taq DNA polymerase.

Qiagen/Operon: Oligonucleotides and biotinylated oligonucleotides.

Santa Cruz Biotechnology Inc.: Antiserum for ELF-1 (C-20, sc-631), NERF-2 (V19, sc-6829), ELF-5 (C-18, sc-9647), c-Jun D (sc-44), AP2γ (sc-8977).

Sigma Chemical Company: DNAse I (D-4527), β-mercaptoethanol, sodium pyruvate, dimethysulfoxide (DMSO), pepstatin, leupeptin, aprotinin.

The following reagents were received as gifts:

Human AP2γ cDNA clone: Dr. Trevor Williams, University of Colorado, Denver CO. and Dr. Helen Hurst, Imperial College, London, U.K.

Rat c-*jun* and c-*fos* cDNA clones: Dr. Tom Curran, St. Jude Children's Research Hospital, Memphis, TN.

Human MEF cDNA clone: Dr. Stephen Nimer, Sloan-Kettering Institute for Cancer Research, New York, N.Y.

Human ESX (ESE-1) cDNA clone: Dr. C. Benz, University of California San Francisco, CA.

Human NERF-2 cDNA clone: Dr. Towia Libermann, Beth Israel Deaconess Medical Centre, Harvard Medical School, Boston, MA.

Human Ets-2 cDNA expression clone, pTKRenilla expression clone: Dr. Peter Cattini, University of Manitoba, Winnipeg, MB.

Promoterless luciferase expression vector, pTK81Luc: Dr. Robert J. Matusik, Vanderbilt University, Nashville, TN.

2.2. Methods Used in Chapter 3

2.2.1. Construction of Reporter and Expression Plasmids

2.2.1.1. Wild type and mutant enhancer-luciferase constructs

The 43 bp wild type and mutated double stranded oligonucleotides, WTFP1-2, M3, M4, m6, M3m6 and M4m6 (**Table 2.1**), which contained both DNAse I protected regions of the rPLII enhancer element, were cloned into the luciferase reporter vector, pTK81 as follows. Five µg of each complementary oligonucleotide were annealed in 20 µl of 10 mM Tris-HCl pH 8.5, 125 mM NaCl using a standard annealing procedure (5 minutes at 95°C, 10 minutes at 65^0C, 10 minutes at 37°C, 15 minutes at room temperature). Clones were constructed using a two-step ligation procedure. First, the double stranded oligonucleotides were phosphorylated using 5 units of T4 polynucleotide

kinase (New England Biolabs) for 30 minutes at 37°C. Samples were phenol/chloroform extracted and ethanol precipitated. Aliquots were ligated overnight at room temperature to allow formation of concatamers. Ligated samples were separated on a 2% low melting agarose gel; fragments were sliced out and DNA was isolated using a GFX™ PCR DNA and Gel Band Purification Kit.

Two μg of pTK81 vector was digested with Sma I and dephosphorylated using 0.5 units of calf intestinal alkaline phosphatase for 15 minutes 37°C and 15 minutes at 56°C. A second ligation was carried out overnight at 16°C using varying amounts of the gel-purified oligonucleotide concatamers and the Sma I digested pTK81 vector. Half of the ligation sample was used to transform DH5α competent cells using the standard method in Ausubel *et al*, 1989. Transformants were selected on LB-ampicillin plates and plasmids were isolated using a Qiagen Miniprep plasmid isolation kit. Since the oligonucleotides contained sites for the restriction enzymes Sml I and BstN I, clones with inserts were identified by digestion with these enzymes. The number of 43 bp oligonucleotides in the inserts was determined by PCR using the WT 84 forward and WT 155 reverse primers (**Table 2.1**); these sequences are located in the TK promoter and a common region in the wild type and mutant oligonucleotides respectively. Clones containing two copies of each wild type or mutant oligonucleotide were sequenced by the University of Calgary DNA Sequencing Facility; a luciferase primer was used as the sequencing primer (**Table 2.1**). Those clones containing both copies in a 5' to 3' orientation were selected for transfection studies (pTK WT Luc, pTK M3 Luc, pTK M4 Luc, pTK m6 Luc, pTK M3m6 Luc, and pTK M4m6 Luc).

Table 2.1 Oligonucleotides used in thesis studies

Oligonucleotide name	Sequence (5' to 3')	Use
WT 84 (F)	TCATGTCTGGATCCAAGC	PCR
WT155 (R)	CATGGGAAGTTCGGGATG	PCR
Luciferase primer	CTTTATGTTTTTGGCGTCTTCCCA	Sequencing
ELF1-F	ATGGATCCGCAATAACTTCACAGACCTGCCC	PCR, cloning
ELF1-R	GCTCTAGACCACCCATAATTCCTTTGGTGTAG	PCR, cloning
WTFP1-2	CCAGGGTTATTTGCTCAAGGGTAAACAGGAAGTAGGGCTTGAA	EMSA, cloning
M2	CCAGGGTgccgTGCTCAAGGGTAAACAGGAAGTAGGGCTTGAA	EMSA
M3	CCAGGGTTATTctacCAAGGGTAAACAGGAAGTAGGGCTTGAA	EMSA, cloning
M4	CCAGGGTTATTTGCTacgtGGTAAACAGGAAGTAGGGCTTGAA	EMSA, cloning
M5	CCAGGGTTATTTGCTCAAGtcgcAACAGGAAGTAGGGCTTGAA	EMSA
m6	CCAGGGTTATTTGCTCAAGGGTAAACActgAGTAGGGCTTGAA	EMSA, cloning
M2m6	CCAGGGTgccgTGCTCAAGGGTAAACActgAGTAGGGCTTGAA	EMSA

M3m6	CCAGGGTTATTctacCAAGGGTAAACActgAGTAGGGCTTGAA	EMSA, cloning
M4m6	CCAGGGTTATTTGCTacgtGGGTAAACActgAGTAGGGCTTGAA	EMSA, cloning
M5m6	CCAGGGTTATTTGCTCAAGtcgcAAACActgAGTAGGGCTTGAA	EMSA
ChIP1 (F)	AGATAGGTTAAGGGAGTCACTGGA	PCR
ChIP1 (R)	CGTCTTATGGGTTCTGTAAGGGTA	PCR
ChIP2 (F)	AGAGCAAAGGAGGGACTAG	PCR
ChIP2 (R)	CTGCTCTTCCAGAAGACTG	PCR
ChIP3 (F)	GCCAGGCTTGTAAAAATAGTG	PCR
ChIP3 (R)	TCCATGTTCAGGGTAAGAAC	PCR
IIF-1262	CCAGGGTTATTTGCTCAAGG	PCR
IIR-1549	TTCTACCTCCATTCTGTCTGTGACTG	PCR
B-Intron DF	GTAACTGCAGTATGGAGGAACCCCCTGATG	PCR
B-Intron DR	GCAACTCGAGTGCCAGTTCATTCCACATAGG	PCR
X6F	CCAATCAAGCTCTTACATTTCCAAG	PCR
X6R	CATCAAGTATCTTTGAGTGCCAACC	PCR
RIH-BF	GTACCCGGGGGAGAATGTTATATCCCATGCCC	PCR
RIH-BR	GACTGTCGACGAGGTGTAATGAGCCGTGACAATAG	PCR
IIF1-RNA	GCT—TCTCTGGGACACTCCTTATGC	RT PCR
IIR2-RNA	TCCTCCAACTCCTTTGTTCTTGAC	RT PCR
BF1-RNA	CTCCTGaTGCTGTTGatgTCAAAC	RT PCR
BR9-RNA	GCAAGtATGCCAGTCATTCCAC	RT PCR

C/EBP	TGCAGATTGCGCAATCTGCA	EMSA
C/EBP-U	TGTTTTCAATGTTGCAACAAGTCAGT	EMSA
C/EBP-D	ACTGACTTGTTGCAACATTGAAAACA	EMSA
WT 33 (F)	GGGTTATTTGCTCAAGGGTAAACAGGAAGTAGG	EMSA
WT 33 (R)	CCTCCCCCCCCTTCCTGTTTACCCTTGAGCAAATAACCC	EMSA
mETS (F)	GGGTTATTTGCTCAAGGGTAAACAttcAGTAGG	EMSA
mETS (R)	CCTACTGAATGTTTACCCTTGAGCAAATAACCC	EMSA
AP-1 consensus	CGCTTGATGACTCAGCCGGAA	EMSA
AP-2 consensus	GATCGAACTGACCGCCCGCGGCCCT	EMSA
T7 primer	TAATACGACTCACTATAGGGAGA	Sequencing
HSS4 (F)	AAACTGAGACCTTCAGGATG	PCR
HSS4 (R)	TTCTGCAGGATGGAATGTC	PCR
HSS5 (F)	AGAGCAAAGGAGGGACTAG	PCR
HSS5 (R)	CTGCTCTTCCAGAAGACTG	PCR

(F) Forward, (R) Reverse; lower case letters represent changes in nucleotides from the wild type sequence; underlined sequence represents restriction enzyme sites.

2.2.1.2. Construction of the AP2γ expression vector

The human AP-2 γ cDNA clone was digested with Hind III. The 1.2 Kb insert was subcloned into the mammalian expression vector pcDNA3 (Invitrogen) for *in vitro* transcription and translation assays and co-transfection studies. The orientation was defined by BamH I digestion and DNA sequencing by the University of Calgary DNA Sequencing Facility.

2.2.1.3. Construction of the Rat ELF-1 expression vector

cDNA was synthesized from Rcho cell total RNA using Molony murine leukemia tumour virus reverse transcriptase (MMuLV) and standard reaction conditions according to the manufacturer's protocol. A 1995 bp rat ELF-1 PCR product containing the entire coding region (Genbank NM_053520) was amplified from this pool using the ELF-1F and ELF-1R primer pair (**Table 2.1**), which respectively contained Bam HI and Xba I restriction enzyme sites. The amplification product was digested with Bam HI and Xba I and ligated to a similarly cut pcDNA3 mammalian expression vector. Plasmids were transformed into DH5α. The orientation and DNA sequence information were verified by sequencing using a T7 primer.

2.2.2. Plasmid Preparation and Transformation

Small-scale plasmid DNA samples were obtained using the alkaline lysis buffer method (Current Protocols in Molecular Biology, Volume 1, Unit 1.6.1-1.6.3, 1989) or DNA miniprep isolation kits (Qiagen). Large-scale preparation of plasmids for transfection studies was carried out using Qiagen Maxi Prep isolation kits according to the manufacturer's instructions. *Escherichia coli* DH5α competent cells were prepared according to the methods in Promega Protocols (Promega, Protocols and Applications Guide, 3rd Edition, ©1996, Cloning in Plasmid Vectors, page 45-46) or Current Protocols in Molecular Biology, Volume 1, Unit 1.8.1-1.8.3, 1989. A standard heat shock transformation protocol was applied as indicated within the same references.

2.2.3. *In vitro* Transcription and Translation

Human AP2γ, MEF, NERF-2, ESX/ESE-1, Ets2 and rat ELF-1were expressed *in vitro* using a T7 transcription and translation reticulocyte kit system (T7-TNT) according to the manufacturer's protocol. Rat c-*jun* and c-*fos* were expressed using the TNT SP6-coupled reticulocyte kit. Protein expression was evaluated by incorporating ^{35}S-methionine into the reactions. Expressed recombinant proteins were separated on 10% denaturing acrylamide gels and dried. Autoradiography was carried out to determine the size of the expressed proteins, which were compared to protein markers of known size. Reactions containing vector alone were used as controls. The optimum concentration for a 50μl reaction volume was determined to be 0.5 to 1μg of expression vector.

2.2.4. Cell Culture

The rat choriocarcinoma Rcho cell line was cultured in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, 50 mM β-mercaptoethanol, 50 U/ml streptomycin, and 50 mg/ml penicillin. This medium favoured growth over differentiation to giant cells and was used routinely to maintain and expand cultures. For experiments where differentiation to the rPLII-expressing giant cell type was required, Rcho cultures were switched at day 3 after plating to supplemented RPMI 1640 medium containing 10% FBS, and maintained on this medium for a specified number of days. The rat anterior pituitary GC cell line was cultured in DMEM containing high glucose supplemented with 50 U/ml streptomycin and 50 mg/ml penicillin (Cattini and Eberhardt, 1987).

2.2.5. Transfections and Reporter Gene Expression Studies

The Rcho rat choriocarcinoma cell line was cultured as described, for 14 to 16 days by which time rPLII-expressing giant cells represented the major cell population. Transfections of wild type and mutant rPLII enhancer firefly luciferase constructs in Rcho cells were carried out in 60 mm dishes using Lipofectamine Plus reagent, according to the supplier's protocol. Four μg of wild type or mutant enhancer construct were transfected with 10 ng of the pTK Renilla luciferase vector. After 48 hours, cell lysates were prepared as previously described (Sun and Duckworth, 1999), and luciferase activities were measured in a Berthold Lumat LB 9507 luminometer using a Promega

dual luciferase reporter assay system according to the manufacturer's protocol. Firefly luciferase activity was normalized to Renilla luciferase activity for each assay. The rat anterior pituitary cell line, GC, was cultured in DMEM with high glucose, supplemented with 10% fetal calf serum and antibiotics (100 U of penicillin/ml and 0.1 mg/ml streptomycin). For co-transfection studies GC cultures were grown to approximately 50% confluency, replated at $2x10^5$ cells per 35 mm dish and transfected 24 hours later using Lipofectamine Plus reagent. To optimize for AP2γ expression levels, cultures were co-transfected with 3 μg of the wild type enhancer luciferase constructs, 10 ng of pTK Renilla and 0, 10, 25, 50, 100, 500 ng, 1 or 2 μg of pcDNA3.AP2γ. DNA concentrations were kept constant by the addition of pcDNA3 vector. Luciferase activity was measured after 48 hours and normalized to Renilla activity. The optimum effect of AP2γ was seen at 50 ng, which was used for further transfection assays.

Statistical analysis of the transfection and co-transfection data was performed using a two-tailed unpaired t test and ANOVA with post hoc Bonferroni test (InStat, version 2.03). A value of $P < 0.05$ was considered statistically significant.

2.2.6. RNA Isolation, Blotting and Hybridization

Total RNA was isolated from frozen cultured cells and tissue using the Trizol reagent (Chomczynski and Sacchi, 1987). RNA samples were prepared for electrophoresis on 1.2% agarose/2.2M formaldehyde gels according to standard procedures outlined in Current Protocols in Molecular Biology. In general samples

contained 20-30 μg of total RNA in a final volume of 30 μl. Following electrophoresis, gels were incubated with sterile 20xSSC buffer (3M NaCl/0.3M sodium citrate) for 30 minutes with gentle shaking. RNA was transferred to Nitroplus membrane by capillary transfer using 20XSSC as the transfer buffer. Membranes were rinsed in 2xSSC and baked for 2 hours at 80°C in a vacuum oven. For hybridization, 25-50 ng of DNA insert was labeled with [α-^{32}P]dCTP using a random prime labeling kit. Excess [α-^{32}P]dCTP was removed using G25 spin columns. Membranes were prehybridized for 3 to 4 hours at 42 °C in buffer containing 4XSSC, 0.4X Denhardt's solution, 50% (v/v) deionized formamide, 10 mMNa_2HP0_4 pH 7.4, 0.1%SDS, 1mM EDTA, pH 8.0, 250 μg/ml sonicated salmon sperm DNA (Ausubel *et al*, 1989). Denatured labeled probe was added to this solution and hybridization proceeded overnight. Membranes were washed twice with 2XSSC, 0.1%SDS for 15 minutes and twice with 0.2XSSC, 0.1%SDS for 10 minutes, all washes at 65 °C. The blots were covered with plastic wrap and exposed to Kodak XAR film at –80°C with an intensifying screen.

2.2.7. Isolation of Nuclear Extracts

Nuclear extracts were prepared from frozen day 14 Rcho cultures according to a slightly modified Dignam protocol (Dignam, *et al.*, 1983). Lysis of cells was extended and the concentration of protease inhibitors was doubled. A large scale Rcho nuclear extract preparation was prepared as follows:

Starting material was usually cell pellets from fifteen 100mm plates of cells that

had been collected, washed twice in 6 ml of ice-cold calcium-magnesium free phosphate buffered saline (PBS-CMF), once in 1 ml of ice cold PBS-CMF containing 50 mM EDTA, pH 8.0 and stored at –80°C. The pellet was thawed on ice for 10 minutes and the packed cell volume (PCV) marked. The thawed cell pellet was resuspended with 5 times the PCV of ice cold Buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol (DTT), 2 mM phenylmethylsulfonylfluoride (PMSF) and 10 µM aprotinin. Cells were transferred into an ice-cold flat-bottomed glass homogenizer (40 ml capacity) and left on ice for 25 minutes to swell. This is an additional 10 minutes to the original Dignam protocol. Cells were homogenized first with 5 to 7 strokes using a loose teflon pestle; a 10 µl aliquot diluted in 100 µl of Buffer A was checked under phase contrast microscopy for cell membrane breakage. If required, cells were homogenized further. Homogenized cells were transferred into 15 ml Corning tubes and centrifuged at 3000 rpm for 10 minutes at 4°C in a Beckman J2-21 centrifuge. The supernatant was removed and the pellet was resuspended in 2 PCVs of Buffer A that included 10 µM pepstatin, 10 µM leupeptin, before transferring into a 20 ml Dounce homogenizer. The pellet was homogenized with 10 strokes using a tight pestle B; a 50 µl aliquot was checked under a microscope. An additional 5 to 10 strokes were given if required to give clean nuclei. The sample was transferred into a cold 30 ml transparent Oakridge tube and centrifuged at 3000 rpm for 15 minutes using a JA-20 rotor in a J2-21 Beckman centrifuge. The supernatant was removed and the pellet was transferred into a new ice-cold 15 ml Corning tube and centrifuged at 12,000 rpm for 35 minutes at 4°C in a JA-21 rotor to remove remaining cytoplasmic debris. Following the removal of the supernatant, the nuclei pellet was resuspended in 0.5 PCV of Buffer C (20 mM HEPES-KOH pH 7.9,

1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 1 mM DTT, 2 mM PMSF, 25% (v/v) glycerol). The resuspended sample was transferred into another cold 15 ml Corning tube and gently mixed on a rotating Nutator for 30 minutes at 4°C. To eliminate nuclear membranes, the sample was transferred into an ice-cold Oakridge tube, and centrifuged at 15,000 rpm for 30 minutes at 4°C using a JA20 rotor. The supernatant was transferred to dialysis tubing (1000 MW cut-off), which had been equilibrated overnight in Buffer D (20 mM HEPES-KOH pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 1 mM DTT, 2 mM PMSF, 20% (v/v) glycerol); dialysis was against Buffer D at 100 times the volume of the recovered supernatant for 3 hours at 4°C. Following dialysis, the sample was aliquoted into ice-cold 1.5 centrifuge tubes and centrifuged at 12,000 rpm for 10 minutes at 4°C before rapidly freezing to –80°C for storage. Protein concentrations were determined using a Bradford's protein assay reagent according to the supplier's protocol.

GC, HepG2, and JEG cell nuclear extracts were prepared using a short Dignam protocol (Andrews and Faller, 1991). Rat placental extracts were prepared as previously described (Sun and Duckworth, 1999), using day 16 to day 18 frozen rat placenta; protease inhibitors were added as for the Rcho preparations. Protein determinations were carried out using a Bradford's reagent according to the protocol supplied by the manufacturer.

2.2.8. Electrophoretic Mobility Shift Assays (EMSA)

Complementary, single-stranded 43bp oligonucleotides, containing either wild type or mutated sequences of the rPLII enhancer element, were synthesized and annealed using a standard procedure as outlined in the plasmid construction section. Annealed oligonucleotides were end-labelled by [γ-^{32}P] ATP and T4 polynucleotide kinase (New England Biolabs) and excess label was removed using a Sephadex G50 fine column. The DNA-protein binding reactions were carried out at room temperature for 20 minutes in 20 µl reaction volumes containing 5mM HEPES pH 7.9, 25 mM NaCl, 1.25 mM $MgCl_2$, 50 µM EDTA, 125 µM DTT, 250 µM PMSF, 5% glycerol and 0.1 µg/µl poly dI-dC. Reactions included 0.8 – 1.0 ng of end-labeled oligonucleotides with 6 µg of Rcho or 10 µg of placental nuclear extracts. Protein–DNA complexes were resolved on 6% non-denaturing polyacrylamide gels in 0.5X Tris-borate-EDTA buffer at a constant current of 25 mAmps. Gels were pre-run at 200V for 30 minutes before samples were loaded. For competition studies, specific oligonucleotide competitors were incubated with nuclear extracts in EMSA reaction buffer on ice for 15 minutes before adding the labeled probe. For supershift studies, 1 µg of specific or non-specific antisera were added to the binding reactions 30 minutes prior to the probe, and incubated on ice. Studies with *in vitro* transcribed and translated proteins were carried out in 10 µl reaction volumes using identical buffer conditions; in this case incubation times were 15 minutes on ice. For supershift experiments using *in vitro* translated proteins, antiserum was added 45 minutes prior to the addition of probes. *In vitro* protein–DNA complexes were resolved

on 6-10% non-denaturing polyacrylamide gels. For all EMSA reactions, gels were dried following electrophoresis and exposed for autoradiography with Kodak Biomax MS film.

2.2.9. DNA-Protein Binding Reactions

Streptavidin-linked M-280 magnetic Dynabeads were initially washed according to the manufacturer's protocol (10 mM Tris-HCl, pH 7.5, 2 M NaCl, 1 mM EDTA), but with additional washes to reduce amounts of bovine serum albumin, present in the storage buffer. After each manipulation beads were collected using a Dynal MPC-S magnetic separation apparatus. Two final washes were performed in coupling buffer (10mM Tris-HCl, pH 7.5, 1M NaCl, 1mM EDTA). Complementary 43 bp wild type oligonucleotides were annealed to form the double stranded enhancer. The sense oligonucleotide included a 5' biotin at the end of a triethylene glycol (TEG) spacer. Oligonucleotide/Dynabead coupling reactions contained 50 pmoles (144 ng) of the double-stranded biotinylated 43bp enhancer oligonucleotide with 50 µg of magnetic beads in coupling buffer to a final volume of 50 µl. Tubes were mixed by gentle rotation at room temperature for 20 minutes. Beads were collected and washed twice in EMSA buffer (5 mM HEPES, pH 7.9, 25 mM NaCl, 1.25 mM MgCl, 50 µM EDTA, 5% glycerol, 250 µM PMSF, 125 µM DTT). Protein-DNA binding reactions were carried out in a final volume of 200 µl of EMSA buffer containing 0.1 µg/µl poly dI/dC and 120 to 150 µg of Rcho nuclear protein. Protein was incubated with the EMSA buffer for 5 minutes on ice before adding the conjugated DNA-Dynabeads. Binding reactions were incubated at room temperature for 30 minutes, with gentle rotation. Five separate

reactions were prepared as described. To remove non-specifically bound proteins, beads were collected and washed once in two volumes of EMSA buffer, containing 1.0 μg/μl poly dI/dC, with gentle mixing for 2 minutes and twice in EMSA buffer without poly dI/dC. After this first set of washes, the five binding reactions were pooled into one tube and washed three times in 0.1 M and twice in 10 mM ammonium bicarbonate, each wash (650 μl) for 2 minutes at 4^0C with gentle rotation. The beads with bound proteins were resuspended in 70μl of 10 mM ammonium bicarbonate; trypsin dissolved in 10 mM ammonium bicarbonate was added to a final concentration of 0.25 ng/μl and the protein-bead sample was digested overnight at 37^0C. Beads were removed after trypsin digestion using the magnet.

2.2.10. Mass S pectrometry

Tryptic digests were divided into three aliquots and dried in a Savant Speed Vac concentrator. Each aliquot represented bound protein from approximately 250 μg of crude Rcho nuclear extract. Samples for MS analysis were treated essentially as outlined in Krokhin *et al*, 2004. Briefly, pellets were dissolved in 6 μl of 0.2% trifluoroacetic acid and chromatographic separation was carried out on a micro-Agilent 1100 series HPLC. Five μl samples were injected onto a 150mm x 150mm column (Vydac 218 TP C18, 5mm) and eluted with a 1 – 80% acetonitrile gradient containing 0.1% TFA; column effluent (4 μl/min) was mixed on-line with 0.5μl of MALDI matrix solution (160 mg/ml 2,5-dihydroxybenzoic acid in 3:1 water:acetonitrile containing 2% formic acid) and deposited by a computer-controlled robot onto a movable gold target at forty, one minute

intervals. Each fraction was analysed by single mass spectrometry (m/z range 560-5000) on a Manitoba/SCIEX prototype quadrupole time of flight (QqTOF) mass spectrometer (Loboda *et al.*, 2000). Ions with sufficient intensity were selected for tandem mass spectrometry (MS/MS) on the same instrument.

Acquisition and analysis of spectra were carried out manually using an in-house program, "TOFMA" (University of Manitoba Time of Flight Laboratory). In some analyses automatic peak assignment was made using the program "M/z" with a signal to noise ratio (S/N) of 2.5. Peptide mass fingerprinting was carried out on selected ions using "ProFound" with S/N of 2.5 and an error limit of 30 ppm. MS/MS spectra were analyzed using "Sonar msms", with S/N of 1.3 and with a 2 Da error for the parent ion and 0.1 Da for fragments. Some MS/MS data files were further analyzed by Global Proteomics Machine software (http://www.thegpm.org) (Craig *et al.*, 2004). Visual inspection of the spectrum was used to confirm identity. Both rodent and mammals databases were routinely searched (National Centre for Biotechnology Information and SwissProt) to ensure maximum probability of identification.

2.2.11. Chromatin Immunoprecipitation Assay (ChIP)

ChIP assays were carried out following procedures outlined in Spencer *et al*, 2003, with adaptations for Rcho cells. Briefly, two 150mm plates of day 14 differentiated Rcho cell cultures (approximately 2-3 x 10^7 cells) were crosslinked with 20 ml of 1% formaldehyde in RPMI 1640 medium per plate for 10 minutes at room temperature,

followed by a 5 minute wash at room temperature in PBS containing 125mM glycine and two further rinses in ice cold PBS. Cells were harvested with a rubber policeman into 5ml/plate of ice cold PBS, pelleted at 500 x g for 10 min at 4^0C and washed once in ice cold PBS. At this stage cell pellets could be stored at -80^0C. All PBS used for these procedures was Ca^{+2}/Mg^{+2}-free.

For ChIP assays frozen pellets were thawed on ice and lysed in 1ml of 50mM Tris, pH8.1, 10mM EDTA, 1%SDS and protease inhibitors (20 μM leupeptin, 18 μM pepstatin, 2 mM sodium vanadate, 20 mM sodium fluoride, 10mM PMSF) for 30 minutes. To fragment chromatin to an average length of 500 bp, samples were sonicated on ice, six times for 15 seconds each, with a Fisher Scientific Sonic Dismembrator Model 100 sonicator at 40% power output. To establish fragment size, approximately 20 μl of sonicated sample was boiled for 1 hour and extracted using a Qiaquick PCR purification kit. DNA was electrophoresed on a 2% agarose gel with markers to establish the size range. After verification of fragment size, samples were centrifuged at 13,000 rpm in a microcentrifuge for 10 minutes at 4^0C. Supernatants were collected and diluted 5-fold in ChIP dilution buffer (16.7 mM Tris pH8.1, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA with 1mM PMSF and 1μg/ml each of leupeptin, aprotinin, and pepstatin. An A_{260} measurement was taken and 0.5 A_{260} units of diluted sample was set aside to represent DNA input sequences. Preliminary antibody pull-down assays and western blots established that 1μg of AP2γ antiserum (Santa Cruz Biotechnology) specifically and efficiently immunoprecipitated target protein at 2 and 4 A_{260} units of input DNA. This was the same antiserum as was used for EMSA studies. For ChIP

assays, 1μg of antiserum was added to $2A_{260}$ or $4A_{260}$ units of DNA and incubated with rotation overnight at 4^0C. Eight ($2A_{260}$) or four ($4A_{260}$) separate reactions were set up. Twenty μl of Protein A/G agarose beads (Santa Cruz Biotechnology) pretreated with 0.1 mg/ml sonicated salmon sperm DNA were added to each tube and incubated for 30 min at 4^0C with gentle rotation. Control samples of identical inputs were similarly incubated with 1μg of non-immune rabbit serum replacing AP2γ antiserum. A further control in which the cross-linked sample was treated with beads alone was also carried out. Beads were collected and washed in 1 ml each of low salt buffer (20 mM Tris, pH8.1, 2 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100), high salt buffer (20 mM Tris, pH8.1, 2 mM EDTA, 500 mM NaCl, 0.1% SDS, 1% Triton X-100), LiCl buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 0.25 M LiCl, 1% NP40, 1% sodium deoxycholate) and twice in 10 mM Tris, pH 8.0, 1 mM EDTA. At this point, the individual $2A_{260}$ or $4A_{260}$ reactions were pooled and protein/DNA bonds were reverse crosslinked by incubation in 70 μl of 10 mM Tris pH 8.1, 1 mM EDTA, 0.5% SDS, 0.5 μg/μl proteinase K at 60^0C overnight followed by boiling for 5 minutes and digestion with 250 ng/μl of RNAse A for 30 min at 37^0C. The DNA fragments were purified on QiaQuick spin columns according to the manufacturer's protocol. The presence of the target rPLII enhancer fragment in the samples was assessed by PCR using a platinum Taq PCR kit and the ChIPF and ChIPR primers (Table 1), which amplify a 335 bp fragment containing the rPLII enhancer element. PCR was performed for 35 cycles. To assess the specificity of the ChIP reactions, PCR was also carried out using the Chip2 Forward and Chip2 Reverse primers that amplified a region of genomic DNA in intron of the rPLII gene.

2.3. Methods Used in Chapter 4

2.3.1. Subclones of P1 12830 rPLII-containing Genomic Clone

P1 12830 was previously isolated by Genome Systems using a PCR primer pair that amplified a region in the 5' flanking region of the rPLII gene. Preliminary mapping and hybridization to known members of the rat prolactin gene family showed that it was approximately 80 Kb in size and contained genes for rPLII and rPLP-B (Öztürk *et al.*, 2004). An internal 9.5 Kb Sac I fragment from P1 12830 was subcloned into the Sac I site of the plasmid vector pBluescript SK for further mapping. The ends of the Sac I subclone were sequenced using the T7 and T3 primer sites in the vector. Restriction enzyme mapping of this clone was carried out and further Bam HI subfragments were also cloned into pBluescript for sequencing using the T3 and T7 primers. Primers IIvX4 and IIvX5 were designed for sequence analysis of an internal region of the Sac I clone.

2.3.2. RNA Expression Analysis

BLASTX analysis of the DNA sequence from the 9.5 Kb Sac I fragment showed limited homology to exons 4 and 5 of rat, mouse and hamster PLII genes. To determine whether this region of the clone contained a transcribed gene for an unidentified member of the rat prolactin gene family, primers were developed for RT-PCR using sequences from these homologous regions. Single-stranded cDNA was synthesized from 1 μg of days 12, 14 and 16 rat placental using RNA MMuLV-RT) (Invitrogen). One μl of this

reaction was amplified using PCR primer pair X5F and X5R. The primer pair, IIF1-RNA/IIR2-RNA that amplified rPLII cDNA served as a positive control for the RT reaction. The PCR program used was as described for the analysis of P1 12830. When no RT-PCR product was obtained, the X5F/X5R primers were tested for the ability to amplify the Sac I DNA fragment. Extension times were increased from 1 min to 5 min, since the priming sites were separated by approximately 6 Kb in this clone, and 35 cycles of amplification were carried out. Small amounts of a high molecular weight fragment of the predicted fragment size were produced. To verify that this was the expected fragment, 2 μl of PCR product was reamplified with the nested primer set X6F and X6R. Amplification was for 35 cycles. PCR product was electrophoresed on a 2% agarose gel, blotted and hybridized to a 2 kb Bam HI subfragment of the Sac I clone, which contained the region amplified by the X6F and X6R primers.

To examine the expression of rPLP-I in placenta, the primer pair RIH-BF/RIH-BR was used for RT-PCR with day 11, 12, 14, 16, 18, 21 rat placental RNA, and RNA from differentiated Rcho trophoblast cells collected at various days after plating. The rPLII primer pair, IIF1-RNA/IIR2-RNA was used as a positive control for the RT reactions.

2.3.3. Generation of P1 12830 Transgenic Mice

All protocols involving animals were approved by the University of Manitoba, Protocol Management and Review Committee.

P1 12830 DNA was prepared using the protocol provided by Genome Systems. DNA was isolated from a 75 ml culture using a NucleoBond^R BAC Maxi Kit with a BAC 100 column, according to the supplier's instructions. In all subsequent steps the DNA was dissolved in polyamine buffer containing 10mM Tris, pH 7.5, 0.1mM EDTA, 30 μM spermine, 70 μM spermidine, 100 mM NaCl. DNA was linearized at a unique Not I vector site and isolated in 1% low melt agarose by pulse field gel electrophoresis using a CHEF-DR11 apparatus. Pulse field gels were run at 200 V for 20 hours (switch interval conditions: initial time 1 sec; final time 10 sec). DNA was purified from gel slices using β-agarase according to the supplier's protocol (Invitrogen). DNA was concentrated by a Centricon YM-30 filtering device, dialyzed against polyamine buffer and adjusted to 3 ng/μl in polyamine buffer without NaCl, for injection into the pronucleus of CD1 fertilized mouse eggs according to standard procedures. Transgenic mice were produced in the University of Manitoba Transgenic Facility, Genetic Models Centre. Pregnant female recipients were sacrificed at day 14 of pregnancy and the extraembryonic membranes, placenta and head, trunk and abdomen were collected separately for each of 17 fetuses.

2.3.4. Identification of Transgenic Fetuses

To identify transgenic conceptuses, DNA was isolated from fetal membranes and analyzed for the presence of the transgene by PCR, using primer pairs for three regions of P1 12830: 5' (IIF-1262, IIR-1549), middle (X6F, X6R; RIH-BF, RIH-BR), and 3' (B-Intron DF, B-Intron DR). Amplification with HotStar Taq was carried out using the

following PCR program: 15 minutes at 94^{0}C for enzyme activation; 30 cycles of a 1 minute 94^{0}C denaturation step, 1 minute 55^{0}C annealing step, 1 minute 72^{0}C extension step; a final 10 minute 72^{0}C extension step. For further assessment of transgene integrity, 10 μg of genomic DNA from each conceptus was digested with either Sac I or Bam HI, blotted, and hybridized to ^{32}P-labeled rPLII and rPLP-B cDNA clones respectively.

2.3.5. Transgene Copy Number Determination

For transgene copy number estimation, 10 μg of genomic DNA from each of the transgenic and representative non-transgenic fetuses was digested with Pst I. P1 12830 DNA was mixed with control non-transgenic digested genomic DNA to give the equivalent of 1, 2, 5, and 10 copies per genome based on a transgene size of 100 kb and a haploid genome size of 3 x 10^9 Kb and also digested with Pst I. All samples were electrophoresed on the same 0.8% gel, blotted and hybridized to a P^{32}-labeled rPLII cDNA probe. Autoradiograms were exposed to Kodak XAR film at –70^{0}C with an intensifying screen and analyzed by densitometry.

2.3.6. Transgene Expression Analysis

Expression of rPLII, rPLP-B and rPLP-I mRNA, in the placentas of transgenic fetuses was determined using RT-PCR. Total RNA was isolated from transgenic and non-transgenic mouse placentas and day 18 rat placenta using the Trizol method and was transcribed using MMuLV reverse transcriptase. One μl of each reaction was used for

PCR. Primer pairs were selected that would amplify both the rat and mouse cDNAs, producing PCR products that could be distinguished by restriction enzyme digestions. In all cases the primers represent the rat sequence. PLII cDNA was amplified using the primers IIF1-RNA (nucleotides 26-49) and IIR2-RNA (nucleotides 431-408). The rat-specific fragment contained a unique Pvu II site; the mouse-specific fragment a unique Cla I site. The PLP-B cDNA was amplified using the primers BF1-RNA (nucleotides 304-327) and BR9-RNA (nucleotides 829-807); the rat-specific fragment contained a unique Sph I site, the mouse-specific fragment a unique Pst I site. The rPLP-I cDNA was amplified using the primer pair RIH-RNAF (nucleotides 382-405) and RIH-RNAR (nucleotides 609-633). The rat-specific PCR fragment contained a unique Taq I site, the mouse-specific fragment a unique Bcl I site. Primer pairs are shown in Table 1. Lower case, underlined nucleotides indicate differences and dashed lines extra bases, in the mouse sequence. PCR conditions were the same as those for assessing genomic DNA and 25 or 30 cycles were used. Fragments were separated on 2% agarose gels.

Total RNA was also isolated separately from the head, trunk and abdomen of the transgenic fetuses and non-transgenic littermates. Fetal RNA was analyzed by PCR for 30 cycles using the same primers and conditions as for the placental RNA.

To estimate the relative amounts of the rat and mouse PLII and PLP-B mRNAs in each transgenic sample, PCR reactions were carried out in which one primer of each primer pair was end-labeled using T4 polynucleotide kinase and $\gamma P^{32}ATP$. For assessment

of PLII mRNA levels, the forward primer was end-labeled; the reverse primer was labeled for assessment of PLP-B expression. After 25 or 30 cycles the PLII reactions were digested with Pvu II, generating labeled rat and mouse fragments of 225 bp and 404 bp respectively. The PLP-B PCR reactions were digested with Pst I, generating labeled rat and mouse fragments of 526 bp and 373 bp respectively. Samples were electrophoresed on 2% agarose gels, dried, autoradiographed and analyzed by densitometry.

2.3.7. DNAse I Hypersensitivity Assays

2.3.7.1. Cell culture

Rcho cells were cultured as previously described. Rat C6 glioma cells were grown on Ham's F10 medium supplemented with 25 mM HEPES buffer, L-glutamine, 15% horse serum, 2.5% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. These cells, which do not express any member of the prolactin gene family, were used as a control cell line.

2.3.7.2. Nuclei isolation from cultured cells

Five to ten 150 mm plates of Rcho or C6 glioma cells, grown to one hundred percent confluence, were harvested as for the nuclear extract preparations and stored at -80^0C. For nuclei preparation, pellets were thawed on ice for 10 minutes and resuspended

in 20 ml of Nuclei Isolation buffer (NIB) (50 mM Tris-HCl, pH7.5, 25 mM KCl, 2 mM $MgCl_2$, 30 mM sodium butyrate, 0.25 M sucrose) plus 1% Triton X-100. Just before nuclei isolation, 25 mg/ml iodoacetamide, 1 mM PMSF, 2 μg/ml aprotinin, were added to the buffer. The cell pellet was resuspended by pipetting up and down about 20 times with a 10 ml pipette and transferred to a sterile, ice-cold glass Dounce homogenizer fitted with a type B pestle. Cells were homogenized 10 to 15 times and a 50 μl sample was examined under a phase contrast microscope to verify production of nuclei. The homogenized samples were centrifuged at 1500 x g for 10 minutes. The crude nuclei pellet was resuspended in 10 ml of cold NIB with additions and transferred to a new cold, sterile homogenizer. Five more strokes were applied; the preparation was again checked under the microscope. Following a further 10 minute centrifugation at 1500 x g, the pelleted nuclei were resuspended in 3 ml of NIB with 1 mM PMSF and 2 μg/ml aprotinin. The DNA concentration was determined by diluting 5 μl of nuclei in 1 ml of 5 M urea/2 M NaCl, and taking an OD_{260} reading.

2.3.7.3. Nuclei isolation from rat placental tissue

Starting material was pieces of frozen day 17/18 rat placental tissue that was finely crushed on dry ice. Tissue fragments were collected into 25 ml of Ca^{+2}/Mg^{+2} free PBS containing 1 mM EDTA in a 50 ml Corning tube and centrifuged at 1000 X g. The tissue pellet was washed three times in this solution to reduce contamination from blood. The pellet was resuspended in 8 volumes of ice-cold Tissue Nuclei Isolation buffer (TNIB) (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 30 mM sodium butyrate, 0.25 M

sucrose) plus 1% Triton X-100, 50 mg/ml iodoacetamide, 2 mM PMSF and 4 μg/ml aprotinin. The resuspended sample was homogenized in a Dounce homogenizer with a B pestle for 5 to 10 strokes. This step was repeated until clean nuclei were seen by phase contrast microscopy. Nuclei were collected by centrifugation at 1500 X g for 10 minutes and resuspended in approximately 3 ml TNIB plus 1 mM PMSF and 2 μg/ml aprotinin. DNA concentrations were determined as for cell culture nuclei.

2.3.7.4. DNAse I hypersensitive site mapping

Nuclei were diluted to 20 A_{260} units/ml in TNIB containing a final concentration of 0.1 mM $CaCl_2$ and 400 μl aliquots were pipetted into 1.5 ml microcentrifuge tubes on ice. Nuclei were treated with increasing concentrations of DNAse I, from 1U to 20U as indicated, and incubated for 10 minutes at 37^0C. Reactions were stopped with the addition of 100 μl of 5% SDS, 100 mM EDTA. Fifty μg of proteinase K was added to the samples and incubation was carried out overnight at 55^0C. Following proteinase K treatment, samples were treated with 50 μg of RNase at 37^0C for 1.5 hour. Samples were extracted twice with phenol/chloroform and once with chloroform followed by ethanol precipitation. Precipitated pellets were resuspended in 10 mM Tris-HCl pH 7.9, 1 mM EDTA. Ten to twenty μg of purified DNA was digested overnight with Pst I or Dra III (2 units per μg genomic DNA). Reactions were stopped by the addition of 1 μl 0.5 M EDTA pH 8.0 and samples were electrophoresed on 0.8-1% agarose gels at 25 V overnight. Gels were photographed and Southern blotted on Nitroplus membrane (Southern, 1975).

2.3.7.4. Probe labeling

Indirect end-labelled probes were synthesized by PCR from rat PRL locus containing BAC clones and genomic DNA samples isolated from Rcho cell line. The GeneFisher program was used for selection of PCR primers [http://bibiserv.techfak.uni-bielefeld.de/genefisher]. The HSS5F/HSS5R primer set amplified a 583 bp genomic DNA fragment from 23620 bp to 24202 bp in the rat genome sequence AABR01038619 (these numbers are now changed). The HSS4F/HSS4R primer set amplified a 679 bp genomic DNA fragment from 3735 bp to 4436 bp in clone AABR01038619 of the rat genome sequence. A BLASTN search showed that these genomic probes did not contain highly repetitive sequences and showed no close similarity to other members of the prolactin family.

Fragments were labeled with $\propto^{32}$P-dCTP using a random prime DNA labeling kit. The activity of DNA was 10^7 to 10^8 dpm per µg. The labeled probes was boiled for 5 minutes and then placed on ice prevent reannealing of single stranded DNA fragments.

2.3.7.5 Hybridization of genomic blots

Blots were hybridized in a Robbins Scientific microhybridization incubator. Prehybridization was in 15 ml of 6.6X SCP (0.66 M NaCl, 0.198 M Na_2HP0_4, 6.6 mM Na_2EDTA, adjusted to pH 6.2), 0.4X Denhardt's solution (Bovine serum albumin, Ficoll 400, polyvinyl pyrrolidone at 0.08 % for each), 1% N-lauryl sarcosine and 100 µg/ml

sonicated salmon sperm DNA for 3 hours at 65^0C. 15 ml of the fresh solution pre-warmed to 65^0C, boiled probe and hybridization was performed for 20 hours at 65^0C. Blots were washed twice with 50 ml of 6.6X SCP containing 1% Sarkosyl for 15 minutes at 65^0C and twice with 1X SCP containing 1% Sarkosyl. If the background was found to be high, one additional wash was carried out with 0.2X SCP containing 1% Sarkosyl for 10 minutes. The blots were exposed to Kodak MS film at –70 to -80^0C for period of overnight to two weeks.

CHAPTER 3

IDENTIFICATION OF NUCLEAR FACTORS ASSOCIATED WITH THE RAT PLACENTAL LACTOGEN II (rPLII) GENE ENHANCER ELEMENT

3.1 Identification of Key Nucleotides in the rPLII Enhancer Element

Earlier studies in our laboratory had identified a 65 bp sequence from the rPLII 5' flanking fragment, which was demonstrated to contain an enhancer element that functioned in rat trophoblast Rcho cells, but not rat pituitary GC cells (Sun and Duckworth, 1999). This sequence was located within a 3 Kb rPLII promoter proximal sequence that was able to direct expression of a luciferase reporter gene in the placenta of transgenic mice (Shah *et al*, 1998). As shown in **Figure 3.1**, two adjacent DNAse I protected regions, FP1 and FP2, were identified in the enhancer element by footprint analysis. Cloned wild type and mutated versions of this fragment had been used for EMSA and reporter gene transfection studies. It had been shown that changes to nucleotides in the "GGA" sequence within FP1 or "GCTC" within FP2 were sufficient to cause a partial (when one site only was changed) or complete loss (when both sites were altered) of enhancing activity in the rat trophoblast Rcho cell line. Based on sequence similarities to known transcription factor binding sites, and the effects of the specific nucleotide changes on enhancing activity, the factor that bound FP1 was tentatively identified as being a member of the Ets family, and the FP2 factor as being AP1 (Sun and Duckworth, 1999). Since all activity was lost when changes were made only to these

defined nucleotides in the cloned fragment, we chose to use more defined synthetic oligonucleotides containing only the FP1 and FP2 DNAse I protected regions for further studies (**Figure 3.1**). For most studies a 43 bp oligonucleotide (WTFP1-2) was used; in some early studies, a 33 bp oligonucleotide was used (WT33).

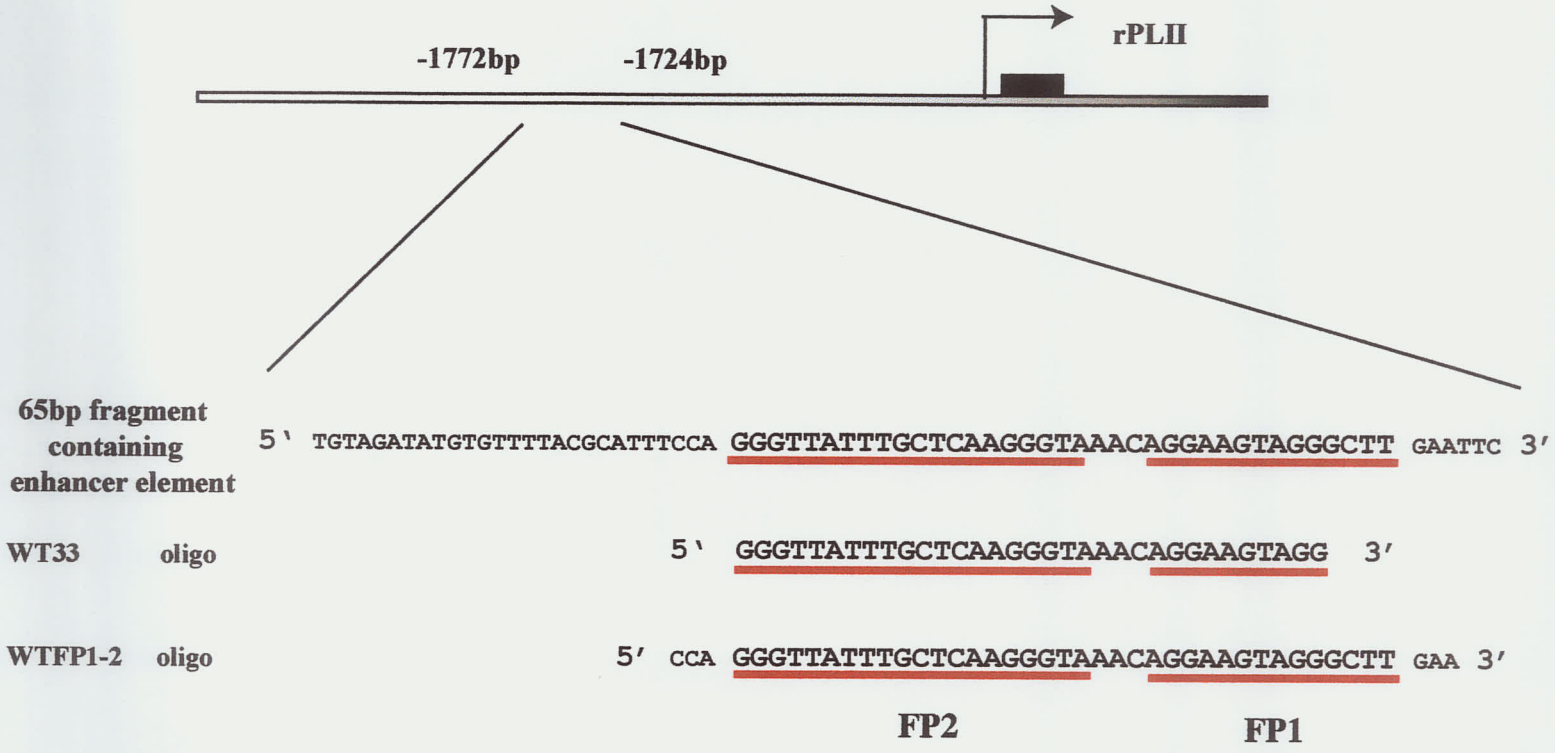


Figure 3.1: The sequence of the rPLII enhancer element at -1772 to -1724 bp and oligonucleotides used for the identification of candidate binding factors and the characterization of specific factors that bind to the core motifs.

The functional 65 bp rPLII enhancer element was used in earlier studies (Hph I- Sac I fragment) (Sun and Duckworth, 1999). Two oligonucleotides, WT33 (33 bp) and WTFP1-2 (43 bp) were used in our studies to characterize protein-DNA interactions and functional studies.

3.1.1 Luciferase Expression Studies using Synthetic Enhancer Oligonucleotides

To determine whether this more defined sequence functioned similarly to the 65 bp cloned enhancer fragment, two copies of the wild type 43 bp oligonucleotide were cloned in tandem 5' to 3' into the luciferase reporter vector, pTK81 as outlined in the Methods section. Oligonucleotides mutated in the FP1 (m6 mutation) and/or FP2 (M4 mutation) regions were also cloned into pTK81. These mutated oligonucleotides are shown in **Figure 3.2A**. The number of cloned copies of each oligonucleotide was determined by PCR and the orientation of the dimers was verified by sequencing analysis. Transient transfection studies were carried out in Rcho cells and luciferase activity for each reporter construct was measured. Results are presented in **Figure 3.2B**.

The wild type 43 bp enhancer construct (WT - pTK Luc) showed enhancing activity when compared to the empty vector (pTK Luc). The enhancing activity of the clone carrying the M4 mutation within the FP2 region (FP2 - M4 pTK Luc) was decreased by approximately 40% when compared to the wild type reporter clone. The reporter clone with the FP1 region GGA mutation (FP1 - m6 pTK81) showed a 70% loss of activity; mutations in both elements (M4 -m6.pTK Luc), showed a complete loss of enhancing activity. All constructs contained two copies of the specific oligonucleotide, both oriented 5' to 3'. These results were similar to previous data with the 65 bp cloned enhancer fragment (Sun and Duckworth, 1999), suggesting that the 43 bp oligonucleotide contained the functional activity of the element. I used the 43 bp oligonucleotide for

EMSA studies designed to determine whether an Ets factor could bind the FP1 region of the enhancer and to identify that Ets factor.

A.

WT	5' CCA GGGTTATTTGCTCAAGGGTA AAC AGGAAGTAGGGCTT GAA 3'
	FP2 FP1
M4	5' CCA GGGTTATTTGCT acgt GGTA AAC AGGAAGTAGGGCTT GAA 3'
m6	5' CCA GGGTTATTTGCTCAAGGGTA AAC ACTGAGTAGGGCTT GAA 3'
M4m6	5' CCA GGGTTATTTGCT acgt GGTA AAC ACTGAGTAGGGCTT GAA 3'

B.

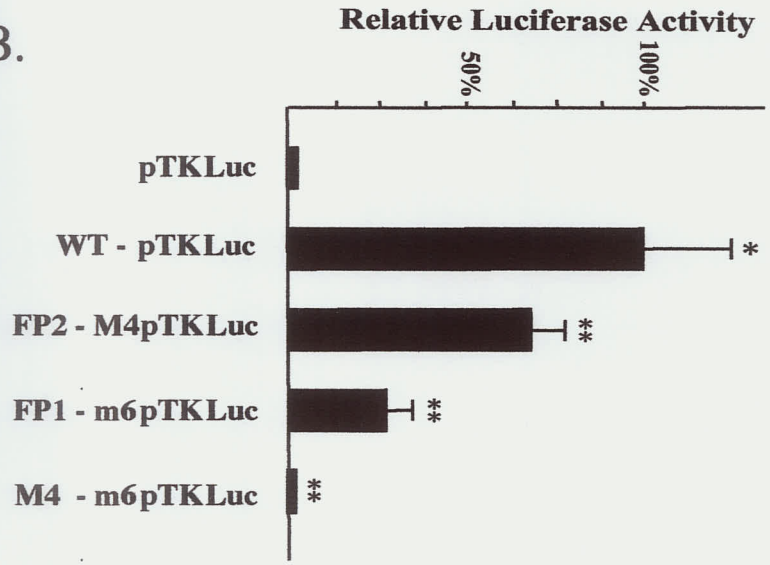


Figure 3.2: Assessment of functional activity of the 43 bp rPLII enhancer oligonucleotide and relative effects of the core binding motifs of FP1 and FP2 region on activity.

(A) The sequences of the wild type and mutated enhancer oligonucleotides. The M4 mutation is in the FP2 DNAse I protected region; the m6 mutation is in the FP1 region. **(B)** Transient transfection studies were carried out in Rcho cells using luciferase reporter constructs. Transcriptional activity of the promoterless reporter vector pTKLuc and enhancer containing constructs containing the mutations in (A) are shown. The firefly luciferase activity was normalized to Renillla luciferase activity for each assay. Luciferase activity is given as a percentage of the WT-pTKLuc which was 100%. The data were analyzed as the mean value and standard deviation of the ratio of firefly luciferase activity/Renilla luciferase activity (n=14). Statistical significance was defined as $p<0.05$ using an unpaired two tail t-test. The activity of WT-pTKLuc was significant as compared to the vector control ($p<0.001$). Constructs containing the M4 mutation in the FP2 region containing vector (FP2-M4pTKLuc) and the GGA-mutated FP1 region (FP1-m6pTKLuc)showed significantly lower activities than wild type (*$p<0.0.016$ and **$p<0.001$, respectively). The construct containing the double mutation (M4-m6pTKLuc) showed a complete loss of activity (**$p<0.0003$) and was similar to the enhancerless vector. Statistical significance of all samples was determined using ANOVA with a *post hoc* Bonferroni test ($p<0.01$).

3.2 Characterization of the FP1 Protein Binding Factor

In previous studies, the FP1 region of the enhancer element was identified as a possible Ets factor-binding site (Sun and Duckworth, 1999). This was based on a consensus Ets core sequence, GGAA, in the FP1 DNAse I protected region, and was supported by database analyses for transcription factor binding sites. Whether an Ets factor actually bound the site, and the possible identity of that factor, was not previously examined in detail. Approximately 45 members of the Ets family of transcription factors have now been identified (Obika *et al.*, 2003). Since the Ets family of transcription factors is so large, I first looked for possible candidates from among those Ets factors that had been identified in the literature as being expressed in placenta or associated with a defect in placental development.

3.2.1 Assessment of Ets Transcription Factor Interactions with the rPLII Enhancer

Placental expression has not been investigated for the majority of Ets factors, but a number have been shown, mainly by RNA blots, to be expressed in human and mouse placenta. These include Ets-2, ESX/ESE-1/Elf-3, ESE-2/Elf-5, Elf-1, MEF-1/Elf-4, NERF-1 and NERF-2/Elf-2 (Yamamoto *et al.*, 1998; Chang *et al.*, 1997; Choi *et al.*, 1998; Zhou *et al.*, 1998; Oettegen *et al.*, 1999; Rao *et al.*, 2002; Miyazaki *et al.*, 1996; Gaspar *et al.*, 2002 and Elk-1 (Savoie, A. and Duckworth, M.L., unpublished data).

A null mutation of Ets-2 in mice results in very early embryonic lethality due to

the inability of trophoblast giant cells to invade maternal decidua at the implantation site (Yamamoto *et al*, 1998). This affect is associated with a specific requirement for Ets-2 in the expression of the matrix metalloprotease gene, MMP-9 (gelatinase B). There were no other reports of embryonic lethal placental effects in Ets factor null mice during our studies. Recently, however, analysis of an ESE-2/Elf-5 mutation in mice has been reported to result in the loss of extraembryonic ectoderm; ESE-2/Elf-5 also appears to be required for the maintenance of trophoblast stem cells (Donnison *et al*, 2005). My main purpose in the following studies was to analyze possible interactions of the rPLII enhancer element with individual *in vitro* expressed recombinant proteins and then to assess potential interactions with endogenous nuclear proteins of candidate Ets factors.

3.2.1.1 *In vitro* Transcription and Translation of Ets Factors

As a first step I aimed to determine whether the FP1 site could interact with *in vitro* transcribed and translated proteins of any Ets family members reported to be expressed in placenta. By RNA blot, I confirmed that Elf-1 and NERF-2 were expressed in both Rcho cells and rat placenta (**Figure 3.3**). In earlier studies (Sun and Duckworth, published data), Ets-2 mRNA was detected in developing rat placenta and Rcho cells but shown not to interact with the 65 bp rPLII enhancer element; ESE-1/ESX expression was also shown by RNA blot to be present in Rcho cells and preliminary data suggested that it could bind this element (Yuxiang Sun, unpublished data). We obtained expression vectors of Ets-2, MEF-1/Elf-4, ESX/ESE-1/Elf-3, NERF-2/Elf-2, Elf-1 and produced proteins using an *in vitro* transcription and translation system. To verify that the

recombinant protein products were synthesized, ^{35}S-methionine was included in the reactions for protein synthesis. Representative gels showing the *in vitro* expressed products are shown in **Figure 3.4**. Among the clones tested, only ESE-2/Elf-5 was not expressed. Sequencing this clone did not explain why there was no expression.

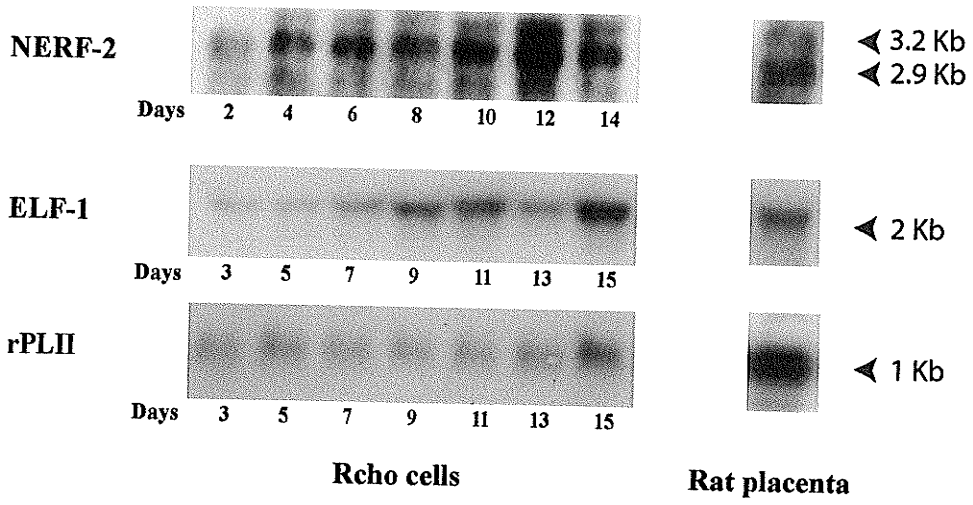


Figure 3.3: Expression analysis of ELF-1, NERF-2 in the Rcho trophoblast cell line and rat placenta.

Total RNA was isolated from Rcho cells, from day 2 to day 15 after plating, and analyzed by northern blot for expression of rat ELF-1 and NERF-2. Rcho cells replicate as small cells that differentiate into the rPLII - expressing giant cell type after several days in culture. On the right are shown total RNAs from day 16 / 17 rat placental tissue hybridized to the same probes.

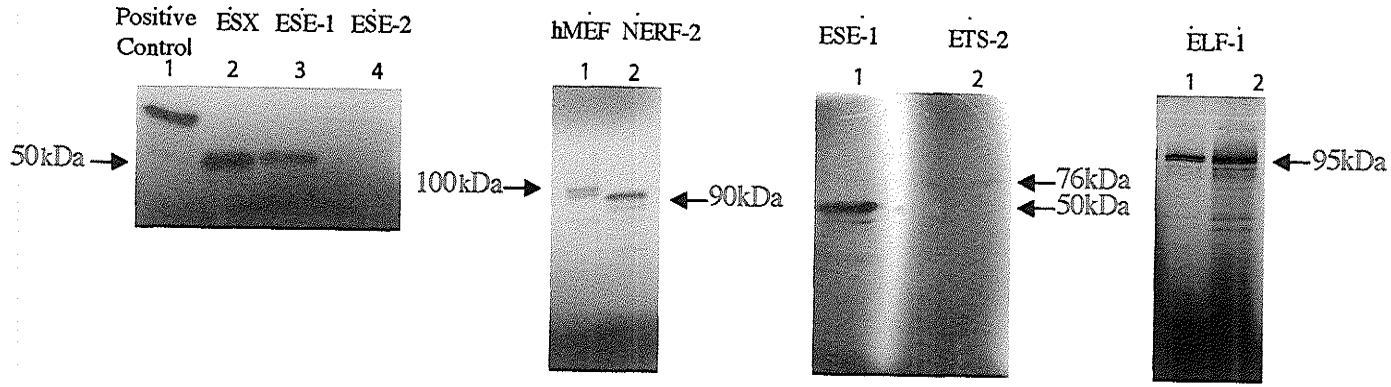


Figure 3.4: **S^{35} methionine labelling to demonstrate *in vitro* protein expression.**

ESX, ESE-1, ESE-2, MEF, NERF-2, ELF-1 and Ets-2 cDNA-containing expression vectors were utilized for recombinant protein production using an *in vitro* transcription and translation reticulocyte system. Proteins were translated in the presence of radioactive methionine and resolved by electorphoresis on 10 % SDS poly-acrylamide gels. The gels were dried and protein samples were visualized by autoradiography. The size of the sythesized bands are shown by arrows. Labelled bands are of the expected protein sizes.

3.2.1.2 EMSA Studies using Recombinant Ets Proteins

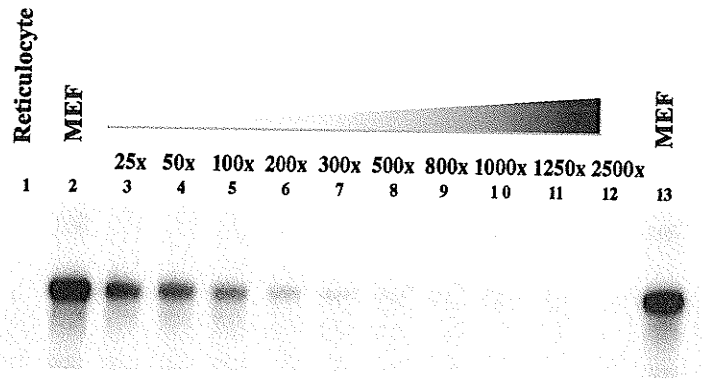
^{32}P end-labelled - double stranded WT33 and WTFP1-2 synthetic oligonucleotides (sequences shown in **Figure 3.1**) were used in these studies. The boundaries of the DNAse I protected regions were not rigorously defined in previous studies. Initially, I designed a 33 bp oligonucleotide (WT33), which covered the more central region of the enhancer element, including the key nucleotides previously defined by mutation. This oligonucleotide was used for some studies with *in vitro* synthesized protein. Later we decided to extend the boundaries of the oligonucleotide probe to include more of the nucleotides 3' of the putative Ets core GGAA sequence and synthesized the 43 bp oligonucleotide (WTFP1-2). Since this was the sequence that was tested for enhancing activity in reporter assays, most EMSA studies, in particular those which used endogenous nuclear proteins, were carried out with the 43 bp oligonucleotide.

MEF-1/Elf-4 binding : *In vitro* synthesized hMEF/Elf-4, a member of the Elf subgroup of Ets factors, showed specific binding to the WT33 oligonucleotide (**Figure 3.5A**). The importance of the Ets core GGA within the FP1 region for this binding was confirmed by using the wild type (WT33) and GGA mutated (mETS) oligonucleotides as competitors (**Figure 3.5A** and **B**). Only excess WT33 oligonucleotide successfully eliminated interaction with the recombinant MEF protein, while the GGA mutated mEts oligonucleotide had no effect on binding. When the mEts oligonucleotide was used as a probe, no interaction with MEF protein was observed (**Figure 3.5B,** lane 10). These data suggested that the GGA sequence was essential for MEF binding; this was the first direct

indication that an Ets factor could in fact bind the FP1 region of the rPLII enhancer element.

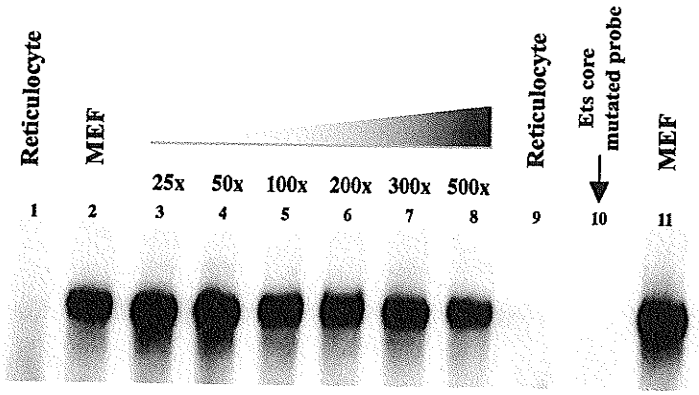
MEF interaction

A.



Competition with unlabelled WT33 oligo

B.



Competition with unlabelled Ets core/GGA mutated oligo

C.

WT33 33 bp 5′ GGG TTA TTT GCT CAA GGG TAA ACA GGA AGT AGG3′

mETS 33 bp 5′ GGG TTA TTT GCT CAA GGG TAA ACA ttc AGT AGG3′

Figure 3.5: Assessment of recombinant MEF protein interaction in gel shift studies.

(A) In EMSA reactions labelled rPLII enhancer oligo, WT33, formed a DNA-protein complex with *in vitro* synthesized MEF (lanes 2, 13). The specificity of complex formation was defined by the ability to compete with unlabelled WT33 oligo with 25-, 50-, 100-, 200-, 300- 500-, 800-, 1000-, 1250-, 2500- times molar excess.
(B) Unlabelled mETS oligo did not compete with 25-,50-, 100-, 200-, 300-, 500- times molar excess of cold oligos against to the WT33 probe. MEF recombinant protein did not show binding to mETS oligo probe (lane 10). As a control, reticulocyte also applied in binding reactions (lane 1 and 9).
(C) Sequence element order and changes in the WT33 and mETS oligos are shown.

However, in spite of the fact that this protein had been detected in human placenta (Miyazaki *et al.*, 1996) we were unable to detect MEF expression in rPLII-expressing Rcho cells or rat placenta using RNA blot analysis, suggesting that this would not be the endogenous factor that binds the rPLII enhancer *in vivo*.

NERF-2/Elf-2 : *In vitro* synthesized NERF-2 specifically bound the wild type 43bp enhancer oligonucleotide (WTFP1-2) as shown in **Figure 3.6**. Addition of unlabelled WTFP1-2 eliminated the interaction of recombinant NERF-2 protein (**Figure 3.6A**). The NERF-2 binding was lost when changes were made to the GGA sequence (**Figure 3.6B**, lane 3). A WTFP1-2/NERF-2 complex was formed and this interaction was competed with unlabelled probe (**Figure 3.6A**); the recombinant protein interaction was lost when the m6 GGA-core mutated oligonucleotide used as a probe (**Figure 3.5B**, lane 3).

Elf-1 binding : *In vitro* synthesized rat Elf-1 protein bound the WTFP1-2 element (**Figure 3.7A**, lane 1). A faint lower complex was also competed, but this was present in a control binding reaction made without the addition of DNA and therefore was likely non-specific (**Figure 3.7A**, lane 9). Elf-1 binding was eliminated with 100 times and higher molar excess of the unlabelled WTFP1-2 oligonucleotide (**Figure 3.7A**, lanes 2 to 7). When an oligonucleotide containing only the FP1 region was used as a probe, an interaction was still seen (**Figure 3.7B**, lanes 2). An Elf-1 specific antiserum produced a supershift complex with the wild type probe, suggesting the specificity of this interaction (**Figure 3.7B**, lanes 4).

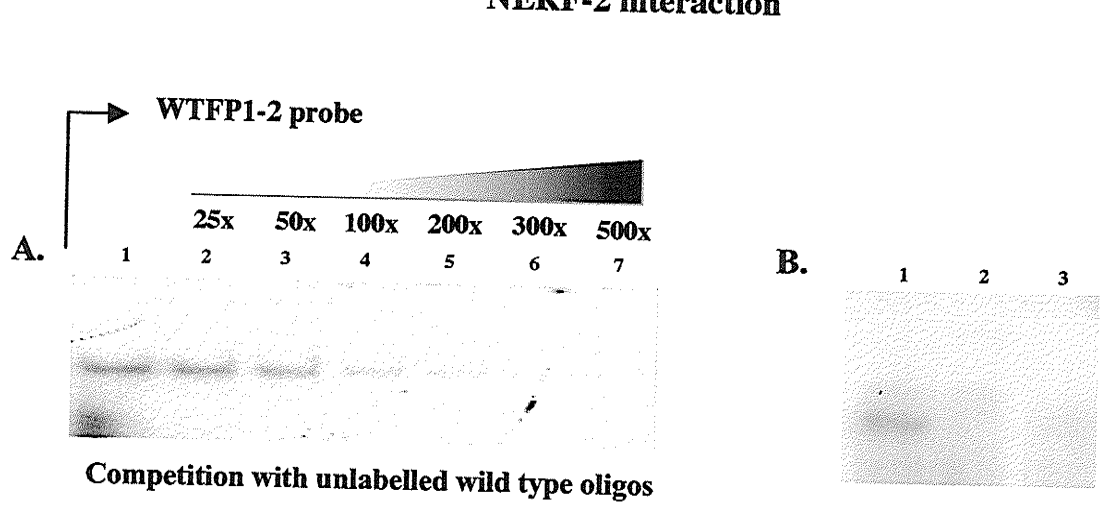
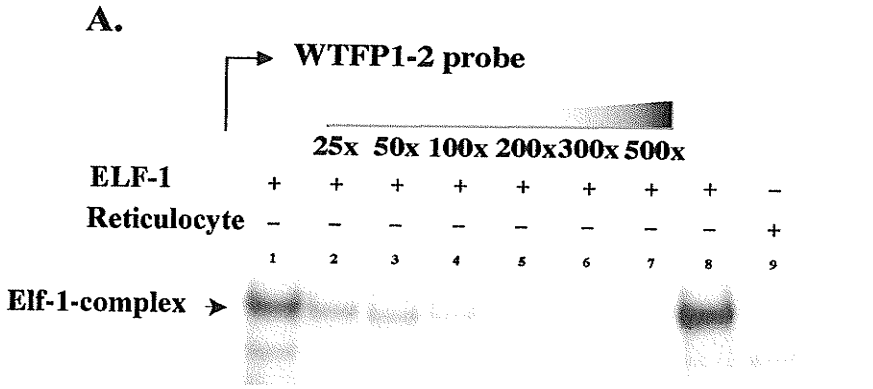


Figure 3.6: Recombinant NERF-2 interacts with the 43 bp enhancer element.

Gel shift reactions were carried out using *in vitro* synthesized NERF-2 with the 43 bp WTFP1-2 oligonucleotide. (**A**) Interaction was seen (lane 1) which was competed with increasing concentrations of unlabelled WTFP1-2 oligo (lanes 2 to 7). (**B**) NERF-2 protein interacts with WTFP1-2 (lane 1) and is competed by 200 times molar excess of cold probe (lane 2), but does not bind when an oligo with the m6 mutated GGA sequence is used as a probe (lane 3).

ELF-1 interaction



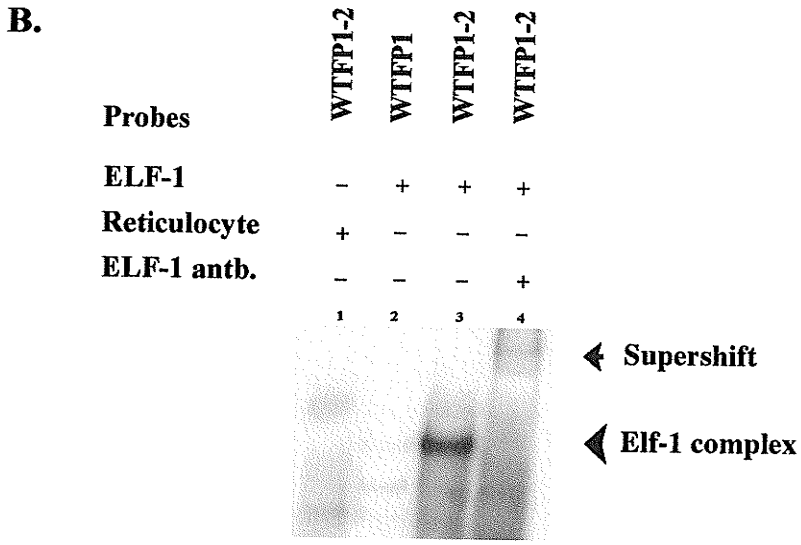


Figure 3.7: Recombinant ELF-1 interacts with the rPLII enhancer element.

(A) *In vitro* transcribed and translated ELF-1 protein was incubated with the WTFP1-2 probe for EMSA studies. Increasing molar excess concentrations of cold probe were added as indicate in competition assays (lanes 2 to 7). Reactions with no competitor are shown in lanes 1 and 8. Lane 9 represents a control binding reaction where no RNA was present in the in vitro translation reaction.
(B) Oligonucleotides WTFP1-2 and WTFP1, which contains only nucleotides in the FP1 region, were used as probes to determine the binding specificity of recombinant ELF-1. Only the WTFP1-2 probe showed strong binding (lane1). The ELF-1 complex was supershifted with ELF-1 antiserum (lane 4). WTFP1 showed only a weak interaction with the ELF-1 protein, suggesting that nucleotides outside this sequence are required for robust binding.

MEF-1/Elf-4, NERF-2/Elf-2 and ELF-1 have been classified as members of the Elf subgroup of Ets family members, based on amino acid sequence homology outside of the Ets DNA-binding domain (Sharrocks, 2001; Oikawa and Yamada, 2003). Although previous preliminary studies appeared to indicate an interaction between ESX/ESE-1/Elf-3 and the 65 bp cloned enhancer element by EMSA (Y. Sun, unpublished data), in my studies *in vitro* synthesized ESX/ESE-1/Elf-3 did not interact with the 43 bp enhancer oligonucleotide (data is not shown). *In vitro* synthesized Ets-2 protein, a member of a different Ets subgroup, also did not show interaction with the WTFP1-2 oligonucleotide. These data suggested that there is some specificity in Ets factor binding to the FP1 region of the rPLII enhancer element.

3.2.1.3 EMSA studies using nuclear extracts from the rat trophoblast Rcho cell line

The evidence of direct interactions between the rPLII enhancer element and several Elf-related Ets transcription factors prompted me to assess whether these endogenously expressed candidate proteins could interact with the rPLII enhancer element. As shown in **Figure 3.8**, the native complexes of nuclear proteins with the FP1 region of the enhancer element were examined in EMSA studies with Rcho nuclear extracts and the 43 bp probe (lane 1). At least six DNA-protein complexes were formed and competition with unlabelled WTFP1-2 demonstrated the specificity of several of these (lane 2). The m6 oligonucleotide probe, containing a GGA to CTG change in the FP1 region, did not compete complex 3 (lane 3), suggesting that this may be an Ets factor-associated complex.

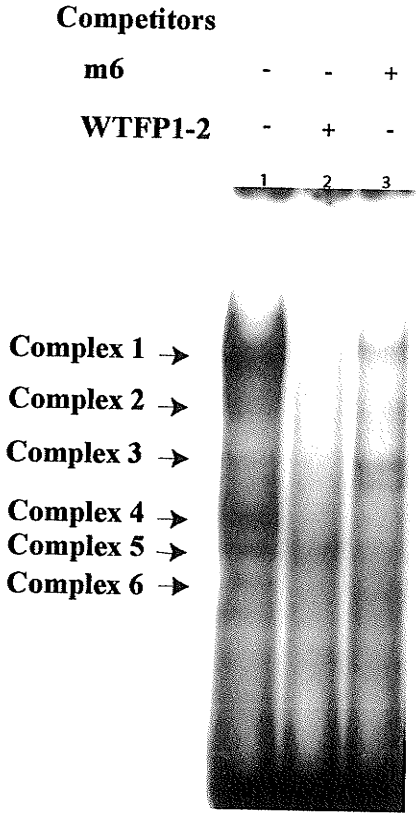


Figure 3.8 : EMSA complexes associated with the GGA sequence in the FP1 region of the rPLII enhancer.

The labelled 43bp WTFP1-2 oligonucleotide was incubated with the nuclear extracts from differentiated D14 Rcho cells in the presence and absence of WT and m6-GGA competitor oligos. There are 6 complexes associated with binding to the WT probe (lane 1). In the presence of a 200 fold excess of cold probe essentially all complexes are lost (lane 2); in the presence of a 200 fold excess of the m6 oligo mutated in the GGA sequence, complex 3 is not competed (lane 3). Complex 3 is sometimes seen as a double band, as seen here.

To reveal possible direct interactions of endogenous Ets proteins shown to bind in our *in vitro* studies, specific antibodies were utilized in EMSA supershift studies. **Figure 3.9** shows these results. NERF-2 antiserum, but not Elf-1 antiserum showed a higher molecular weight complex (**Figure 3.9**, lanes 3 and 2, respectively). Elf-1 antiserum was shown to supershift *in vitro* synthesized Elf-1 protein; NERF-2 antiserum did not supershift the *in vitro* synthesized NERF-2, making it unclear what endogenous protein the antiserum was recognizing in these reactions. ESE-2/ELF-5 antiserum was also tested. This factor is closely related to ESX/ESE-1/Elf-3, and is expressed in mouse and rat placenta, and Rcho cells (Chang *et al.*, 1997; Sun and Duckworth, unpublished data). We were not able to get *in vitro* expression of this protein. The available ESE-2/ELF-5 antiserum is reported to cross-react to a lesser extent with the ESX/ESE-1, ESE-3a and –3b proteins of mouse, rat and human origin (Santa Cruz Biotechnology Inc). When used in EMSA studies, this antiserum also produced a higher molecular weight complex similar to that seen with NERF-2 antiserum (data not shown); in neither case, however, where a supershift was seen, was there a visible decrease in any of the lower complexes. **Figure 3.10** shows the effect of increasing concentrations of these antisera on complex formation; although the supershift complex becomes stronger with increasing antiserum concentration, changes in the amounts of the lower complexes are not obvious. Nonetheless, similar concentrations of non-immune rabbit serum had no effect on complex formation (lanes 8 to 10), suggesting that the higher molecular weight complex was due to specific interactions.

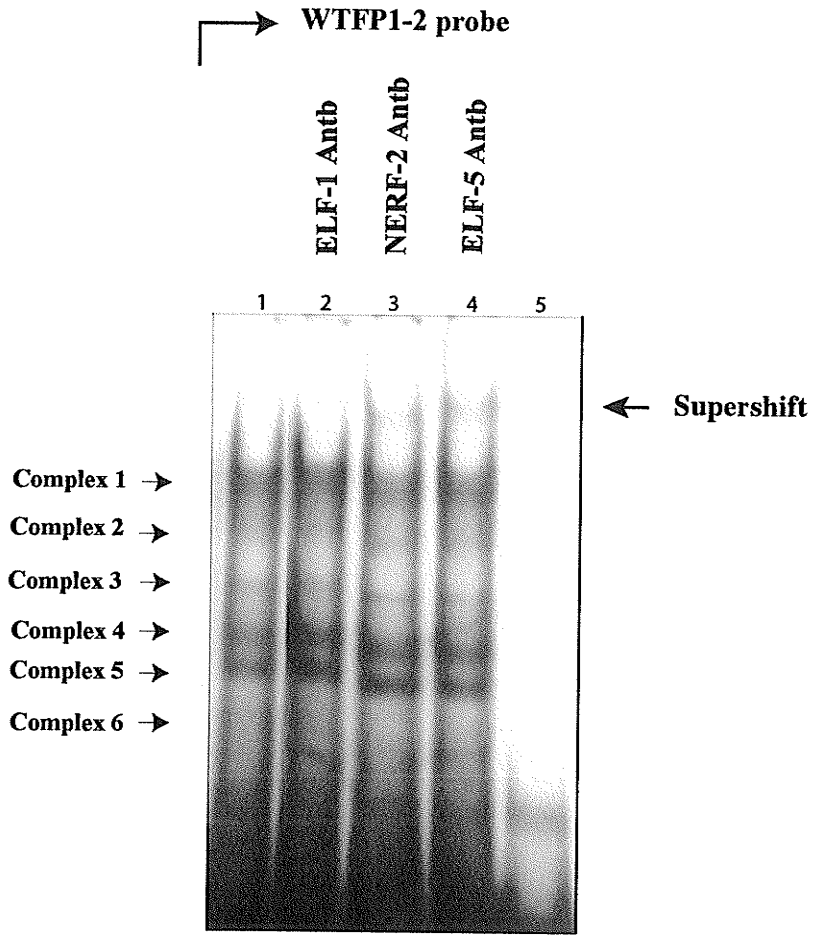


Figure 3.9: EMSA-supershift studies using ELF-1, NERF-2 and ELF-5 antibodies to assess endogenous Rcho protein interactions with the rPLII enhancer element.

EMSA studies were performed using the wild type oligo enhancer probe WTFP1-2 with Rcho nuclear extracts and ELF-1, NERF-2 and Elf-5 specific antisera. Complexes formed without antiserum are shown in lane 1. ELF-1 antiserum did not supershift any complexes (lane 2). NERF-2 and ELF-5 antiserum produced a higher molecular weight complex (lanes 3 and 4 respectively), but no loss of a lower molecular weight complex was evident. Lane 5 represents free probe.

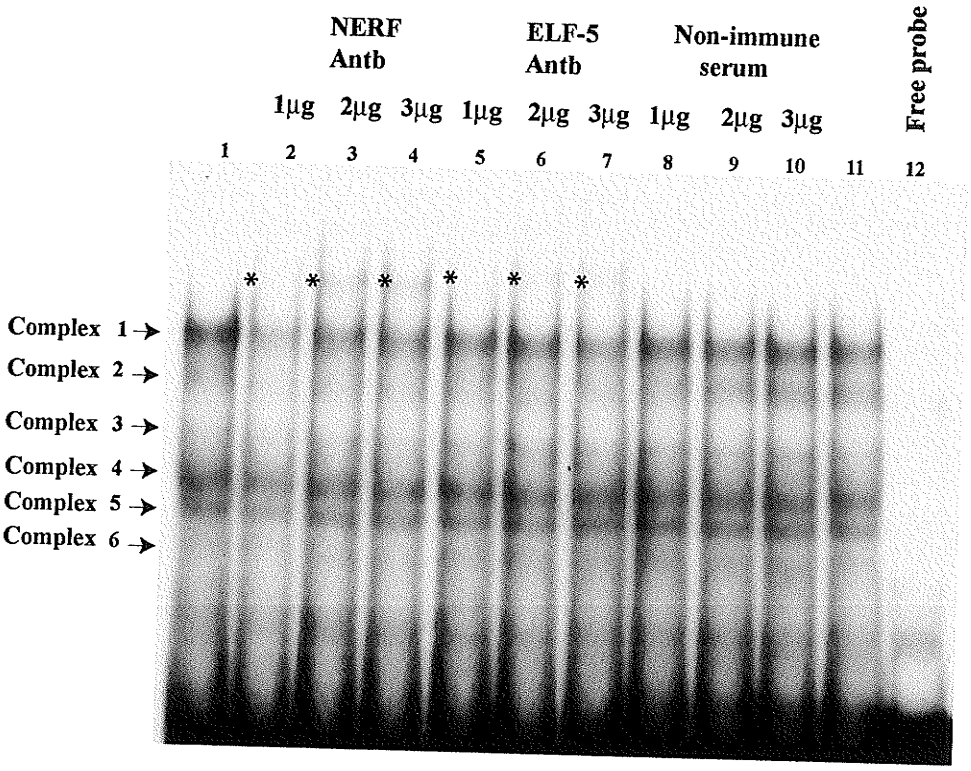


Figure 3.10: EMSA supershift studies of the rPLII enhancer with NERF-2 and Elf-5 antisera.

EMSA studies were carried out with Rcho nuclear extracts and the wild type (WTFP1-2) enhancer probe using various concentrations of NERF-2 and Elf-5 antiserum as indicated. Specific complex formation without antiserum is shown in lanes 1 and 11; complexes are numbered. A higher molecular weight complex is seen at all concentrations of both specific antisera (lanes 2 to 7) as marked by asterisks, but in particular at the higher concentrations. The non - immune rabbit serum showed no higher complex formation (lanes 8 to 10). There was no clear loss of any lower molecular weight complex.

These data suggested that more than one Ets factor could bind the rPLII enhancer and that these factors were present in Rcho nuclear extracts. To confirm that the individual factors were binding the FP1 element, the m6 oligonucleotide was used as a probe in EMSA supershift studies with NERF-2 and ESE-2/Elf-5 antisera. The results are shown in **Figure 3.11**. As expected for this probe, EMSA reactions without antiserum did not show complexes 3 and 4 (lane 2). However, when the antisera were added, higher molecular weight complexes similar to those formed with the WTFP1-2 probe (lanes 3 and 5) were also formed with the m6 probe (lanes 4 and 6). These results using the mutated Ets core oligonucleotide are difficult to interpret. The higher molecular weight complex may be due to non-specific interactions, although the fact that non-immune serum does not produce a similar complex makes this less likely. Potential protein-protein interactions between the factors that bind to the FP1 and FP2 region may have a role in the assembly of the supershift complex even with the GGA-core mutated probe. Nucleotides outside the GGA core may be involved in the formation of these protein-protein complexes.

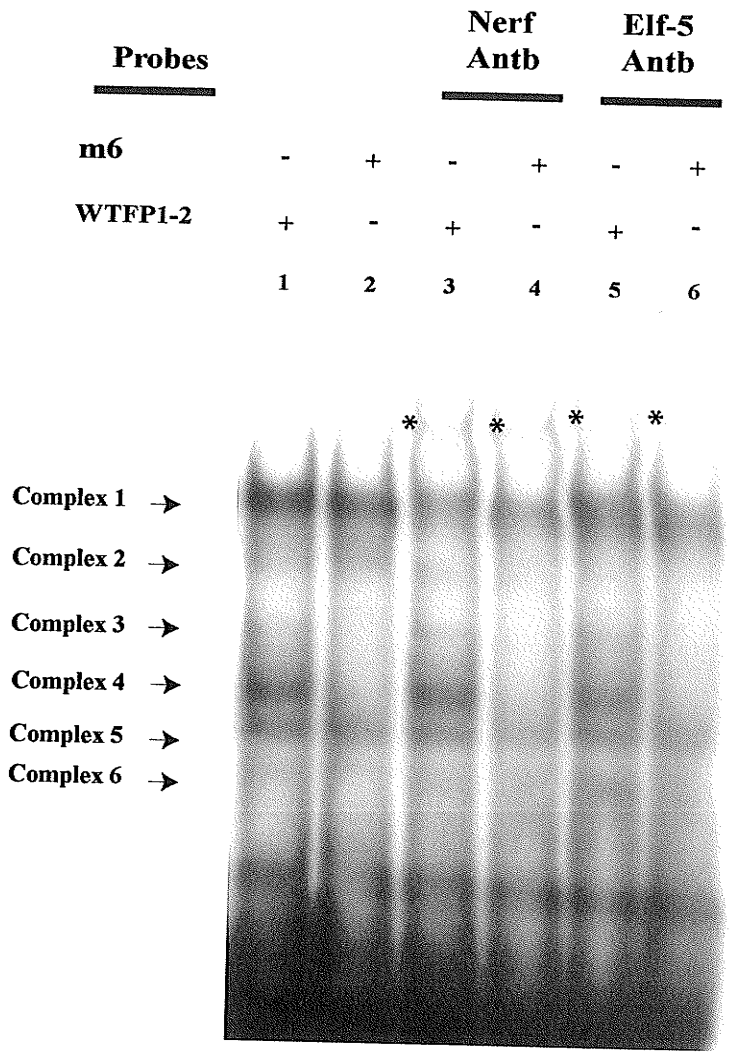


Figure 3.11: NERF-2 and Elf-5 antisera form supershift complexes with GGA-core mutated oligonucleotide probes.

EMSA studies were performed using the wild type and GGA - core mutated m6 oligos as probes with Rcho nuclear extracts and NERF-2 and Elf-5 specific antisera. Complexes formed with the WTFP1-2 probe are seen in lane 1. The m6 mutation from GGA to CTG within the FP1 region of wild type probe affected the formation of complexes 3 and 4 as seen in lane 2. Supershift assays with NERF-2 and Elf-5 antiserum were carried out using the wild type and mutated probes. A higher molecular weight complex is formed with NERF-2 antiserum and both the wild type (lane 3) and m6 probes (lane 4). A similar complex is formed using the Elf-5 antiserum with both wild type (lane 4) and m6 (lane 6) probes.

3.2.1.4 Conclusions

These *in vitro* DNA-protein EMSA studies provided the first direct evidence that a member of the Ets family of transcription factors, and in particular members of the Elf subfamily of Ets factors, may have a role in the rPLII enhancer activity. It is likely that these factors show direct interactions with the GGA sequence in the FP1 region of the rPLII enhancer; this is a core consensus recognition site for Ets factors. It became clear, however, that the identification of the specific Ets protein that functioned in rPLII gene regulation would not be readily identified using EMSA studies alone. A different and more direct approach was needed; such an approach is outlined in **Section 3.4**.

3.3. Characterization of the FP2 Protein Binding Factor

3.3.1. Reexamining formation of an AP1 complex on the FP2 region of the rPLII enhancer element

Based on sequence similarity, the FP2 DNAse I protected region of the rPLII enhancer was previously hypothesized to be an AP1 [*c-jun/c-fos*] DNA binding site (Sun and Duckworth, 1999). *In vitro* synthesized *c-jun/c-fos* dimers had been shown to bind to a 65 bp cloned enhancer fragment in EMSA studies, and addition of a *c-jun* antiserum resulted in the loss of complex formation (Sun and Duckworth, 1999).

Initially the 33bp oligonucleotide (WT33), which contained the complete FP2 region, was used for these studies. DNA-protein reactions were carried out using *in vitro* synthesized *c-jun/c-fos* proteins. Synthesis of each recombinant protein was verified by ^{35}S-methionine labeling (**Figure 3.12**). EMSA studies were carried out to demonstrate interactions with an AP1 consensus-binding site and the WT33 oligonucleotide, which contained the complete FP2 region (**Figure 3.13**). A complex was formed when the consensus AP1 binding site was used as a probe (**Figure 3.13**, lane 4). This complex was supershifted with *c-jun* specific antiserum (lane 3) and competed by excess cold probe (lane 6), confirming that the *in vitro* synthesized *c-fos/c-jun* dimers were able to bind specifically to the consensus sequence. Unexpectedly, given the previously reported results, no complex was formed when the WT33 enhancer oligonucleotide was used as a probe (lane 9). In addition, the WT33 oligonucleotide did not compete complex

formation with the AP1 consensus probe (lanes 7 and 8). When we conducted EMSA studies using the 43 bp oligonucleotide probe (WTFP1-2) with Rcho nuclear extracts and *c-jun* antiserum, no supershift formation was detected (**Figure 3.14**, lanes 2 and 3). Similar results were obtained with the WT33 probe (data not shown).

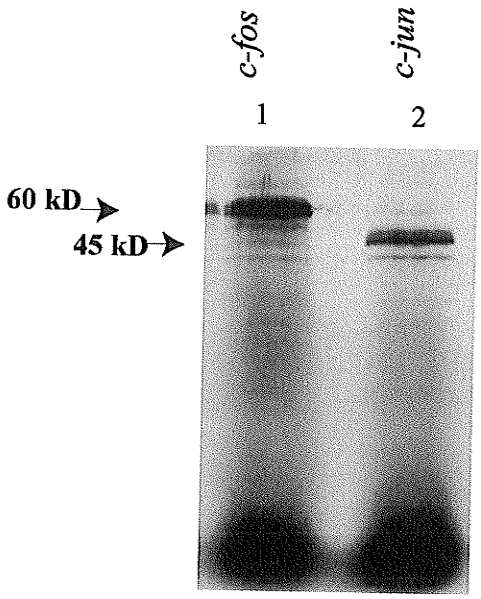


Figure 3.12: *In vitro* recombinant *c-fos* and *c-jun* protein expression.

c-*fos* and c-*jun* cDNA expression clones were transcribed *in vitro* using a TNT reticulocyte e xpression system containing S-35 methionine. Labelled protein was resolved by electrophoresis on a 10% SDS polyacrylamide gel. Expression products were visualized by autoradiography as shown.

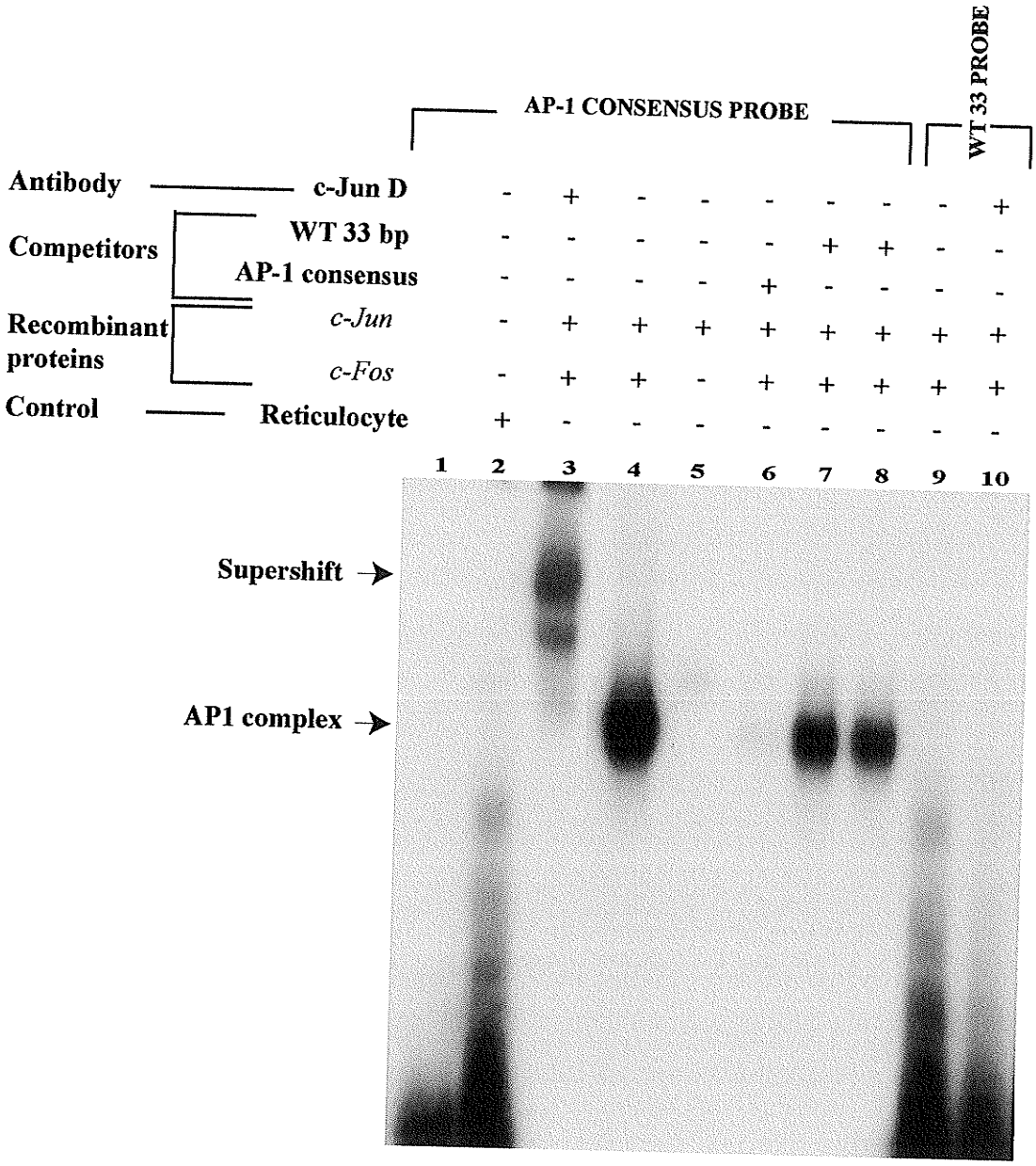

AP-1 CONSENSUS PROBE
WT 33 PROBE
Antibody
c-Jun D
Competitors
WT 33 bp
AP-1 consensus
Recombinant proteins
c-Jun
c-Fos
Control
Reticulocyte
1 2 3 4 5 6 7 8 9 10
Supershift
AP1 complex

Figure 3.13: ***In vitro*** **assessment of AP-1 interaction with a 33 bp rPLII enhancer element.**

EMSA studies were carried out to assess the binding ability of *in vitro* synthesized c-fos and c-jun proteins with a 33 bp enhancer oligonucleotide. To demonstrate specific interactions with the recombinant proteins an AP-1 consensus oligonucleotide was used as a probe (lanes 1 - 8). An AP-1 complex made up of c-fos/c-jun dimers shifted the AP-1 consensus oligonucleotide probe (lane 4); this complex was supershifted with c-jun D antiserum (lane 3). A binding reaction with only c-jun does not form a complex with the consensus probe (lane 5). A 100 fold molar excess of the AP-1 oligonucleotide competes binding (lane 6). The 33 bp rPLII element at 100 and 200 fold molar excess does not compete the AP1 consensus oligonucleotide (lanes 7 and 8). The 33 bp enhancer element does not form a complex with c-fos/c-jun dimers (lanes 9 and 10). Free probe and a reticulocyte lysate control are shown in lanes 1 and 2, respectively.

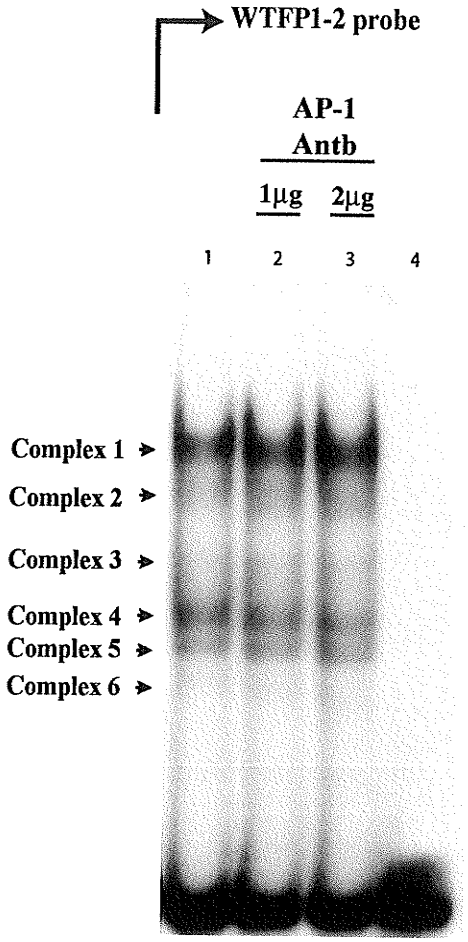


Figure 3.14: EMSA supershift studies to assess AP-1 association with the 43 bp WTFP1-2 oligo.

Six micrograms of Rcho nuclear extract were incubated with the rPLII wild type enhancer oligo WTFP1-2 as a probe. Lane 1 shows the complexes formed with probe and extract only. Lanes 2 and 3 contain 1 and 2 µg respectively of a c-*Jun*-specific antiserum. No supershifts were seen. Free probe is shown in lane 4.

3.3.1.2 Conclusions

The 43 bp oligonucleotide contained all the nucleotides within the DNAse I protected regions, including the putative AP-1 binding site in FP2; it also functioned similarly to the larger cloned fragment as an enhancer in reporter gene expression studies (**Figure 3.2**). Our results suggested that the previously observed *c-fos/c-jun* dimer binding to the cloned 65 bp enhancer may not have occurred within the enhancer sequence itself, but perhaps to a cryptic site within the additional sequence present in the cloned DNA. No EMSA studies were previously carried out using the FP2-mutated 65 bp probe, which would have addressed this possibility. Nonetheless, the loss of enhancing activity following mutation of these specific nucleotides within the FP2 region could only be explained if some or all of these nucleotides were involved in binding the authentic transcription factor. Determining the identity of this factor was the focus of further studies.

3.3.2 Computational Analysis of Potential Transcription Factor Binding Sites in the 43 bp Enhancer Sequence

I compared the 43 bp enhancer sequence to databases containing known transcription factor binding sites using software programs available at three web sites (**Chapter 2, Methods**). No consecutive matches larger than 4 bp were found anywhere within the complete sequence; five transcription factor binding sites showed nucleotide

matches within regions of the enhancer in which mutations had produced a loss in enhancing activity (**Figure 3.2** and Sun and Duckworth, 1999). One of these factors, Ets-1, had a binding site similar to the FP1 region. Previous experiments had shown that Ets-1 did not bind or trans-activate the rPLII enhancer (Y. Sun, unpublished data). Examination of the FP2 region revealed a partial sequence in common to binding sites associated with a number of factors including AP1, a zinc finger protein, ZID (Huynh and Bardwell, 1998), CCAAT/enhancer-binding protein-β (C/EBP-β) (Toda and Shzuta, 1996; Chen and Chou, 1995; Chen and Liu, 2000; Bamberger and Löning, 2004), and the PAX2/5/8 group of transcription factors (Van Renterghem and Christophe, 1996; Kozmik and Busslinger, 1993).

Of these potential binding factor candidates, both C/EBP-α and -β are expressed in human and mouse placenta, and binding sites have been described in the promoters of a number of placentally-expressed genes (Begay *et al.*, 2004; Bamberger *et al.*, 2004). To test whether a C/EBP factor might bind the rPLII enhancer element, EMSA studies were carried out using the 43 bp oligonucleotide with Rcho nuclear extracts and a C/EBP rabbit polyclonal antiserum that recognized both C/EBP-α and -β. Double stranded 20 bp and 26 bp C/EBP consensus oligonucleotides were also used in EMSA studies to demonstrate the presence of C/EBP proteins in placental cells and to confirm C/EBP antiserum-protein interactions (**Chapter 2, Methods, Table 2.1**).

As shown in **Figure 3.15**, both C/EBP consensus sequences formed weak complexes with endogenous nuclear factors from Rcho cells (lanes 5 and 7). Specific

interaction of the C/EBP proteins with the consensus elements was verified using the C/EBP specific antiserum (lanes 6 and 8), which supershifted the complex. The weakness of the complex formation may be due to the amount of C/EBP present in the Rcho nuclear extracts. None of the complexes formed with the 43 bp rPLII enhancer oligonucleotide were supershifted by the C/EBP antiserum (lane 2) or by rabbit IgG, which was utilized as a control for non-specific interaction (lane 10). Neither C/EBP consensus binding site oligonucleotide competed with the 43 bp probe (data not shown). We concluded that although C/EBP proteins were present in Rcho cells, this family of transcription factors did not bind the FP2 region.

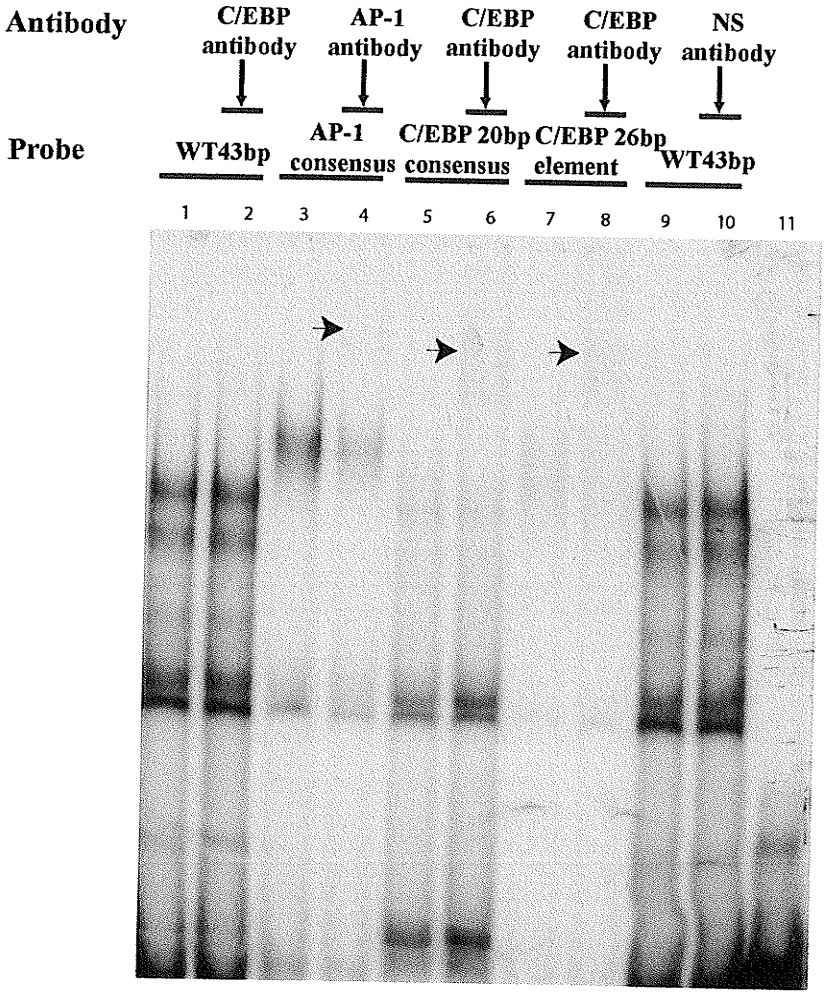


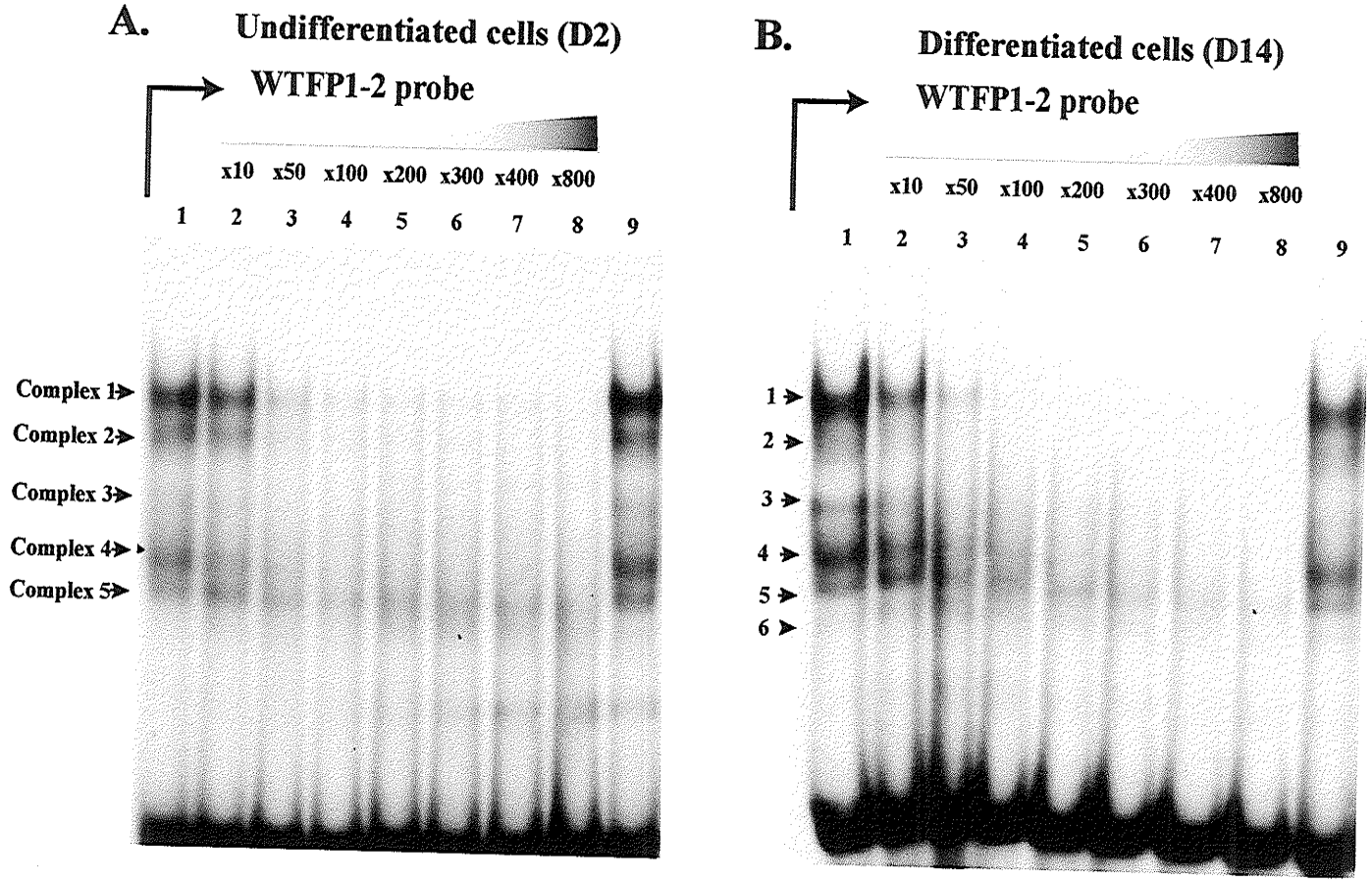
Figure 3.15: Identification of endogenous C/EBP and AP1 proteins and analysis of their association with the enhancer element in EMSA studies.

A 20 bp C/EBP consensus oligo, a 26 bp C/EBP consensus binding element, AP1 consensus oligo and the rPLII 43 bp wild type enhancer element were used as probes in EMSA with Rcho nuclear extracts. Lanes 1, 3, 5, 7, 9 show the interactions between each probe as indicated. C/EBP antiserum (lanes2, 6 and 8) and *c-Jun* antiserum (lane 4) were added to the binding reactions to determine specificity of the interactions. The supershifted complexes are indicated by arrows. Weak supershifts are seen in reactions with the consensus oligos, but not with the rPLII enhancer oligo. Non - immune IgG serum was used as a control (lane 10). Free unbound probe is shown in lane 11.

3.3.3 Defining Core DNA Binding Motifs in the rPLII Enhancer Element

3.3.3.1 Characterization of DNA-protein complexes formed with the 43 bp oligonucleotide

To characterize in detail the nuclear protein complexes formed with the complete enhancer element, EMSA studies were carried out using the wild type 43bp oligonucleotide (WTFP1-2) as a probe with nuclear extracts from the Rcho cell line and rat placental tissue. To test whether there might be differences between cells that highly expressed rPLII and those which did not, Rcho nuclear extracts were made from cultures grown for 14 days (D14), which contained rPLII-expressing trophoblast giant cells, and from freshly plated cultures that contained mainly rapidly dividing, undifferentiated trophoblast cells (D2). Unlabelled 43 bp oligonucleotide was used as a competitor at 10 to 800 fold molar excess. Results for the Rcho cell extracts are shown in **Figure 3.16A** and **B**. Five distinct complexes were seen, of which complexes 1 to 4 were specifically competed by excess cold probe (lanes 2 to 8). The complexes formed were similar between differentiated and undifferentiated cultures, suggesting that the factors that bound the enhancer element were also present in trophoblast cells prior to giant cell differentiation. Four less well-defined complexes were formed with day 17 nuclear extract from rat placental tissue (**Figure 3.16C**); complexes 1 and 2 were similar to those formed with Rcho nuclear extracts, but weaker; complexes 3 and 4 were very strong and appeared to be specific, based on competition by cold probe, but it was difficult to directly compare these with complexes 3 and 4 formed with Rcho nuclear extracts.


A.
Undifferentiated cells (D2)
WTFP1-2 probe
x10 x50 x100 x200 x300 x400 x800
1 2 3 4 5 6 7 8 9
Complex 1
Complex 2
Complex 3
Complex 4
Complex 5
B.
Differentiated cells (D14)
WTFP1-2 probe
x10 x50 x100 x200 x300 x400 x800
1 2 3 4 5 6 7 8 9
1
2
3
4
5
6

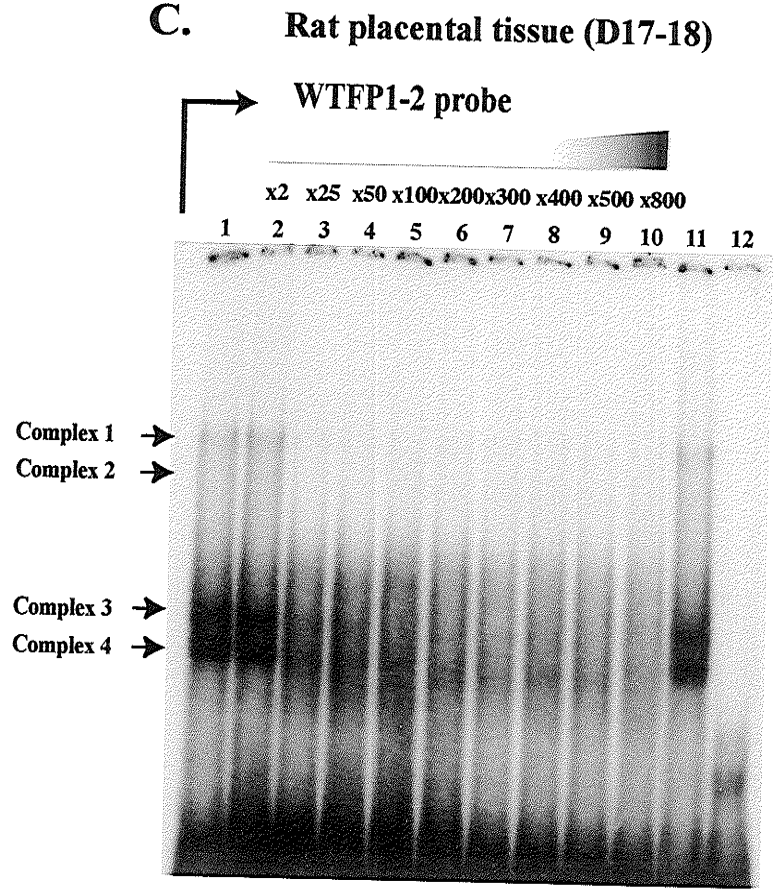

C.
Rat placental tissue (D17-18)
WTFP1-2 probe
x2 x25 x50 x100 x200 x300 x400 x500 x800
1 2 3 4 5 6 7 8 9 10 11 12
Complex 1
Complex 2
Complex 3
Complex 4

Figure 3.16: EMSA comparison of the DNA-protein complexes formed with differentiated and undifferentiated Rcho cells and rat placenta.

The WTFP1-2 oligonucleotide was used as a probe with nuclear extracts from undifferentiated (day 2) and differentiated (day 14) Rcho cells **(A and B)** and day 17/18 rat placental **(C)**. Complexes formed with each of these extracts are shown in lanes 1 and 9 of panel **A**, lanes 1 and 11 of panel **C**. To identify specific complexes, unlabelled probe was added as a competitor at the molar excess concentrations indicated. Rcho complexes 1 to 4 appear to be specific. The complexes formed with the two Rcho extracts are similar although complexes 3 and 4 are less intense with the undiffereniated cells **(A)**. The individual complexes formed with the placental extracts may not be exactly equivalent with those of the Rcho cells.

3.3.3.2. Mutational analysis defines FP1 and FP2 core binding site nucleotides

I had not been able to identify the FP2 binding factor using transcription factor database searches. Thus, it was decided to define more precisely the core nucleotides within the FP2 region that were involved in protein binding, with the hope of providing further information that would allow identification of the factor. EMSA studies were carried out using a series of 43 bp enhancer oligonucleotides with 4 bp nucleotide changes across the FP2 region with or without an altered GGA sequence in the FP1 region. These oligonucleotidess are shown in **Figure 3.17A**. Results in which mutated oligonucleotides were used as competitors for the wild type probe WTFP1-2 are shown in **Figure 3.17B**.

As shown previously, the 43 bp WTFP1-2 oligonucleotide competed complexes 1 to 4 (lane 2). The M2 oligonucleotide (lane 4) was also able to compete these complexes, suggesting that the nucleotides in these positions within the FP2 region were not involved in a protein-DNA interaction. When mutant oligonucleotides M3 (lane 6), M4 (lane 8) and M5 (lane 10) were used, competition was seen primarily with complexes 3 and 4. Conversely, the m6 oligonucleotide with an altered GGA in the FP1 region competed complexes 1, 2 and 4, but not 3 (lane 3). When the M3, M4, M5 mutations were combined with the m6 mutation, no complexes were competed (lanes 5, 7, 9 and 11). These results suggested that M3, M4, M5 associated nucleotides were involved in complex 1 and 2 assembly. The nucleotide changes within the FP1 region did affect these complexes.

A.

WT	5′	CCA GGGTTATTTGCTCAAGGGTA AACAGGAAGTAGGGCTT GAA 3′
		FP2 **FP1**

Oligos including sequential mutations in FP2 site:

M2	5′	CCAGGGT gccg TGCTCAAGGGTAAACAGGAAGTAGGGCTTGAA 3′
M3	5′	CCAGGGTTATT ctac CAAGGGTAAACAGGAAGTAGGGCTTGAA 3′
M4	5′	CCAGGGTTATTTGCT acgt GGTAAACAGGAAGTAGGGCTTGAA 3′
M5	5′	CCAGGGTTATTTGCTCAAG tcgc AACAGGAAGTAGGGCTTGAA 3′

Oligos including sequential mutations in FP2 site and mutation of GGA-Ets core in FP1 site:

m6	5′	CCAGGGTTATTTGCTCA AGGGTAAACA CTG AGTAGGGCTTGAA 3′
M2m6	5′	CCAGGGT gccg TGCTCAAGGGTAAACA CTG AGTAGGGCTTGAA 3′
M3m6	5′	CCAGGGTTATT ctac CAAGGGTAAACA CTG AGTAGGGCTTGAA 3′
M4m6	5′	CCAGGGTTATTTGCT acgt GGTAAACA CTG AGTAGGGCTTGAA 3′
M5m6	5′	CCAGGGTTATTTGCTCAAG tcgc AACA CTG AGTAGGGCTTGAA 3′

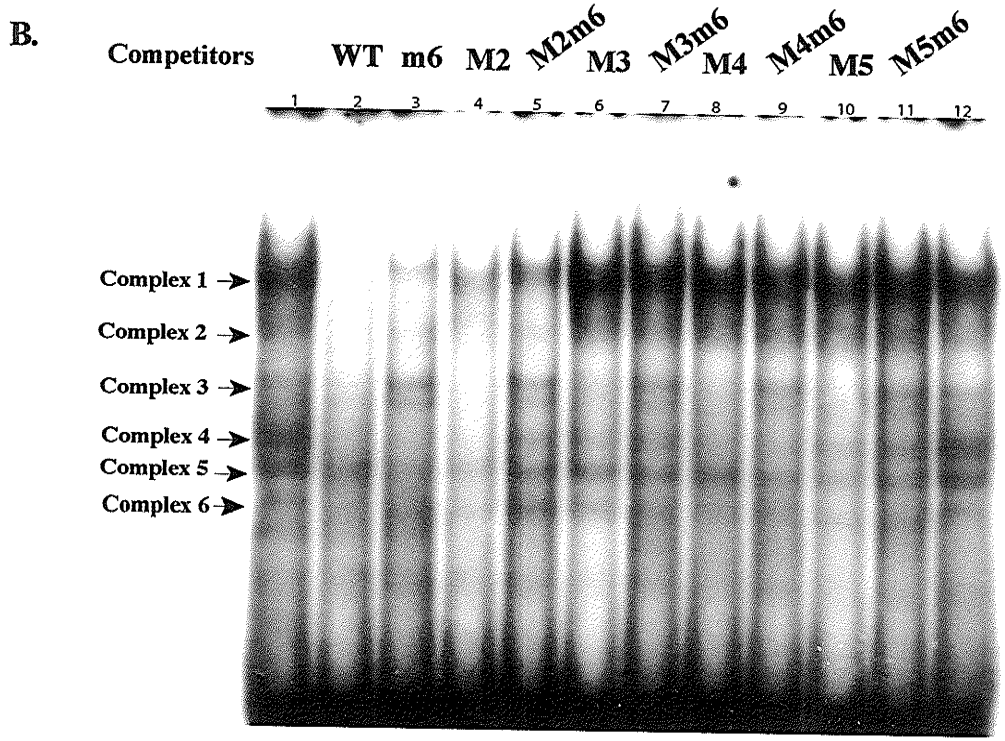


Figure 3.17: Competition of WTFP1-2 complexes by oligonucleotides mutated in the FP1 and FP2 regions of the rPLII enhancer.

(**A**) Oligonucleotides containing four nucleotide changes within the FP2 region of the rPLII enhancer and a GGA change in FP1. Changes are shown in colour and lower case (FP2). (**B**) The WTFP1-2 oligonucleotide was used as a probe in EMSA studies and competition was carried out using WTFP1-2 (lane 2) or mutated oligonucleotides, as indicated, at 200 fold molar excess (lanes 3 to 11). Lanes 1 and 12 show complexes formed with WTFP1-2. Complexes 1 and 2 appear to be associated with specific nucleotides in the FP2 region; complexes 3 and 4 are affected by the m6 mutation.

EMSA results using these mutant oligonucleotides as probes are shown in **Figure 3.18**. Oligonucleotides with individual mutations showed binding results that were consistent with the competition studies. The M3, M4 and M5 oligonucleotides did not form complexes 1 and 2 (lanes 5, 8 and 10) and the m6 oligonucleotide did not form complexes 3 and 4 (lane 2). When the M3 to M5 FP2 mutations were combined with the m6 FP1 mutation there appeared to be a more complete loss of complexes 1 and 2, perhaps suggesting a role for protein-protein interactions in stabilizing complex formation. Surprisingly, complexes 5 and 6 appeared to become stronger when the M4m6 and M5m6 oligonucleotides were used as probes (lanes 9 and 11). Since the individual mutations affected the formation of the known complexes, these stronger complexes may be an artifact, perhaps reflecting the formation of a new or more robust protein binding site as a result of the combined nucleotide changes. Together these data suggested that the formation of complexes 3 and 4 required the GGA triplet found in the FP1 site and that complexes 1 and 2 resulted from proteins binding to nucleotides within a 12 bp region as defined by the M3, M4 and M5 mutations (**Figure 3.17**).

Although these EMSA experiments helped to define the key binding residues within the FP2 region more precisely, and confirmed the importance of the GGA sequence in the FP1 region, this additional information was still not sufficient to identify the rPLII enhancer binding proteins based on transcription factor binding site database information.

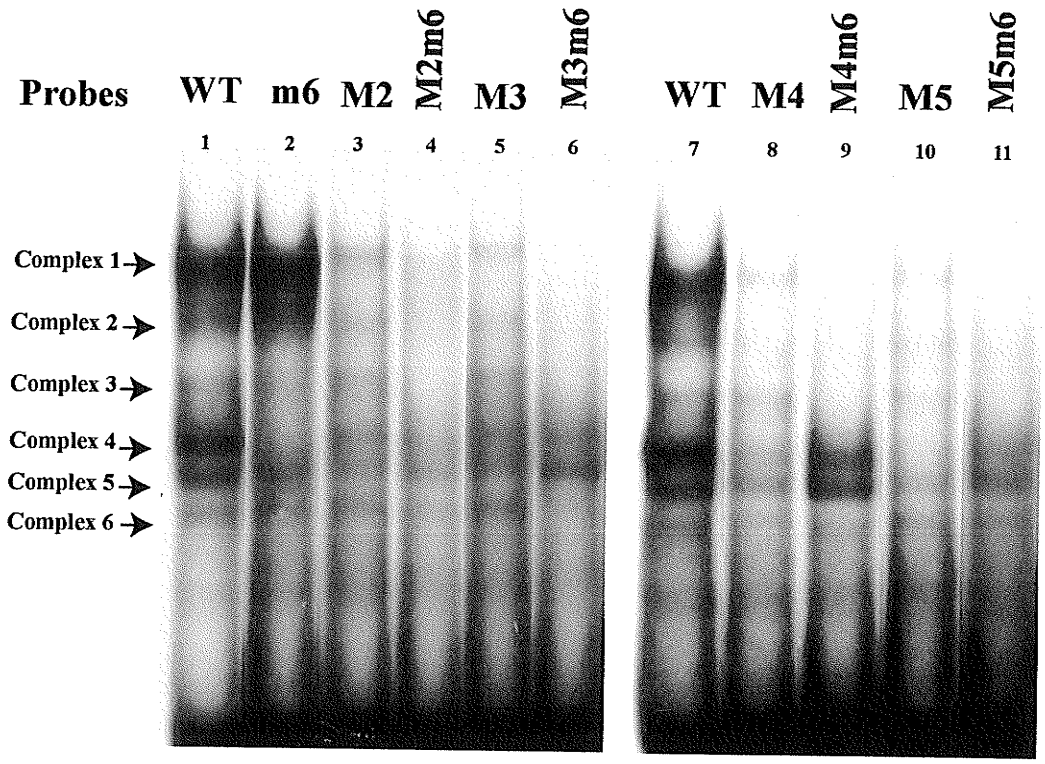


Figure 3.18: EMSA studies using mutated FP1 and FP2 oligonucleotides as probes identify FP specific complexes.

The mutated oligonucleotides are those described in Figure 3.17. Oligonucleotides were labelled and used as probes in EMSA studies with differentiated Rcho nuclear extract. The specific probe is indicated above each lane. As in the competitor EMSA studies, the m6 mutation affects the formation of complexes 3 and 4; mutations M3, M4, and M5 have a marked effect on complexes1 and 2. Double mutations affect M3m6 - M4m6 have a marked effect on the formation of complexes 1 through 3.

3.4. Proteomic Identification of Candidate rPLII Enhancer-Binding Factors

3.4.1. Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS):

We decided to use a direct, unbiased proteomics approach to isolate and identify the protein factors that bound the rPLII enhancer element. Proteomics can be defined as the identification and study of the structure and function of proteins, including how they work and interact with each other. Proteomics utilizes a powerful set of technologies among which mass spectrometry (MS) has become an important recent addition (Park and Russell, 2001; Shevchenko *et al.*, 2001; Bauer and Kuster, 2003; Steen and Mann, 2004). Our choice of matrix assisted laser desorption ionization (MALDI) time of flight (TOF) mass spectrometry for the identification of the rPLII enhancer binding proteins was based on two innovations developed in the University of Manitoba Time of Flight Laboratory, Department of Physics and Astronomy. One was the availability of a state-of-the-art prototype Manitoba-Sciex quadrupole time of flight (Qq-TOF) instrument; the other was the development of off-line coupling of high performance liquid chromatography (HPLC) to MALDI MS (Verhaert *et al.*, 2001; Krokhin *et al.*, 2003). The combination of a highly sensitive instrument that could detect less than 100 femtomoles of peptide and a means of coupling this instrument to an HPLC system that could simplify small amounts of complex protein mixtures without significant losses, was crucial for experiments in which only very small amounts of material would be available for analysis. Our collaborators for these experiments were Dr. Lynda Donald and Dr.

Harry Duckworth from the Department of Chemistry, University of Manitoba. A schematic diagram of the instrument used in these studies is shown in **Figure 3.19**.

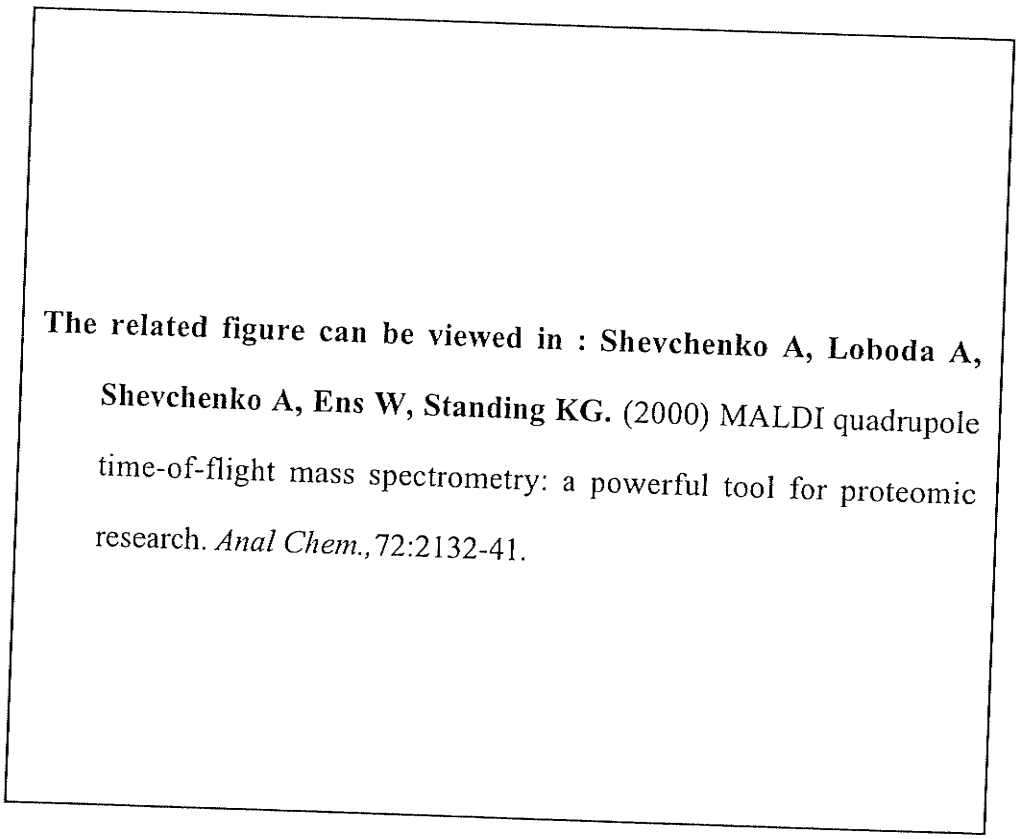
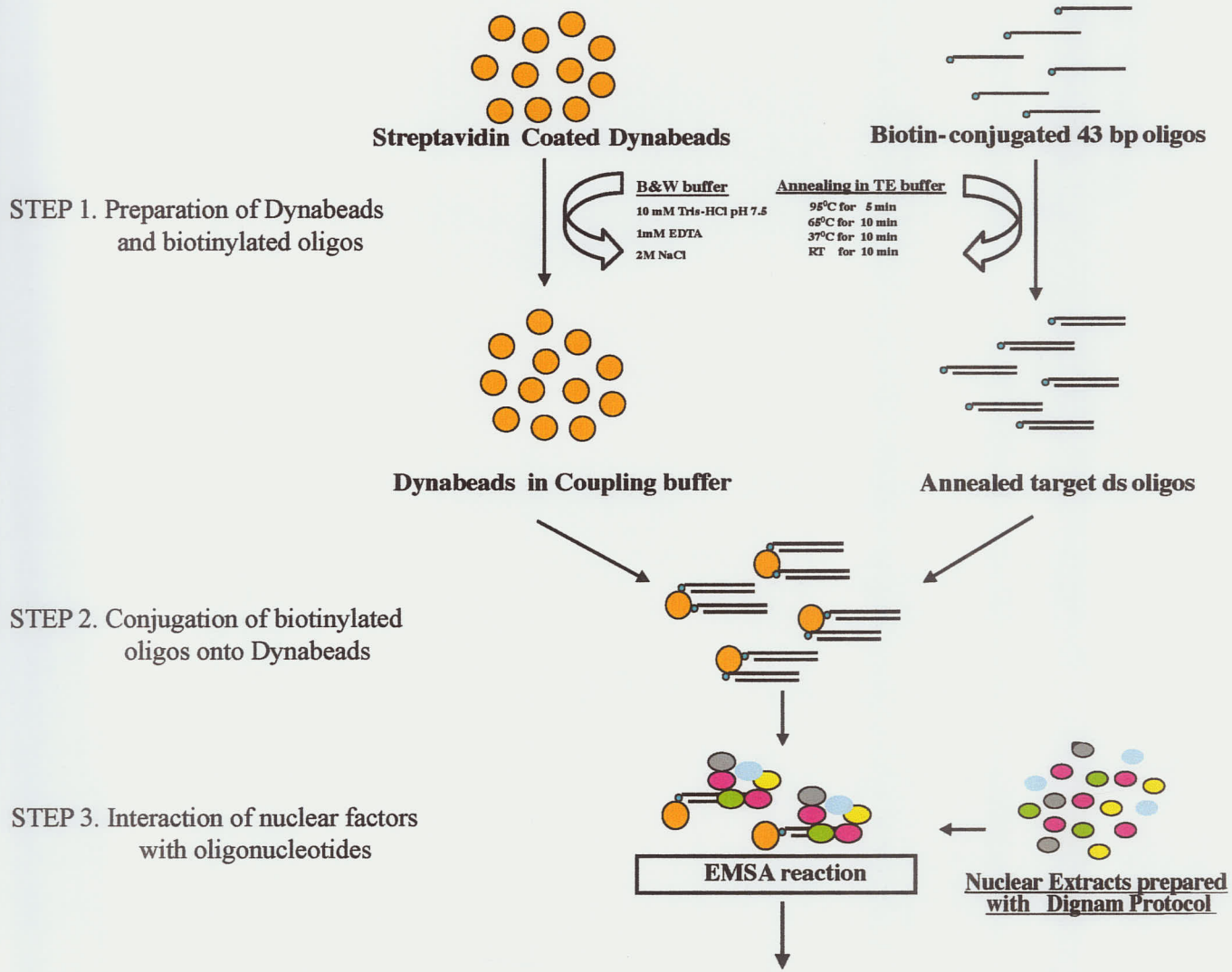


Figure 3.19: MALDI quadrupole time-of-flight mass spectrometry.

3.4.2. rPLII enhancer binding protein selection

The experimental procedures used for the binding factor selection, purification, trypsin digestion and MS analysis are outlined in detail in the Methods and illustrated in **Figure 3.20**. Our method differed from similar previous approaches (Zimber-Strobl and Furukawa, 1994; Nordhoff and Kristiansen, 1999) in that it used HPLC to separate peptides before MS analysis instead of an SDS-polyacrylamide gel that requires much larger amounts of protein. Briefly, double-stranded biotinylated 43 bp WTFP1-2 oligonucleotide was bound to paramagnetic streptavidin coated Dynabeads. The oligonucleotide-bead mixture was incubated with nuclear extract from Rcho cells in EMSA binding buffer, followed by washes in binding buffer with and without the general competitor, poly dI/dC, and finally in 0.1 M NH_4Cl. Several trials were carried out before the final procedure, as outlined, was established. Volumes of reactions and washes were limited because of the size of the magnetic stand used to collect the beads after each procedure. This made it necessary to pool several reaction mixtures to ensure that enough bound protein was available for analysis. Washes in EMSA buffer with and without poly dI/dC were important for removal of non-specifically bound proteins; washes in NH_4Cl were initially carried out to remove salt and other components of the EMSA buffer that would interfere with the MALDI MS. Although this became less important once the HPLC step was introduced this wash was kept in the final protocol. A further innovation in our method was the trypsin digestion of bound protein without prior removal from the beads. Although this meant the presence of trypsin and streptavidin peptides in the mixtures, this approach also ensured that no proteins would be left on the beads from

inadequate elution procedures. A portion of each tryptic digest was separated by HPLC and samples were deposited automatically at one minute intervals onto a MALDI target (Loboda *et al.*, 2000). This methodology allowed the entire sample to be analyzed by MS without loss of material.



STEP 1. Preparation of Dynabeads and biotinylated oligos

STEP 2. Conjugation of biotinylated oligos onto Dynabeads

STEP 3. Interaction of nuclear factors with oligonucleotides

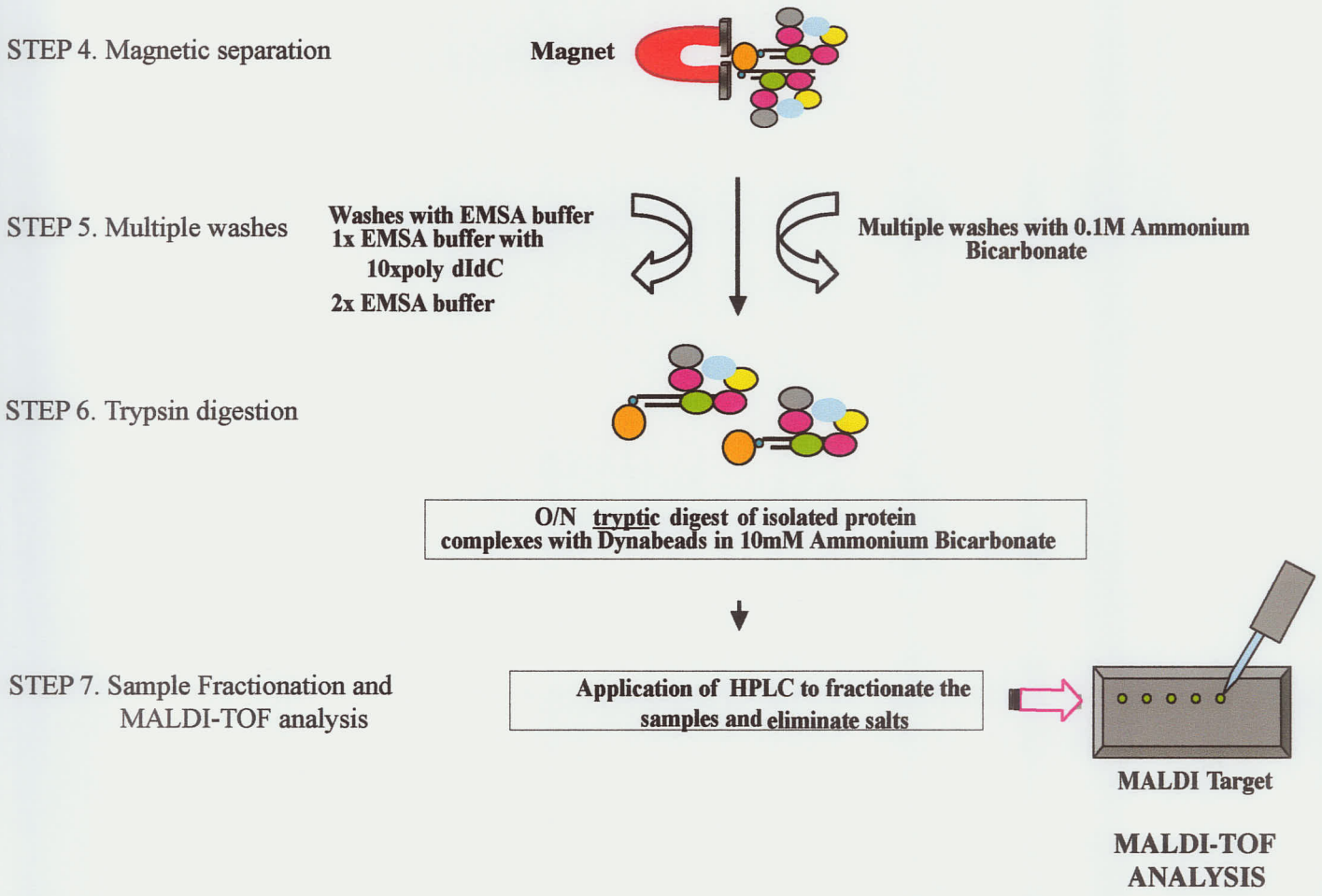


Figure 3.20: Experimental procedure for the preparation of binding factors for MALDI-TOF MS analysis

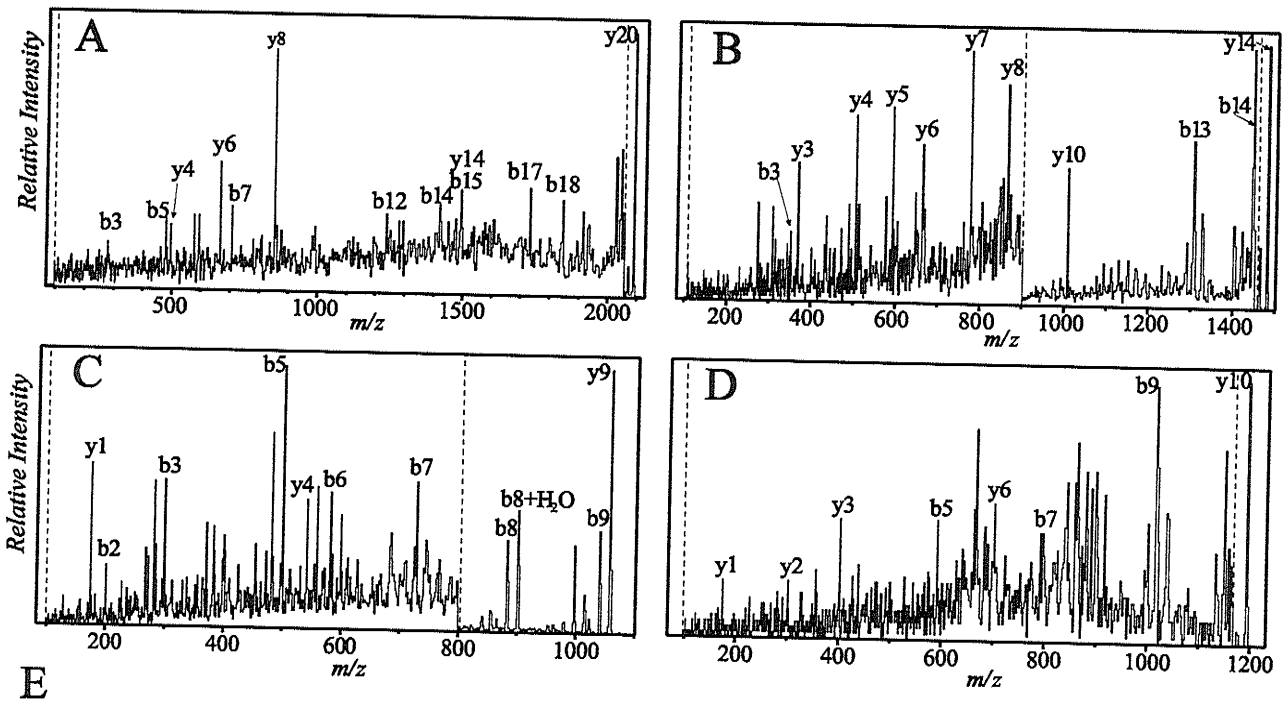
3.4.3. MALDI-TOF MS analysis of rPLII enhancer binding proteins

Dr. Donald carried out HPLC separation of the tryptic peptides, collection and analysis of MS spectra. Initial analysis involved automatic peptide mass matching of the MS spectra with known proteins in the National Center for Biotechnology Information Genbank and SwissProt protein databases using the "ProFound" program. The rat protein databases were poor at the time this work was started; to maximize the probability of identifying mass matches, the human and mouse proteomes were included in our database searches. As a result of our selection procedure, the samples were known to contain tryptic peptides derived from streptavidin, bovine serum albumin (present in the magnetic bead storage buffer), and trypsin itself, those peptides were identified and removed from the data files manually. Dr. Donald provided lists of the statistically best mass matches to screen for likely candidates.

Tandem MS (MS/MS) was also carried out on ions that were present in sufficient quantity to be further fragmented. The set of m/z "daughter" ions generated from a specific "parent" ion allowed peptide sequence to be inferred, giving a greater confidence in the protein identification than simple mass matching. Manual assignment and "Sonar" and "Global Proteomics Machine" software (http://www.thegpm.org) were used to match these sequences with known proteins in databases (Craig and Beavis, 2004).

3.4.4. Identification of AP2γ Peptides

A Sonar analysis of MS/MS spectra initially identified one precursor ion, from HPLC fraction 27 at *m/z* 2087.159, as a human and mouse AP2γ peptide. The MS/MS spectrum of this peptide is shown in **Figure 3.21**. As shown in **Figure 3.22**, the b and y daughter ions formed by fragmentation of this parent ion matched well with the expected residues 295-314 of the mouse AP2γ (Genbank Q61312) and residues 296-315 of the human AP2γ (Genbank Q92754). Although five different AP2 family members have been identified (Williams *et al.*, 1988; Williams and Tjian, 1991; Buettner *et al.*, 1993; Moser *et al.*, 1993; Meier *et al.*, 1995; Bosher *et al.*, 1996; Chazaud *et al.*, 1996; McPherson *et al.*, 1997; Li *et al.*, 2000; Zhao *et al.*, 2001; Tummala *et al.*, 2003; Feng and Williams, 2003), peptide assignment could be made unequivocally to AP2γ, based on the presence of a histidine residue in the third position of this peptide [AAHVTLLTSLVEGEAVHLAR]. The residue at this position is an asparagine in human and mouse AP2α, and AP2β (**Figure 3.23**). At the time of this identification the rat AP2γ was not in the databases. Dr. H. Duckworth used the BLAST program to compare the mouse AP2γ cDNA sequence with the rat genome sequence and identified a partial rat AP2γ coding region that contained the same amino acid sequence as the human and mouse proteins. Subsequent annotation of the rat AP2γ cDNA and protein in Genbank databases (NM_201420 and NP_958823 respectively) have confirmed the identification of this peptide in the rat AP2 γ protein sequence (**Figure 3.23**).

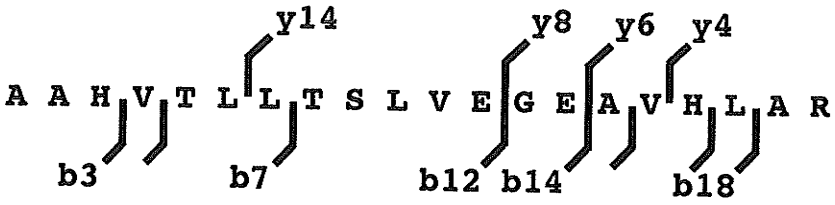


E

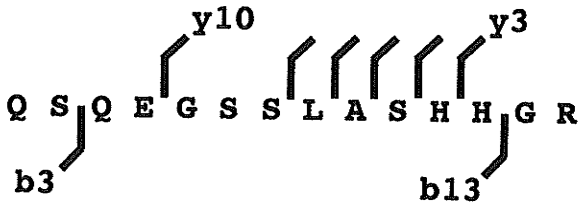
1 mlwkitdnvk yeedcedrhd assngnprip hlssagqhly spapplshtg vaeyqpppyf
61 pppyqqlays qsadhyshlg eayaaainpl hqpaptgsqq qawpgr**qsqe gsslashhgr**
121 **sagliphisg legsavssrr** eayrrsdlll phahaleagl aenlglhema hpieevqnvd
181 dphlllhdqt virk**gpismt knplglpcq**k dlvgvvmnps evfcsvpgrl sllsstsk**yk**
241 **vtvaevqrr**l sppeclnasl lggvlrraks knggrslrek **ldkiglnlpa grrkaahvtl**
301 **ltslvegeav hlar**dfayvc eaefpskava dyltrphlgg rnemaarksv llaaqqvck**e**
361 **ftdllhqdr**t pngnsrptpv letniqncls hfslithgfg sqaicaavsa vqnyikeali
421 tidksymnpg kqspadsnkt mekmekhrk

Figure 3.21: MALDI TOF mass spectrometry identifies AP2γ as an rPLII enhancer binding protein.

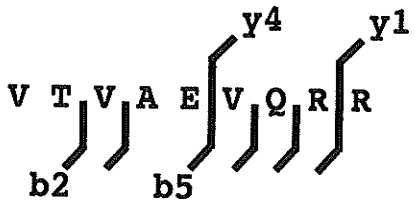
Tandem mass spectra of four peptides that were identified by database searches as rat AP2γ are shown in panels A to D. A, Parent ion at m/z 2087.159 matched amino acids 295-314 of the mouse, 296-315 of the human and 295-315 of the rat AP2γ proteins. B, Parent ion m/z 1480.693 matched amino acids 107-120 of rat AP2γ; C, Parent ion m/z 1057.623 matched amino acids 242-250 of rat AP2γ; D, Parent ion m/z 1192.668 match amino acids 240-249 of rat AP2γ. Dotted lines on the spectra indicate a change in intensity scale. E, shows the locations of all tryptic peptides identified by mass matching in bold; peptidessequenced by MS/MS are shown as shaded boxes. The underlined histidine at amino acid 297 identifies the protein as AP2γ. Other known members contain an asparagine at a comparable location.



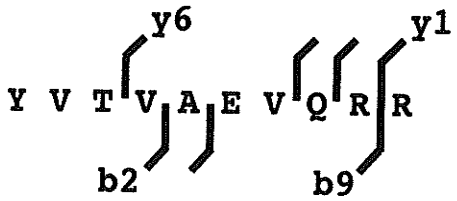
A. Precursor ion m/z 2087.159 (2087.161) from HPLC fraction 27. The ion pattern matches well with that expected from residues 295-314 of mouse (Swiss Prot Q61312), 296-315 of human (SwissProt Q92754), and rat transcription factor AP-2γ.



B. Precursor ion m/z 1480.693 (1480.689) from HPLC fraction 11. The fragmentation patternfits with that expected from residues 107-120 of the rat AP-2γ.



C. Precursor ion m/z 1057.623 (1057.612) from HPLC fraction 13. The ions are a reasonable match to those expected from fragmentation of the peptide with residues 242-250 of rat AP2γ.



D. Precursor ion m/z 1192.668 (1192.668) from HPLC fraction 15. The ions match those expected for the peptide 240-249 of rat AP2γ.

Figure 3.22: Analysis of the MS/MS data that identified rat AP2γ.

Selected MS peaks of sufficient intensity were selected for analysis by MS/MS. The fragments were compared to protein databases and peptide identities were confirmed by manual examination. The numbers in brackets represent the expected masses of these peptides.

A.

```
                 301          315 316          330 331          345
AP2α        EKLDKIGLNLPAGRR KAANVTLLTSLVEGE AVHLARDFGYVCETE
AP2β        ERLEKIGLNLPAGRR KAANVTLLTSLVEGE AVHLARDFGYICETE
AP2γ        EKLDKIGLNLPAGRR KAAHVTLLTSLVEGE AVHLARDFAYVCEAE
```

B.

```
                 106          120 121          135 136          150
AP2α        -----QPQHPGWPGQ RQSQESG-LLHTHRG LPHQLSGLDPRRDYR
AP2β        -----QHPWGQRQRQ EVGSEAGSLLPQPRA ALPQLSGLDPRRDYH
AP2γ        PTGSQQQAWPGRQSQ EGSSLAS-HHGRSAG LIPHISGLEGSAVSS
```

C and D.

```
                 256          270    271          285
AP2α        SSTSKYKVTVAEVQR    RLSSPECLNASLLGG
AP2β        SSTSKYKVTVGEVQR    RLSPPECLNASLLGG
AP2γ        SSTSKYKVTVAEVQR    RLSPPECLNASLLGG
```

Figure 3.23: Comparison of the MS - identified rat AP2γ peptide sequences with rat AP2α and AP2β peptides.

A to **D** refer to the MS/MS peptides shown in the panels with these letters in **Figure 3.21.** The AP2γ peptides identified by MS/MS are shown in pink boxes. Related peptides in AP2α and AP2β proteins are shown in yellow boxes. The AP2γ peptide in **A** shows the underlined histidine at position 3, which is an asparagine in the other proteins. The peptide in **B** is also unique for AP2γ. The overlapping peptides **C** and **D** are conserved in all the proteins.

Three further ions that were sequenced by MS/MS were identified as being from rat AP2γ. Spectra for these ions and the identified b and y fragmentation ions from the parent ions are shown in **Figure 3.21**. Precursor ion *m/z* 1480.693 from HPLC fraction 11 is unique to rat AP2γ, while precursor ions *m/z* 1057.623 and 1192.668, from fractions 13 and 15 respectively, (**Figure 3.22**) represent overlapping peptides that are conserved in all known AP2 family members across species. A comparison of the AP2γ proteins from rat, mouse and human is shown in **Figure 3.24**. Mass matching analysis of spectra to the most recent rat databases, using Profound, has now identified tryptic peptides covering 26.8% of the rat AP2γ sequence. A complete list of the AP2γ peptides identified by mass matching and MS/MS are shown in **Table 3.1** and are noted within the rat AP2γ sequence in **Figure 3.21**.

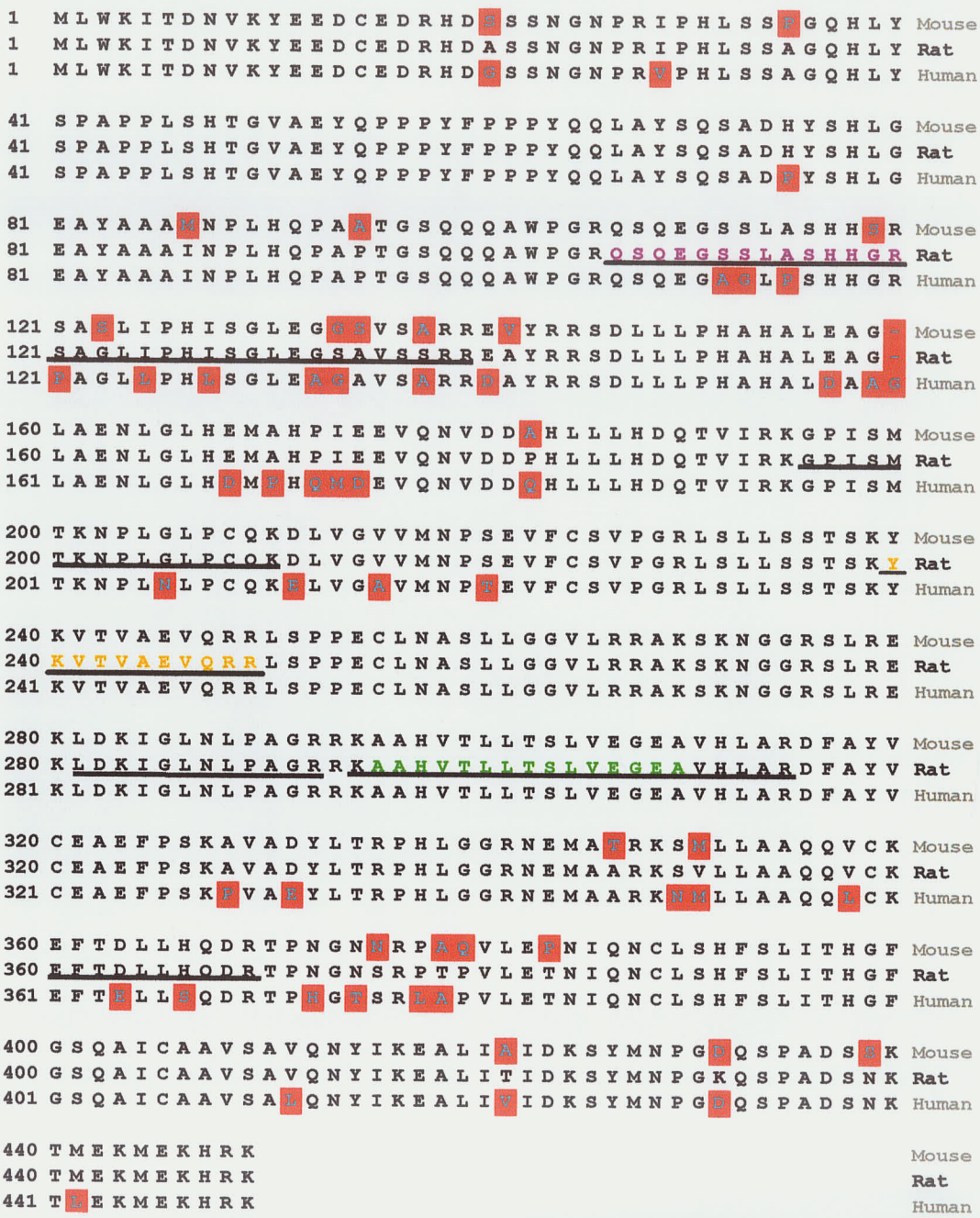

1 MLWKITDNVKYEEDCEDRHDGSSNGNPRIPHLSSFGQHLY Mouse
1 MLWKITDNVKYEEDCEDRHDASSNGNPRIPHLSSAGQHLY Rat
1 MLWKITDNVKYEEDCEDRHDGSSNGNPRVPHLSSAGQHLY Human
41 SPAPPLSHTGVAEYQPPPYFPPPYQQLAYSQSADHYSHLG Mouse
41 SPAPPLSHTGVAEYQPPPYFPPPYQQLAYSQSADHYSHLG Rat
41 SPAPPLSHTGVAEYQPPPYFPPPYQQLAYSQSADFYSHLG Human
81 EAYAAAMNPLHQPAATGSQQQAWPGRQSQEGSSLASHHGR Mouse
81 EAYAAAINPLHQPAPTGSQQQAWPGRQSQEGSSLASHHGR Rat
81 EAYAAAINPLHQPAPTGSQQQAWPGRQSQEGAGLFSHHGR Human
121 SASLIPHISGLEGGSVSARREVYRRSDLLLPHAHALEAG- Mouse
121 SAGLIPHISGLEGSAVSSRREAYRRSDLLLPHAHALEAG- Rat
121 FAGLLPHLSGLEAGAVSARRDAYRRSDLLLPHAHALDAAG Human
160 LAENLGLHEMAHPIEEVQNVDDAHLLLHDQTVIRKGPISM Mouse
160 LAENLGLHEMAHPIEEVQNVDDPHLLLHDQTVIRKGPISM Rat
161 LAENLGLHDMFHQMDEVQNVDDQHLLLHDQTVIRKGPISM Human
200 TKNPLGLPCQKDLVGVVMNPSEVFCSVPGRLSLLSSTSKY Mouse
200 TKNPLGLPCQKDLVGVVMNPSEVFCSVPGRLSLLSSTSKY Rat
201 TKNPLNLPCQKDLVGAVMNPTEVFCSVPGRLSLLSSTSKY Human
240 KVTVAEVQRRLSPPECLNASLLGGVLRRAKSKNGGRSLRE Mouse
240 KVTVAEVQRRLSPPECLNASLLGGVLRRAKSKNGGRSLRE Rat
241 KVTVAEVQRRLSPPECLNASLLGGVLRRAKSKNGGRSLRE Human
280 KLDKIGLNLPAGRRKAAHVTLLTSLVEGEAVHLARDFAYV Mouse
280 KLDKIGLNLPAGRRKAAHVTLLTSLVEGEAVHLARDFAYV Rat
281 KLDKIGLNLPAGRRKAAHVTLLTSLVEGEAVHLARDFAYV Human
320 CEAEFPSKAVADYLTRPHLGGRNEMATRKSMLLAAQQVCK Mouse
320 CEAEFPSKAVADYLTRPHLGGRNEMAARKSVLLAAQQVCK Rat
321 CEAEFPSKPVAEYLTRPHLGGRNEMAARKNMLLAAQQLCK Human
360 EFTDLLHQDRTPNGNMRPAQVLEPNIQNCLSHFSLITHGF Mouse
360 EFTDLLHQDRTPNGNSRPTPVLETNIQNCLSHFSLITHGF Rat
361 EFTELLSQDRTPHGTSRLAPVLETNIQNCLSHFSLITHGF Human
400 GSQAICAAVSAVQNYIKEALIAIDKSYMNPGDQSPADSSK Mouse
400 GSQAICAAVSAVQNYIKEALITIDKSYMNPGKQSPADSNK Rat
401 GSQAICAAVSALQNYIKEALIVIDKSYMNPGDQSPADSNK Human
440 TMEKMEKHRK Mouse
440 TMEKMEKHRK Rat
441 TLEKMEKHRK Human

Figure 3.24: Comparison of the mouse, rat and human AP2γ sequences.

The mouse, rat and human AP2γ proteins sequences are shown. (Genbank Q61312, NP_003222 and NP_958823 respectively). The rat peptides identified by MALDI MS are underlined. The rat peptides that were sequenced by MS/MS are shown in various colours. The boxed amino acids represent residues that are different from those in the rat AP2γ sequence.

TABLE 3.1. Rat AP2γ peptides identified by mass matching and tandem mass spectrometry

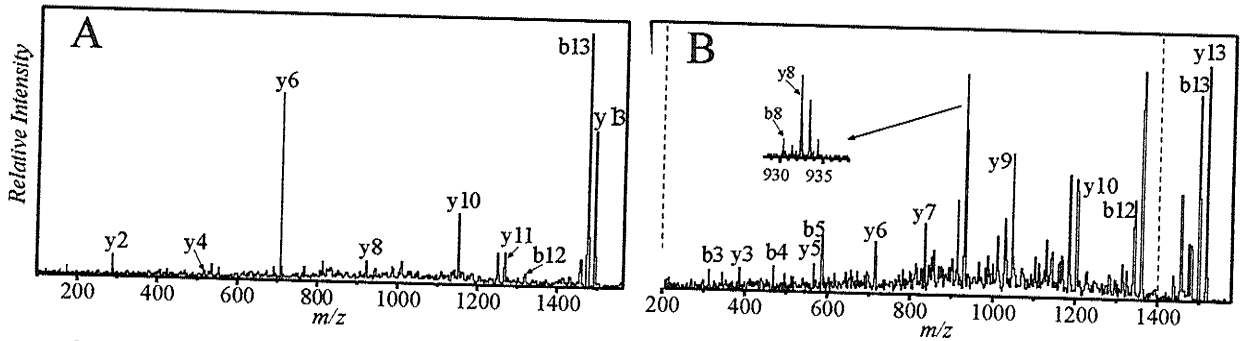
Mass in Daltons		Error in	Predicted sequence[c]	Rat AP2γ Peptide[d]
Observed[a]	Expected[b]	ppm		
909.546	909.539	8	IGLNLPAGR	284 – 292
1056.604	1056.604	1	VTVAEVQRR [C]	241 – 249
1191.679	1191.661	15	YKVTVAEVQR [D]	239 – 248
1265.749	1265.745	3	LDKIGLNLPAGR	281 – 292
1272.630	1272.610	2	EFTDLLHQDR	360 – 369
1479.697	1479.68	11	QSQEGSSLASHHGR [B]	107 – 120
1682.914	1682.884	18	GPISMTKNPLGLPCQK	195 – 210
1836.947	1836.968	11	SAGLIPHISGLEGSAVSSR	121 – 139
1993.057	1993.070	7	SAGLIPHISGLEGSAVSSRR	121 – 140
2086.176	2086.153	11	AAHVTLLTSLVEGEAVHLAR [A]	295 – 314
2214.287	2214.248	18	KAAHVTLLTSLVEGEAVHLAR	294 – 314

[a] Mass of observed ion; [b] Mass of ion as calculated from ProFound; [c] Sequence of rat AP2γ tryptic peptides; [d] Location of amino acid sequence in the rat AP2 γ protein. [A,B,C,D] Peptides sequenced by tandem mass spectrometry (Fig. 3.21, 3.22) and identified using Sonar msms and Global Proteomics Machine software programs. The rat AP2γ protein sequence is from Genbank Accession number NP_958823.

3.4.5. Identification of PARP-1 and Histone H1 Subtypes

In addition to AP2g, and background peptides derived from streptavidin, trypsin and bovine serum albumin, the mass spectra contained a number of ions that were identified both by mass matching and MS/MS data as peptides derived from PARP-1 and isoforms of histone H1. As illustrated in **Figure 3.25**, ten separate rat PARP-1 peptides (Genbank NP_037195) were identified, representing 13.8% coverage of the protein.

Peptides representing the N-terminal sequences of five different isoforms of rat histone H1 were also present in the sample. Three of these H1 isoforms were identified in the rat protein or genome databases as rat H1.4 (Genbank, NP_579819), H1.0 (Genbank, NP_036710) and an unknown H1 subtype (Genbank, XM_225330). The other two sequences, while highly related to known H1 subtypes, were not in any databases. There is evidence that different histone H1 isoforms may play specific roles in the control of gene expression (Parseghian and Hamkalo, 2001; Brown, 2003), but given the several subtypes identified in our experiments it would seem more likely that histone H1 binds the short rPLII enhancer oligonucleotide because of a general affinity for DNA and proteins and our MS data simply reflect the different H1 subtypes present in the differentiated Rcho cells.



1 maeaterlyr veyaksgras ckkcsesipk dslrmaimvq spmfdgkvph wyhfscfwkv
61 ghsirqpdte vdgfselrwd dqqkvkktae aggvagkgqh ggggkaektl gdfaaeyaks
121 nrstckgcme kiekgqmrls kkmldpekpq lgmidrwyhp tcfvknrdel gfrpeysasq
181 lkgfsllsae dkealkkqlp avksegkrkc devdgideva kkkskkgkdk essklekalk
241 aqnelvwnik delkkacstn dlkellifnq qqvpsgesai ldrvadgmaf gallpckecs
301 gqlvfksday yctgdvtawt kcmvktqnps rkewvtpkef reisylkklk ikkqdrlfpp
361 essapappap pvsitsapta vnssapadkp lsnmkiltlg klsqnkdeak amieklggkl
421 tgsankaslc istkkevekm skkmeevkaa nvrvvcedfl qdvsasaksl qellsahsls
481 swgaevkvep gevvvpkgks aapskkskga vkeegvnkse krmkltlkgg aavdpdsgle
541 hsahvlekgg kvfsatlglv divkgtnsyy klqllesdke srywifrswg rvgtvigsnk
601 leqmpskeda vehfmklyee ktgnawhskn ftkypkkfyp leidygqdee avkklavkpg
661 tksklpkpvq elvgmifdve smkkalveye idlqkmplgk lsrrqiqaay silsevqqav
721 sqgssesqil dlsnrfytli phdfgmkkpp llnntdsvqa kvemldnlld ievaysllrg
781 gsddsskdpi dvnyeklktd ikvvdrdsee aevirkyvkn thatthnayd levidifkie
841 regesqrykp frqlhnrrll whgsrttnfa gilsqglria ppeapvtgym fgkgiyfadm
901 vsksanycht sqgdpiglil lgevalgnmy elkhashisk lpkgkhsvkg lgktapdpsa
961 sitldgvevp lgtgipsgvn dtcllyneyi vydiaqvnlk yllklkfnfk tslw

Figure 3.25: The identification of PARP-1 peptides from MALDI-MS analysis.

Mass spectrometry identified PARP-1 as a protein selected by the rPLII enhancer oligo. **A** and **B** represent MS/MS data for two peptides that were identifed as rat PARP-1. The rat PARP-1 protein sequence is from Genbank NP_037195. The peptide sequences in bold characters represent the peptides that were identified by mass matching alone. The peptide sequences in shaded boxes represent the two peptides that were also sequenced by tandem mass spectrometry confirming that they were from rat PARP-1.

3.5. Studies to Confirm the Relevance of AP2γ Binding to rPLII Enhancer Function

3.5.1. *In vitro* rPLII Enhancer-AP2γ Interactions

3.5.1.1. EMSA studies with *in vitro* synthesized AP2γ protein

As shown in **Figure 3.26**, a comparison of a consensus AP2 binding site with the rPLII enhancer region showed a region of marked homology within the 12 bp sequence identified as important for FP2 factor binding (**Figures 3.17** and **3.18**). The unusual change at position 3 of a C to a T may have been a reason why the FP2 region was not identified as an AP2 binding site in database searches. To confirm that AP2γ could directly bind the rPLII enhancer element, human AP2γ protein was synthesized using an *in vitro* transcription/translation system. Recombinant AP2γ protein production was confirmed by incorporation of radioactive ^{35}S-methionine, and detection by autoradiography after separation on an SDS polyacrylamide gel (**Figure 3.27A**). The ability of the rPLII element to bind AP2γ was tested by EMSA using the WTFP1-2 oligonucleotide as a probe. Although faint, the recombinant AP2γ was able to shift the enhancer oligonucleotide (**Figure 3.27B**, lane 2). Binding to an AP2 consensus oligonucleotide was also faint (data not shown). AP2γ antiserum supershifted the enhancer complex, while non-immune rabbit IgG did not (**Figure 3.27B**, lanes 3 and 4). These data provided further evidence that AP2γ protein could associate with the enhancer element.

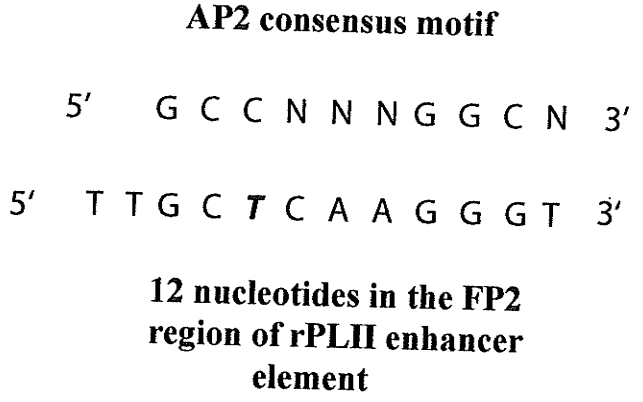
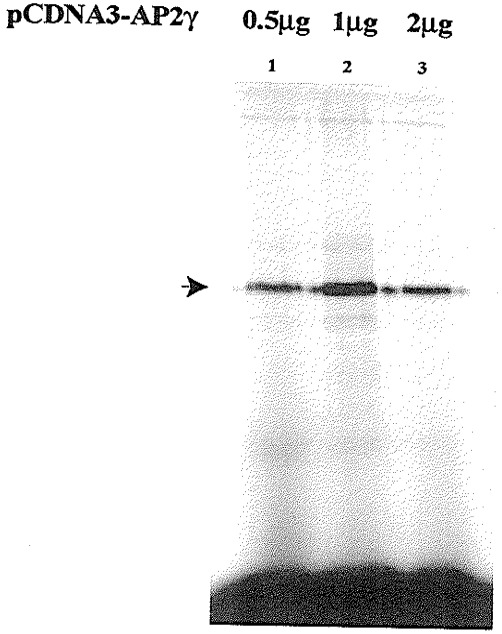


Figure 3.26: Comparative sequence alignment between an AP2 consensus binding motif and the FP2 region of the rPLII enhancer element.

The upper sequence is an AP2 consensus binding site. The lower sequence is the 12 bp region identified by EMSA studies as a potential FP2 factor binding site. The T shown in bold and italics is an unusual substitution and may be a reason why the FP2 region was not identified in transcription factor database searches as an AP2 binding site.

A. *In vitro* **synthesized AP2γ visualized by ^{35}S-methionine incorporation**



B. **Binding of recombinant human AP2γ protein to rPLII enhancer oligo**

WT FP1-2 probe	+	+	+	+
IgG control	-	-	-	+
AP2γ antiserum	-	-	+	-
AP2γ	-	+	+	+
Reticulocyte	+	-	-	-
	1	2	3	4

Supershift ►

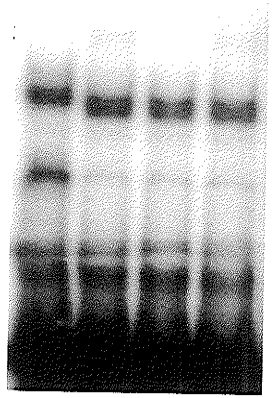


Figure 3.27: Gel shift analysis of recombinant AP2γ interaction with the 43 bp rPLII enhancer element.

(A) AP2γ protein was prepared by *in vitro* transcription and translation utilizing a pcDNA3 - AP2γ expression construct. Three concentrations of expression plasmid, as shown, were tested to obtain optimal synthesis. To demonstrate the protein expression, ^{35}S-methionine was added to thereactions. Reaction mixes were electrophoresed on 10 % SDS acrylamide gels and expressed products were visualized by autoradiography.
(B) The recombinant proteins were tested for binding ability to the wild type rPLII enhancer element, WTFP1-2. A control with no added RNA is shown in lane 1. A faint complex is marked by the lower arrow in lane 2. This band disappears and a higher molecular weight band appears in lane 3 (upper arrow) when AP2γ antiserum is added. No supershift is seen with non-immune rabbit serum (lane 4).

3.5.1.2. EMSA studies with rat trophoblast cell nuclear extracts

To confirm that AP2γ was the endogenous protein that bound the rPLII enhancer element, EMSA supershift studies were carried out with Rcho or rat placental nuclear extracts and AP2γ antiserum. Results are shown in **Figures 3.28**. Rcho nuclear complexes 1 and 2 were supershifted by the AP2γ antibody (**Figure 3.28A**, lane 1). Mutagenesis studies (**Figures 3.17** and **3.18**) had shown that these complexes were associated with factors binding to nucleotides in the FP2 region. Although complexes were less clear with the placental nuclear extracts (**Figure 3.28B**), the addition of AP2γ antiserum (lane 2), but not non-immune rabbit IgG (lane 3), clearly produced a higher molecular weight complex. These results indicated a specific interaction between the rPLII enhancer and AP2γ present in rat trophoblast cells.

3.5.1.3. EMSA competition studies with an AP2 consensus binding site oligonucleotide

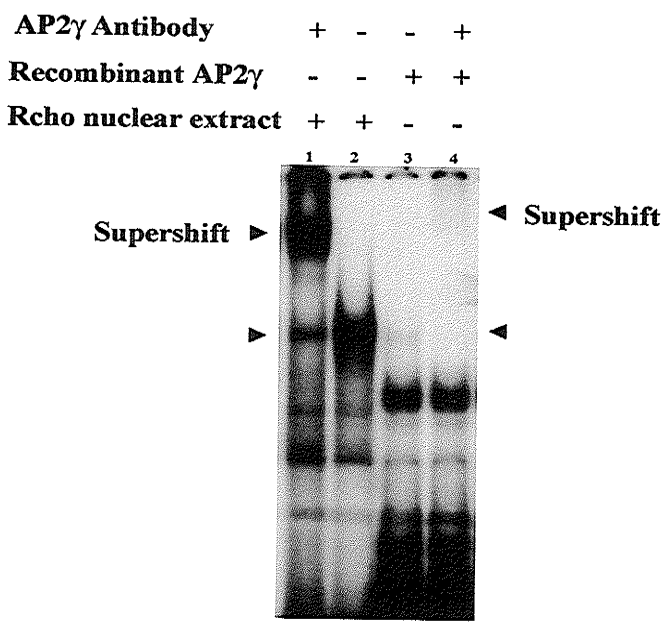
Gel mobility shift assays were also carried out to determine whether an oligonucleotide corresponding to a consensus AP2 binding element could compete the Rcho nuclear factor binding to the enhancer element. As shown in **Figure 3.28C**, the formation of complexes 1 and 2 was reduced by excess unlabelled AP2 consensus oligonucleotide. Some residual complex 1 remained even in the presence of 500 time excess of the competitor oligonucleotide (lane 9); a small amount of complex 1 also did

not shift completely with the AP2γ antiserum, suggesting that some of the complex at this size may not represent an interaction with AP2γ.

These EMSA data supported the MALDI MS identification of AP2γ as the factor in trophoblast cells that binds the rPLII enhancer element *in vitro*.

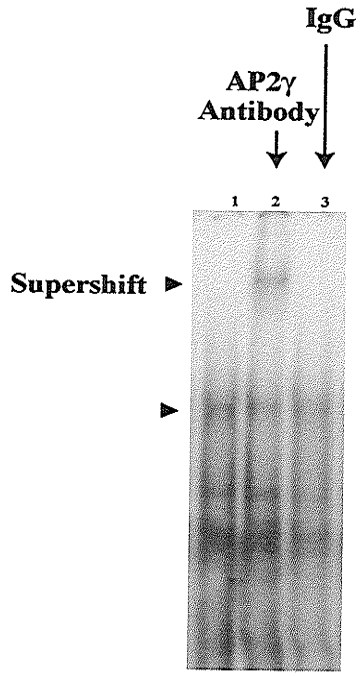
A.

Binding of *in vitro* synthesized AP2γ and Rcho nuclear proteins to WTFP1-2



B.

Binding of rat placental nuclear extracts to WTFP1-2



C.

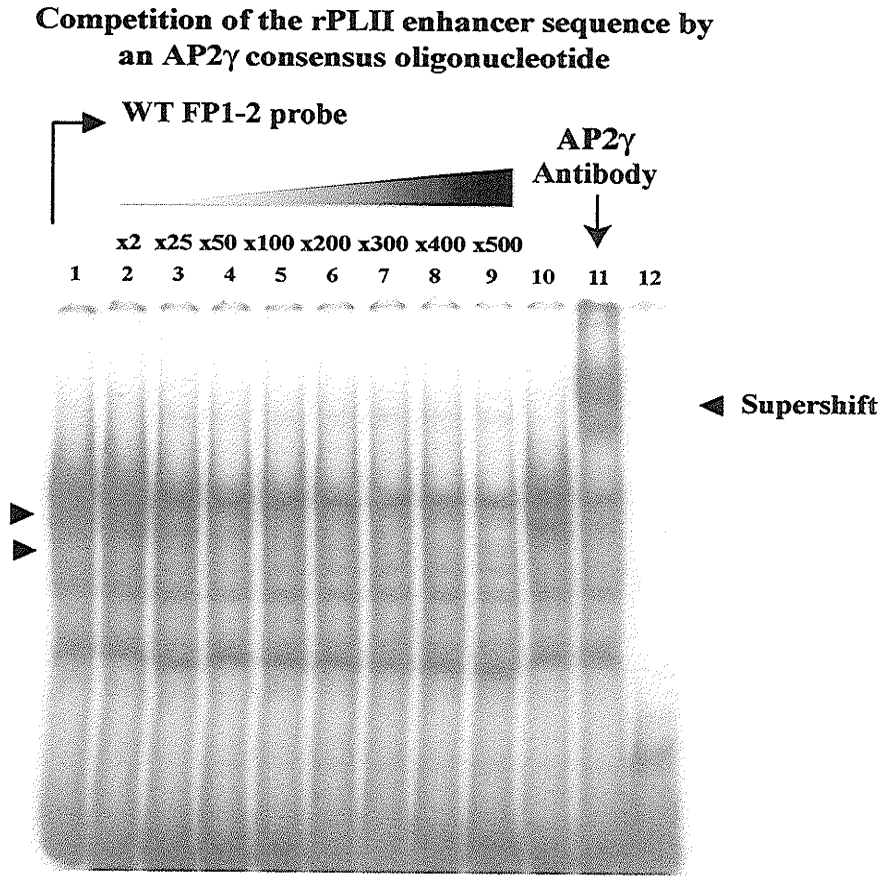

Competition of the rPLII enhancer sequence by an AP2γ consensus oligonucleotide
WT FP1-2 probe
AP2γ Antibody
x2 x25 x50 x100 x200 x300 x400 x500
1 2 3 4 5 6 7 8 9 10 11 12
Supershift

Figure 3.28: EMSA studies to assess specific interactions of endogenous nuclear factors from Rcho cells and placenta with the rPLII enhancer by supershifts and by competition with an AP2 consensus element.

(A) Complexes formed between WTFP1-2 and nuclear extracts from differentiated Rcho cells form high molecular weight complexes (lane 2) that are shifted by AP2γ antiserum (lane 1). A similar complex formed with the *in vitro* synthesized human AP2γ (lane 3) is also supershifted (lane 4). The heavy lower band is an artifact.
(B) Although less clear, nuclear factors isolated from rat placental tissue also form a complex with WTFP1-2 (lower arrow head, lane 1) that is supershifted by AP2γ antiserum (upper arrow head, lane 2) but not by non-immune rabbit serum (lane 3).
(C) A consensus AP2γ binding site oligo at the molar excess concentrations shown, was used as a competitor of WTFP1-2 in binding reactions with Rcho nuclear extracts. Complexes and 2, as previously identified, were competed by this oligo. Lane 11 shows a supershift using the AP2γ antiserum. In both the competitions and the supershifts some complex 1 and 2 is still visible, suggesting there may be other DNA/protein complexes at this size.

3.5.2. *In vivo* assessment of AP2γ and rPLII-Enhancer Interactions

3.5.2.1. Chromatin Immunoprecipitation Assays (ChIP)

The ChIP assay is a technique for assessing the association of DNA binding factors with specific DNA sequences in the context of native chromatin. **Figure 3.29** outlines the procedure used to determine whether AP2γ was associated with the enhancer element in the nuclei of differentiated rPLII-expressing Rcho cells. The quality and fragmentation size of the isolated genomic DNA fragments were assessed following sonication (**Figure 3.30A** and **B**). This control step was important to define the length of the chromatin fragments to be used for immunoprecipitation procedure and to eliminate potential pull-down of larger fragments containing unrelated AP2γ binding sites. The antiserum used in these experiments was the same as was used in the EMSA assays. DNA-protein cross-linking conditions and the ratios of sample to antiserum were optimized for immunoprecipitation as outlined in the Methods. The primer set, ChIP1F and ChIP1R (**Chapter 2, Methods, Table 2.1**), was tested for PCR using an rPLII clone containing the enhancer region of genomic DNA and a BAC clone containing the rPLII and other prolactin-related genes. A 335 bp PCR fragment was predicted and seen in all cases (**Figure 3.31**).

The results of three representative ChIP experiments are shown in **Figure 3.31 B-D**. In each case, "input" represents PCR product from non-immunoprecipitated, cross-linked sample and serves as a positive control. "$2A_{260}$" and "$4A_{260}$" represent PCR

products from immunoprecipitated samples in which different ratios of sample to antiserum were used as described in the **Methods**. "Beads" represent PCR products from control samples to which no antiserum was added and "IgG" represents control samples to which preimmune rabbit IgG was added at a similar DNA:protein ratio as the specific AP2γ antiserum. The specific 335 bp PCR fragment was seen not only in the input samples but also in the samples immunoprecipitated by the specific AP2γ antiserum. This band was missing or very faint in the control samples treated with protein A/G Sepharose beads only (**Figure 3.31C**, lane 5; **3.31D**, lane 6) or a non-specific preimmune rabbit serum (**Figure 3.31C** lane 6; **3.31D**, lane 5), confirming the specificity of the AP2γ interaction with the rPLII enhancer region.

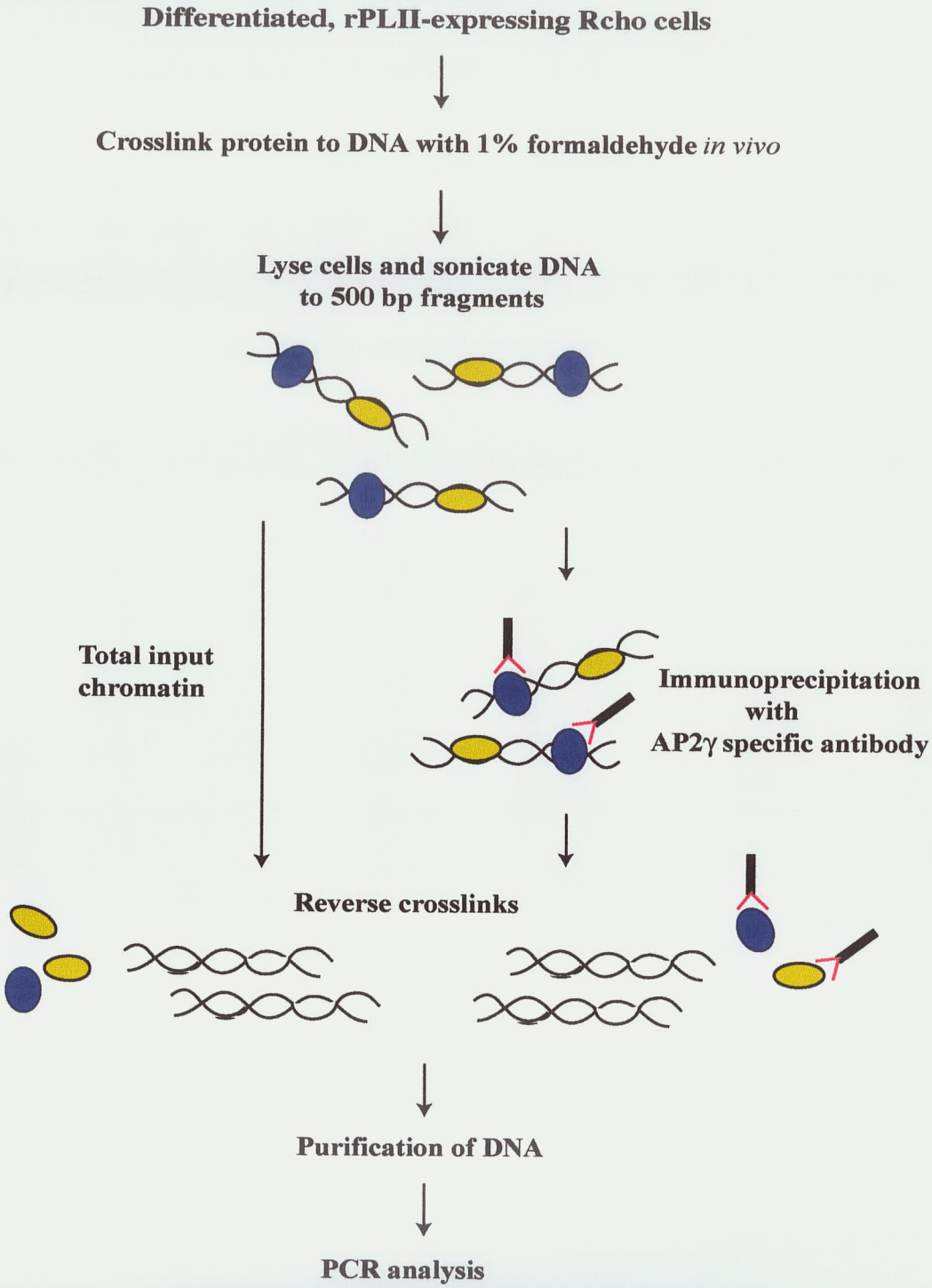


Figure 3.29: Chromatin Immunoprecipitation (ChIP) in Rcho cells.

A schematic overview of the ChIP procedure applied to determine an association of AP2γ with the rPLII enhancer element in chromatin.

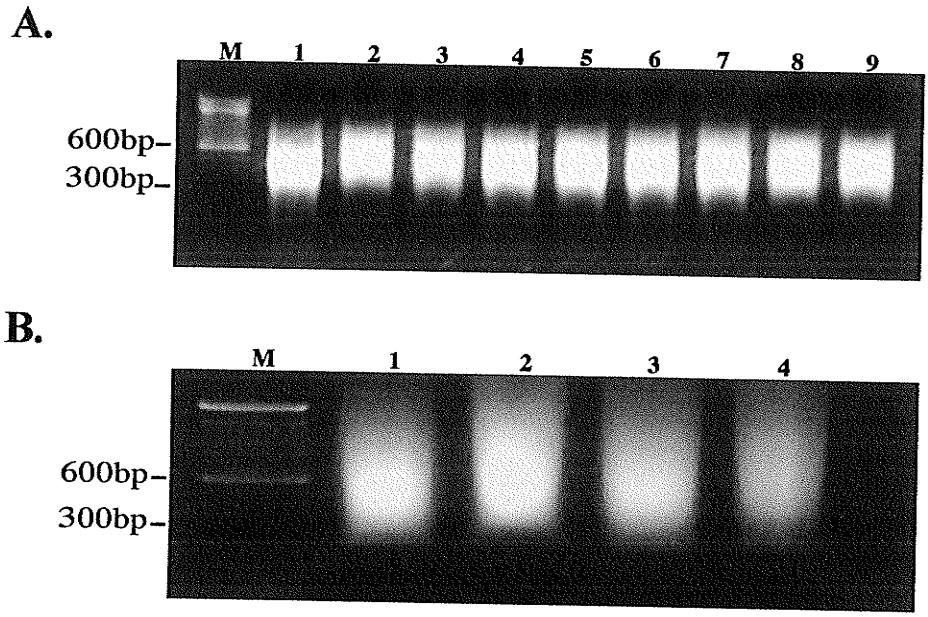


Figure 3.30: Confirmation of genomic DNA fragmentation for ChIP procedure.

(A) Fractionation of nuclei samples from 2^{7} - 3^{7}x10 differentiated rPLII-expressing Rcho cells after six 15 seconds bursts at 40 % power output from the sonicator. **(B)** Partially purified sonicated genomic samples showing an average size of 500 bp. Each plate represents an individual plate at the end of sonication.

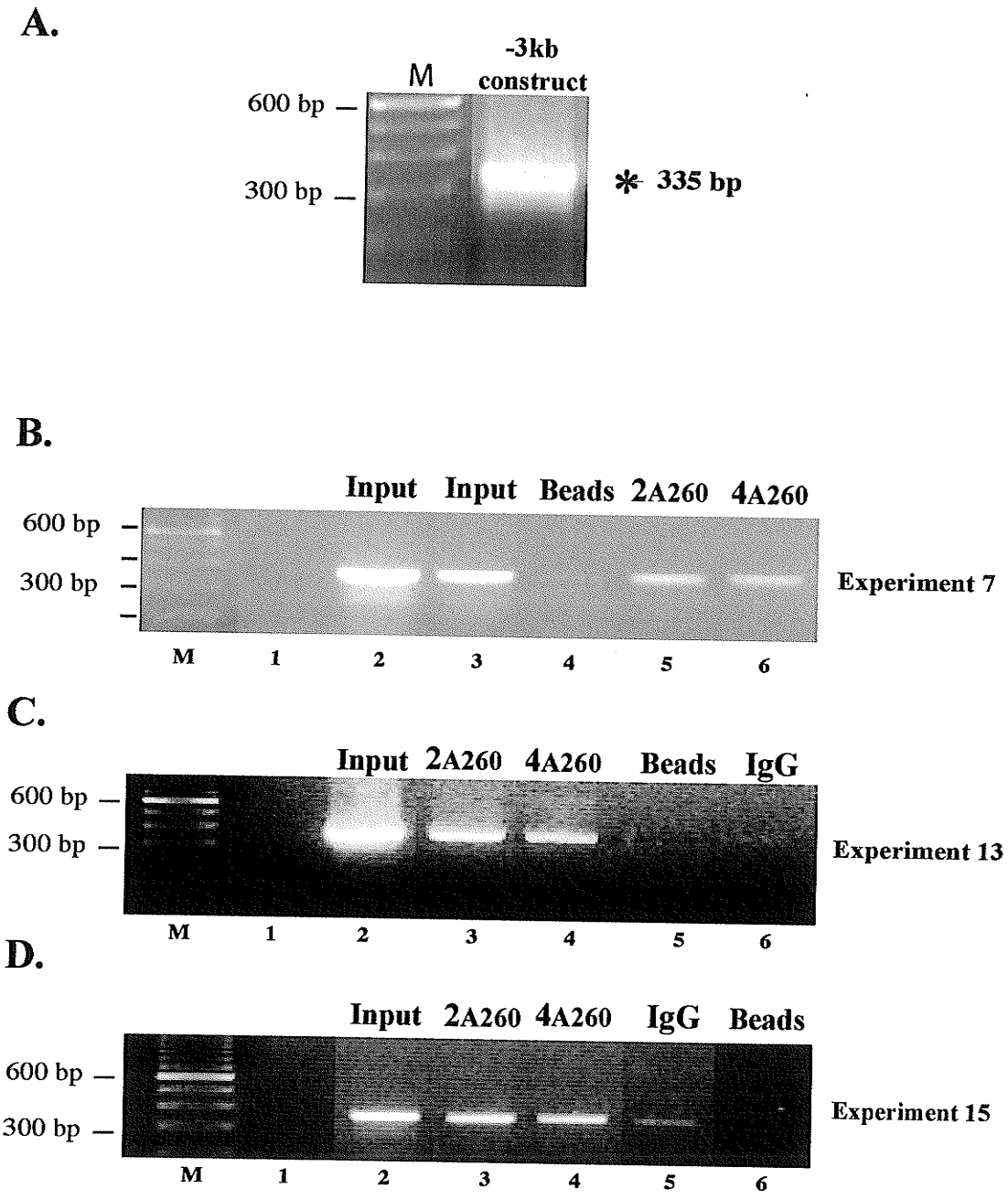


Figure 3.31: ChIP analysis of rPLII enhancer region.

Figure 3.31: ChIP analysis of rPLII enhancer region.

A. Confirmation of PCR primers using a clone containing the 3 kb of rPLII 5' flanking region where the enhancer is located. An expected 335 bp PCR fragment was produced. **B, C,** and **D,** show the results of 3 different ChIP experiments. Experiments were performed as described in the Methods. Input shows a PCR reaction from the DNA that was not immunoprecipitated. 2A260 and 4A260 represent different concentrations of DNA that were used in the ChIP reactions. Beads represents reactions in which no antibody but only Protein A and G Sepharose beads were used in the ChIP reactions. IgG represents reactions in which non-immune rabbit serum was used in place of the AP2γ antiserum. The antiserum was the same as was used for the EMSA studies. Only the input samples and those immunoprecipitated with the specific antiserum showed the expected 335 bp PCR product. Ten μl samples were used in all PCR reactions except the input samples where 4 μl were used. All PCR reactions were for 35 cycles. Lane 1 of each set of PCR reactions represents the control with primers only.

Since we were unable to find a positive rat gene as control for AP2γ binding, we tested whether sequences unrelated to an AP2γ site would also be immunoprecipitated, as a way of testing the specificity of our ChIP reactions. Primers were made to sequences in the rPLII 5′ and 3′ flanking regions to assess the presence of these sequences in the immunoprecipitated pool of genomic fragments. The ChIP3 primer set was designed to amplify a 679 bp fragment located 4669 bp 3' of the enhancer element of rPLII gene; the ChIP2 primer set was designed to amplify a 578 bp genomic fragment located at 15,005 bp 5' of enhancer element as determined from the rat genomic sequence in Genbank. Primer sequences are given in **Table 2.1**. **Figure 3.32** shows the presence of the expected PCR fragments in the input samples, but not in the AP2γ immunoprecipitated DNA. The enhancer specific primers amplified the 335 bp PCR fragment in these samples (data not shown.)

The ChIP experiments provided evidence that AP2γ was bound to the rPLII enhancer fragment in the chromatin of Rcho cells and strongly supported its role in the expression of rPLII in these cells.

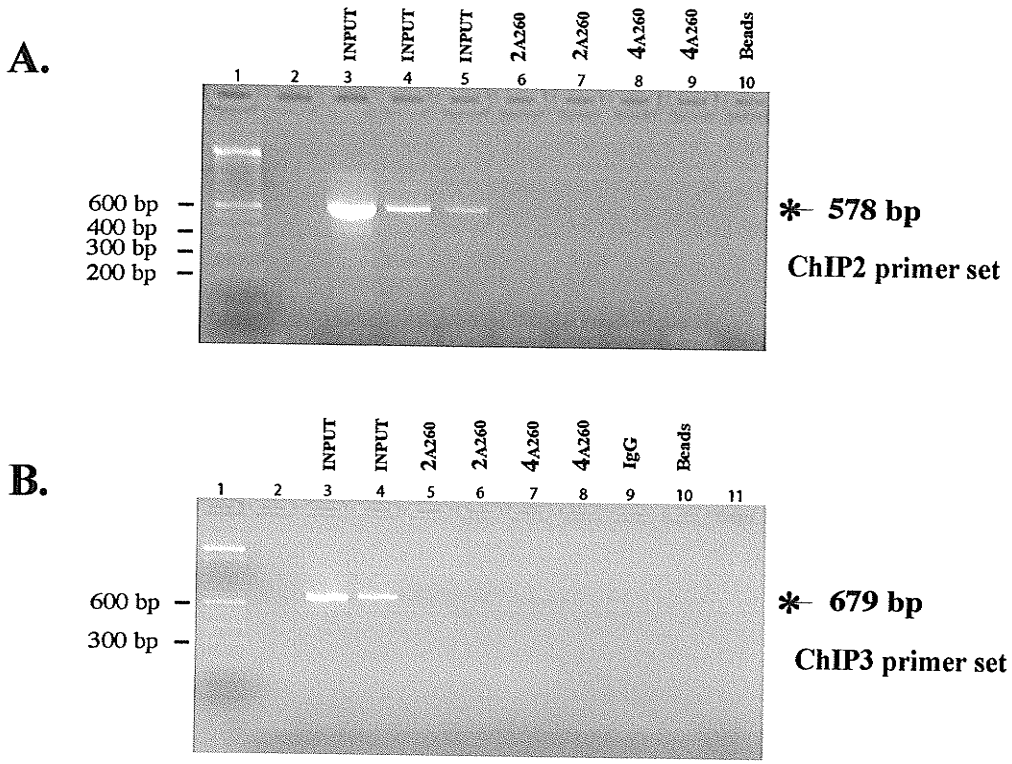


Figure 3.32: Examination of the specificity of AP2γ antiserum immunoprecipitation in ChIP assays

PCR analysis on AP2γ ChIP selected DNA was carried out using primers to two regions of the rPLII gene that were unrelated to the enhancer element. **(A)** The ChIP2 primer set amplified a 578 bp fragment located approximately 15 Kb upstream of the enhancer element. Serial dilutions of the input DNA, 1:40, 1:250, 1:500 were amplified (lanes 3 to 5). ChIP samples at 2A260 and 4A260 concentrations were also amplified (lanes 6 to 9). Lane 10 represents a PCR reactionin which only beads were used. **(B)** The ChIP3 primer set amplified a 679 bp fragment located 5 Kb downstream of the enhancer element; Lanes 3 and 4 show 1:40 and 1:250 dilutions of the input material. Amplification of ChIP samples at two concentrations are shown in lanes 5 to 8. Both non-immune serum (lane 9) and beads only (lane 10) are shown. Only the input samples showed PCR products with both primer sets, suggesting that the AP2γ antiserum was specific for regions of the genomic DNA that bound this factor.

3.5.2.2. AP2γ association correlates with rPLII enhancer activity

Co-transfection studies were carried out to assess the functional role of AP2γ in rPLII expression. When we first began our functional studies, we found that when Rcho cells were co-transfected with the AP2γ expression clone, pCDNA3-AP2γ, and the WTFP1-2/TK promoter reporter construct, enhancing activity was repressed as compared to a control with no added AP2γ (data not shown). It has been previously reported that overexpression of AP2 results in the repression of transcriptional activity possibly by self-inhibition or by sequestering cofactors (Zhong *et al.*, 2003; Kannan *et al.*, 1994; Kannan and Tainsky, 1999; Kannan *et al.*, 1999). The rPLII enhancer had previously been shown to be inactive in the rat pituitary GC cell line (Sun and Duckworth, 1999). When tested by RNA blot analysis we detected no AP2γ expression in GC cells as compared with Rcho cells and the human choriocarcinoma cell line, JEG (data not shown). We therefore chose the GC cell line for co-transfection studies to test for functional activity of AP2γ on the rPLII enhancer.

As described in the Methods, the AP2γ expression clone was first titrated to determine the optimum amount to use in co-transfection experiments. This was determined to be 50 ng pcDNA3-AP2γ per 35 mm dish. Results with wild type and mutated rPLII enhancer reporter clones are shown in **Figure 3.33**. These experiments were carried out in rat pituitary GC cells that do not express AP2γ. A greater than 5 fold increase ($p<0.0001$) was seen with the wild type clone, pTK WT Luc, in the presence of AP2γ (lane 3) as compared to without AP2γ (lane 2). When AP2γ was co-transfected with

a reporter construct containing the M4 mutation in the FP2 region, no enhancement of reporter activity was seen over the activity of pTK M4 Luc clone alone (lane 4). These data confirmed that AP2γ has a positive effect on rPLII enhancer activity as a result of specific binding nucleotides within the FP2 region.

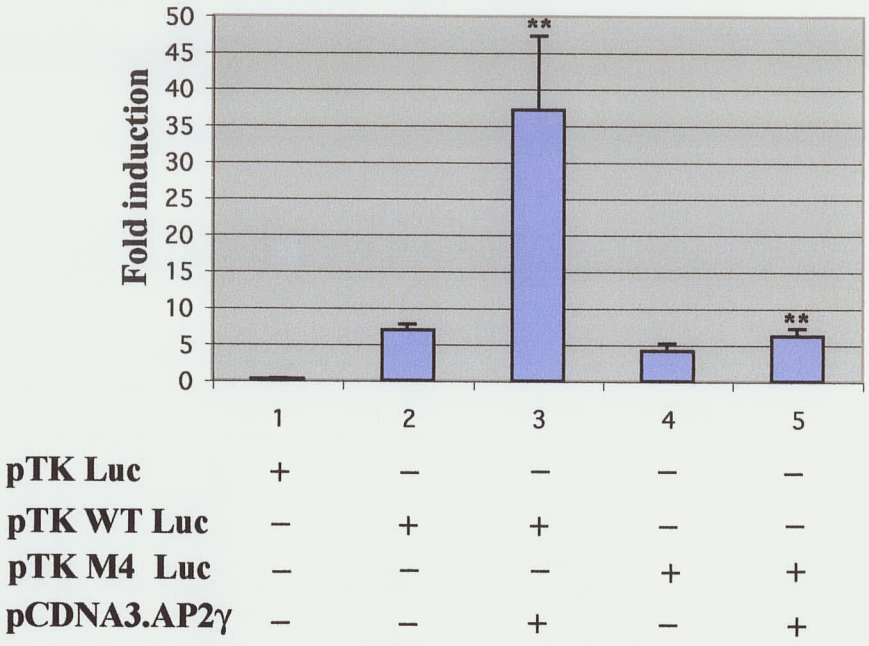


Figure 3.33: Cotransfection studies to assess the functional affect of AP2γ on the rPLII enhancer.

Rat pituitary GC cells were transiently transfected with either the WT enhancer reporter construct, pTK WT Luc, or the FP2 mutant construct, pTK M4 Luc with or without 50 ng of the human AP2γ expression clone, pCDNA.AP2γ. Firefly luciferase activity was normalized to co-transfected Renilla luciferase activity. The data are plotted as the mean value and standard deviation of the firefly/Renilla luciferase ratios (n=6). AP2γ increases the activity of the wild type construct (lanes 2 and 3) (**$p<0.0001$), but has no effect on the activity of the pTK M4 FP2 mutated construct (lanes 4 and 5), which is not significantly different form the wild type construct without co-transfected AP2γ.

3.6 Conclusions

The application of MALDI MS identified AP2γ as a potential transcription factor that could bind the rPLII enhancer element. Further gel shift experiments confirmed its interaction and showed that this binding is specific for the FP2 region of the enhancer sequence. The enhancer AP2 binding site is somewhat atypical and would not be predicted to be a high affinity site based on consensus binding site analysis and PCR based binding site selection studies (McPherson and Weigel, 1999; Mohibullah *et al.*, 1999). Our ChIP experiments confirm, however, that AP2γ is associated with the enhancer site in the chromatin of rPLII expressing Rcho cells. Although our *in vitro* EMSA data suggest that a member of the Ets family of transcription factors is able to bind the FP1 site, we were unable to identify this factor using our binding protein selection proceed and MALDI MS. This could be for a number of possible reasons, including the quantity of the specific protein, strength of binding to DNA, or the quality of current rat protein and genome databases.

CHAPTER 4

IDENTIFICATION OF ADDITIONAL REGULATORY ELEMENTS INVOLVED IN RAT PLACENTAL LACTOGEN II EXPRESSION

4.1. Isolation and Mapping of a P1 Genomic Clone Containing the rPLII Gene

Previously published experiments from our laboratory had demonstrated that a 3 Kb 5' flanking region of the rat PLII gene, which contained the enhancer element, was sufficient to target luciferase expression to the placenta in F0 transgenic mice (Shah *et al*, 1998). Highly variable placental expression levels, and ectopic expression of rPLII mRNA in some fetuses suggested, however, that there were elements outside this 3 Kb region that were required for the complete developmental expression of the rPLII gene. Whether these elements flanked the gene itself, as appears to be the case for the rat PRL gene, which is at the most 5' end of the PRL gene locus, or whether, as in the human growth hormone (hGH) locus, far distal elements are required for appropriate regulation has never been rigorously examined.

To try to better understand the regulation of genes in the rat PRL locus, Dr. M. L. Duckworth isolated a genomic clone from a rat P1 genomic library and partially characterized it by restriction enzyme mapping and hybridization to known members of the PRL gene family. P1 12830 was initially found to contain the rPLII and the rPLP-B genes within approximately 80 Kb. Further mapping and sequencing by Dr. Duckworth

also located the previously described rPLP-I gene on this clone. The work that I carried out on the characterization of this clone was to localize the rPLII and rPLP-B genes within the P1 clone and to identify an rPLII-related pseudogene, the first to be described in this gene family. This work, which is described below, was completed about the time the first rat genome sequence became available (Öztürk *et al.*, 2004). A map of the P1 12830 clone is shown in **Figure 4.1**.

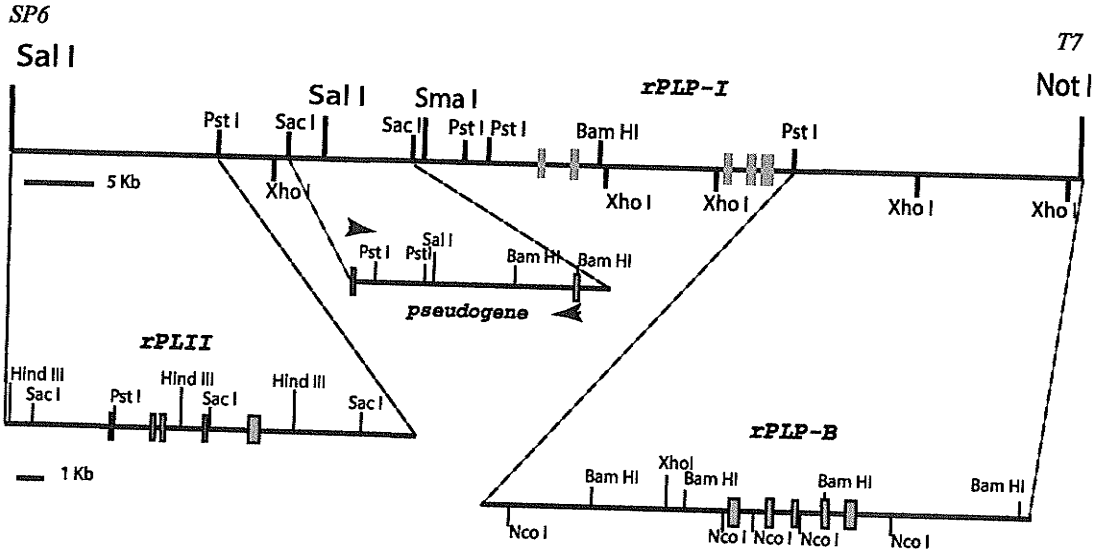


Figure 4.1: A restriction enzyme map of the P1 12830 genomic clone.

Exons of the rPLII, rPLP-I, and rPLP-B gene are shown as shaded boxes. Sequences within the identified pseudogene that have homology to exons in the rPLII gene are also shown as shaded boxes. This Figure is from Öztürk *et al* (2003).

4.1.1. Mapping the locations of the rPLII and rPLP-B genes

The P1 12830 genomic insert had been cloned into Sal I and Not I restriction sites of the multiple cloning site in the pAD10SacBII vector. To determine the size of the genomic insert and the positions of the two genes known to be located on the fragment, a restriction enzyme mapping analysis using the infrequent-cutting enzymes Sal I, Not I and Sma I, was carried out; genomic fragments were separated using pulse field gel electrophoresis (PFGE). These studies demonstrated that the P1 12830 clone contained an approximately 80 Kb insert. Results are shown in **Figure 4.2**. A single Not I site was found only in the vector at the 3' end of the insert; digestion generated a linear fragment around 97 Kb, reflecting the total size of the insert plus vector (**Figure 4.2A**, lane 3).

A Sal I digestion produced fragments of approximately 75 Kb and 23 Kb (**Figure 4.2A**, lane 1); a Sal I/Not I double digest eliminated the 75 Kb fragment, producing fragments of approximately 60 Kb and 14 Kb fragments as shown in **Figure 4.2A**, lane 2. The vector contained a Sal I cloning site at the 5' end of the insert. A radioactively labelled rPLII cDNA hybridized with a 23 Kb fragment present in both the single and double digests (**Figure 4.2B** and **C**, lanes 1, 2, and 3), placing the rPLII gene at the 5' end of the insert. The 75 Kb Sal I fragment and the 60 Kb Sal I/Not I fragments both hybridized with the rPLP-B cDNA probe as indicated by the white arrow in **Figure 4.2B** and **D**, lanes 2 and 3, placing the rPLP-B gene at the 3' end of the insert. The position of the rPLP-I gene was established, later, by Dr. Duckworth.

A.

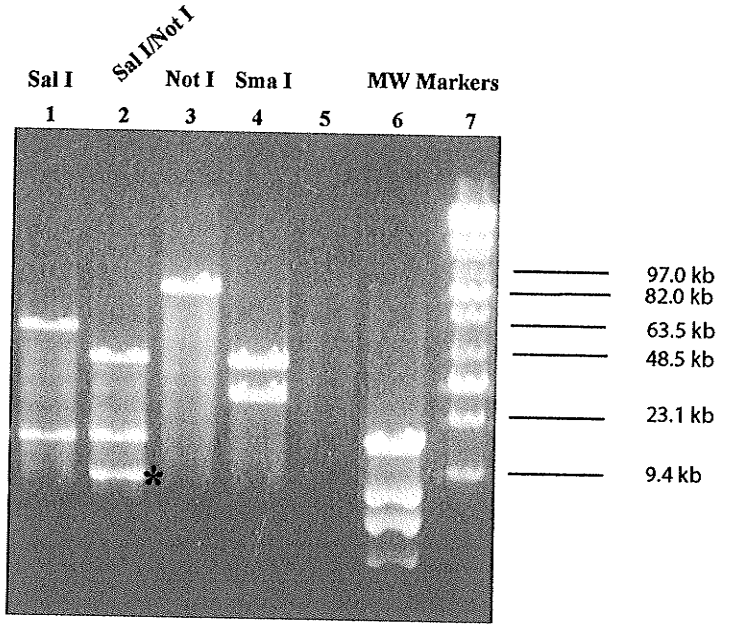


Figure 4.2: Defining the size of rat genomic clone, P1 12830, and determining the relative positions of the rPLII and rPLP-B genes by restriction enzyme mapping and pulse field electrophoresis.

(A) Digests with infrequent cutters, Sal I, Not I and Sma I. Markers are Hind III cut lambda DNA and midrange I pulse field gel markers. An asterisk marks the vector band. Digests indicate that the complete insert is approximately 80 Kb.

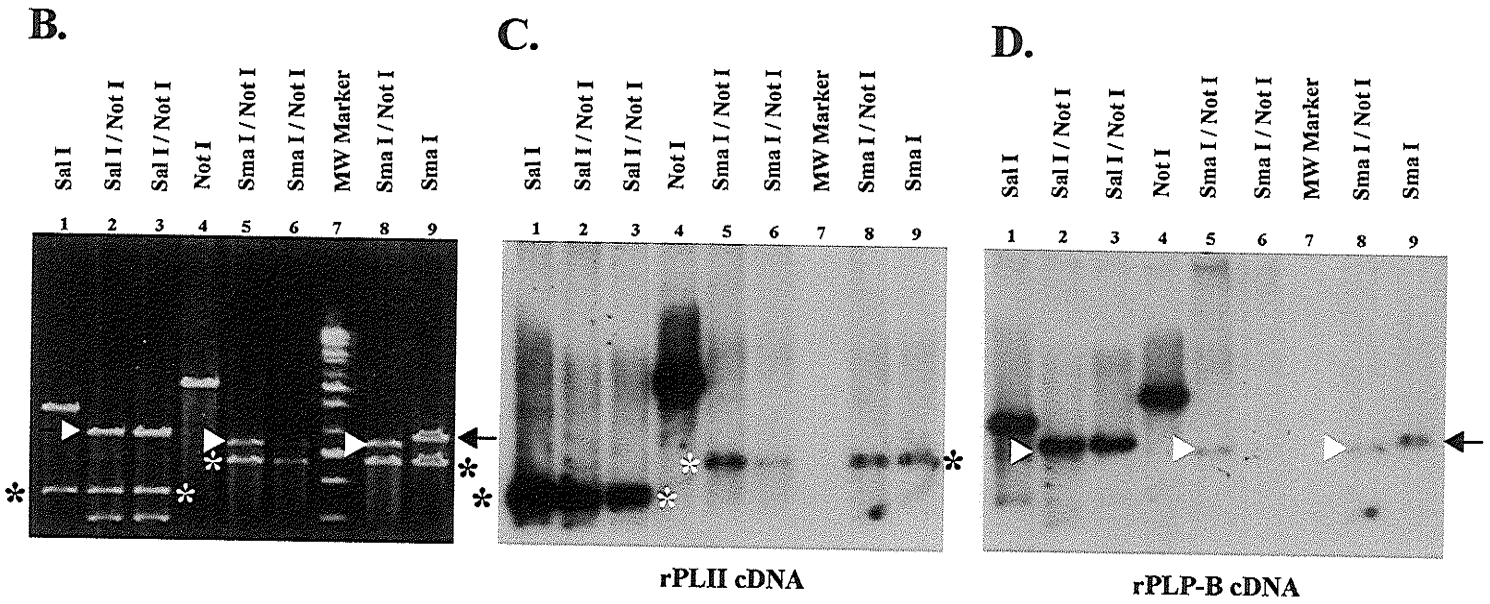


Figure 4.2: Defining the size of rat genomic clone, P1 12830, and determining the relative positions of the rPLII and rPLP-B genes by restriction enzyme mapping and pulse field electrophoresis.

(B) Pulse field gel that was blotted for hybridization to rPLII and rPLP-B cDNA clones. Digests are as indicated. Asterisks mark the bands shown in (C) that hybridize to the rPLII cDNA probe. Arrow heads and arrow mark the bands shown in (**D**) that hybridize to the rPLP-B clone.

A Sma I digestion revealed two fragments of approximately 56Kb and 43Kb (**Figure 4.2A**, lane 4; **Figure 4.2B**, lane 9). The 43Kb Sma I fragment hybridized to the rPLII cDNA probe, as indicated by asterisks in **Figure 4.2B** and **C**, lane 9; This same fragment was present in the Sma I/Not I digest (**Figure 4.2B**, lanes 5, 6, and 8). Since a Sma I site was located in the vector at the 5' end of the insert, this digest also helped to localize the rPLII gene to the 5' end of the clone. An rPLP-B cDNA probe hybridized with an approximately 50 Kb Sma I/Not I fragment (**Figure 4.2B** and **Figure 4.2D**, lane 8), which is slightly smaller size than the 56 Kb Sma I fragment seen in lane 9, again localizing the rPLP-B gene 3' of the rPLII gene in the insert.

Further mapping and comparison to the previously published rPLII gene map restriction (Shah *et al.*, 1998) showed that the clone contained approximately 4 Kb of rPLII 5' flanking sequence, and the rat prolactin-like protein B (rPLP-B) gene contained complete 5' flanking DNA and approximately 6 Kb of 3' flanking DNA, at the 3' end of the clone.

4.2. Identification of an rPLII-related Pseudogene

The large distance between the rPLII and rPLP-B genes in the P1 12830 clone suggested that there could be further genes in the P1 clone. The average size of genes in this family that had been studied to this time were approximately 7 to 10 Kb. In earlier studies, ^{32}P-labeled total cDNA prepared from day 17 of rat placental tissue had been shown to hybridize to a number of restriction enzyme fragments from the P1 12830 clone

(Dr.Duckworth). Most of these fragments could be associated with the rPLII, rPLP-B or rPLP-I genes, but a 9.5 Kb Sst I (Sac I) fragment did not fit with any of these, suggesting the presence of another gene.

4.2.1. Sac I Fragment Mapping Analysis

I isolated the 9.5 Kb internal Sac I fragment and subcloned it into the multiple cloning site of pBluescript SK, as described, for further restriction enzyme mapping and sequence analysis. This clone contained a Sal I site; the P1 clone contained a single Sal I site which helped to locate the 9.5 Kb fragment precisely within the central region of P1 12830 (**Figure 4.1**). I carried out restriction enzyme mapping on two selected subclones, 1-4 and 2-5, which were cloned in opposite orientations, using Sst I (Sac I), BamH I, Xho I, Hind III, and Pst I single and double digestions. The map of this region is shown in **Figure 4.1**.

4.2.2. DNA Sequence Analysis

The ends of the oppositely oriented Sac I subclones, 1-4 and 2-5, were sequenced using T_3 and T_7 primer sites in the pBluescript vector. In addition, the 1-4 clone was sequenced using an internal primer I designed after obtaining the first sequencing results. Sequencing data were compared to the rodent EST and genomic databases using the BLASTN (nucleotide vs. nucleotide) and BLASTX (translated DNA vs. protein database) alignment analysis programs.

The BLASTX search indicated two regions of homology with the rat, mouse and hamster PLII proteins. As shown in **Figure 4.3**, one region near the 5' end of the Sac I fragment showed homology to exon 4 and the other region, approximately 6 Kb more 3' end, showed homology to exon 5. When translated, both the PLII exon 4- and exon 5-related sequences were incomplete and contained frame shifts and stop codons. The DNA sequence was verified using the internal primers, IIvX4 and IIvX5 (**Chapter 2, Methods, Table 2.1**), which were designed for sequencing on both strands in the region of the putative exon 5. At a similar location to the exon 4/intron D boundary in the rat and mouse PLII genes, a GT nucleotide doublet is also present in the novel sequence and may represent a splice donor site. After this putative exon / intron boundary, the sequence immediately 3' of this dinucleotide shows no homology to translated PLII sequences. The putative exon 5 sequence potentially extends sixteen amino acids more 5' than is shown, but these do not represent conserved residues in PLII and were not identified by BLASTX analysis. An AG doublet is located immediately 5' end of a leucine codon, as indicated in **Figure 4.3**, and may represent the remnant of a splice acceptor site.

The DNA sequence data suggested that this region of the P1 12830 clone represented a portion of an rPLII-related pseudogene rather than a new member of the prolactin gene family. Given, however, that the Sac I fragment had been chosen for further analysis based on its hybridization to placental cDNA, RT-PCR studies were carried out to try to identify potential RNA transcripts. cDNA synthesized from day 12 and 16 rat placental mRNA was amplified using the primer pair X5F/X5R (**Chapter 2, Methods, Table 2.1**). These primers are complementary to "conserved" coding

sequences within the putative exon 4 (X5F) and exon 5 (X5R). No product was observed (data not shown). A control reaction using the rPLII primers, IIF1-RNA and IIR2-RNA (**Chapter 2, Methods, Table 2.1**), amplified a fragment of the expected size indicating that there was no problem with the reverse transcriptase reaction. These RT-PCR results support the hypothesis that the Sac I fragment contained a portion of a pseudogene rather than a transcribed member of the rat prolactin family. During our studies, no other pseudogene has been described in the rodent prolactin family, probably because most members have been identified using hybridization to cDNA clones or EST analysis, both of which are dependent on gene transcription. Analysis of the rat genome may reveal more pseudogenes. It has been reported that there are three pseudogenes in the mouse PRL locus (Wiemers *et al.*, 2003; Mallon *et al.*, 2004). Recently, a newly identified rat PLP-P gene and its related pseudogene have been discovered (Alam *et al.*, 2006).

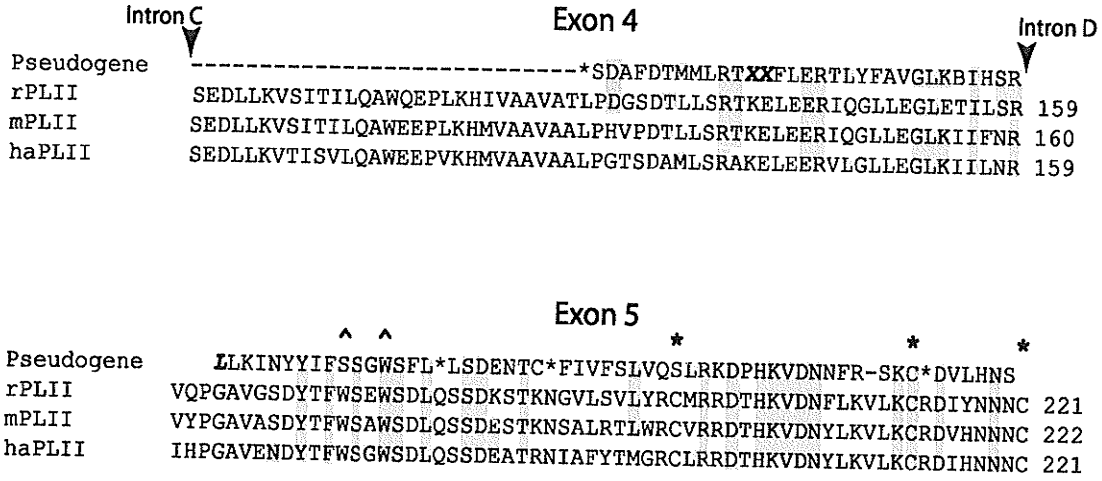


Figure 4.3: Amino acid sequence comparison of the rPLII-related pseudogene fragment with the rat, mouse and hamster PLII proteins.

Nucleotide sequence from the 5' and 3' regions of the 9.5 Kb Sac I fragment were compared by the BLASTX program with the mammalian nucleotide database. Two regions showed homology to translated exons 4 and 5 of the rat, mouse and hamster PLII cDNAs. Identical amino acids that are conserved between the pseudogene and at least one of the PLII genes are shaded. No homology was detected in the pseudogene beyond the regions shown. Conserved cysteines are marked by a*, and conserved tryptophans by an inverted "v". The BLAST program inserted two Xs, shown in bold, where a shift in reading frame was required to maintain the sequence homologies. Asterisks in the sequence indicate stop codons; one insertion, as shown by a hyphen, was introduced by the program to give a better fit. The locations of the introns in the rPLII sequence are shown by arrows. There is GT dinucleotide in the pseudogene sequence at the exon 4/intron D boundary and translated amino acid sequence similarity with the PLIIs is lost at this point. The pseudogene "exon 5" is approximately 6 Kb more 3'. Pseudogene homology with rPLII exon 5 ends at an asparagine (N). Immediately 5' of the leucine residue shown in bold is an AG dinucleotide.

4.3 Generation of P1 12830 F0 Transgenic Mice

Although the P1 12830 genomic clone did not provide much more rPLII 5' flanking sequence than had been previously tested in transgenics (Shah *et al*, 1998), it did contain the complete rPLII 3' flanking region as well as the complete 5' and 3' flanking sequences of the rPLP-I and the complete rPLP-B 5' flanking sequence. To determine whether the immediate proximal flanking sequences, as opposed to distant sequences in the larger gene locus, were sufficient to regulate the complete developmental expression of members of the rat PRL gene family, transient transgenic mice (F0) were created in the University of Manitoba Transgenic Facility using the P1 12830 clone. The P1 clone was linearized by Not I and purified by the pulse field gel electrophoresis before injection into CD1 fertilized one cell embryos. This work was carried out by Agnes Fresnoza. Fetuses and placentas were collected at day 14 of pregnancy and analyzed for the presence of the transgene. This is approximately equivalent to day 16 of pregnancy in the rat, a time when both rPLII and rPLP-B are highly expressed. Three of 17 normally developing fetuses tested positive for the transgene.

4.3.1. Characterization of P1 12830 F0 Transgenic Mice

To normalize expression of the rat genes to the number of copies of the transgene integrated in the mouse placenta, I determined the P1 transgene copy number for each animal as described in the **Methods**. The transgene copy number was assessed by Southern blot analysis of Pst I digested genomic DNA isolated from mouse placentas

using a rPLII cDNA clone as a probe. In addition, 1, 2, 5 and 10 copies of P1 12830 genomic DNA sample was added to non-transgenic mouse DNA samples which was also digested with Pst I. Densitometry analysis estimated that the transgenic animals contained 1 copy (TG 715.3), 8 copies (TG 715.4) and 3 copies (TG715.6) of the P1 transgene per genome (**Figure 4.4**).

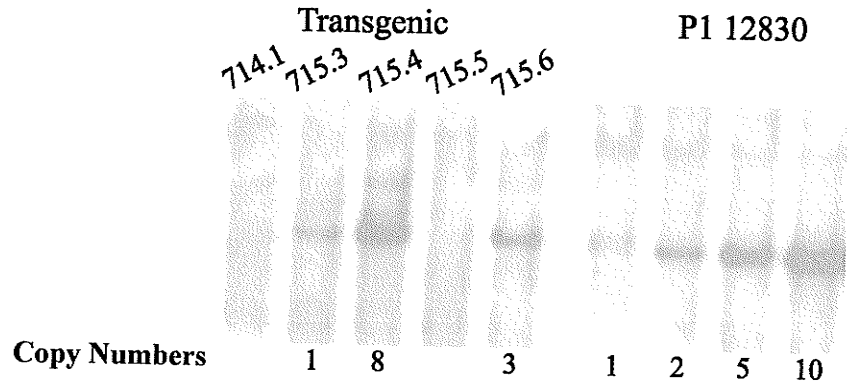


Figure 4.4: Determination of transgene copy numbers.

Ten µg of genomic DNA from each transgenic mouse, from a non-transgenic littermate and from a non - transgenic mouse to which 1, 2, 5 and 10 copies of P1 12830 DNA had been added were digested with Pst I, Southern blotted and hybridized to a labelled rPLII cDNA probe. Comparisons of hybridization intensity were carried out using densitometry. The number of each animal is given above each lane; P1 12830 copy numbers and estimated transgene copy numbers are shown below the lanes.

4.3.2. Comparing transgene expression levels to endogenous expression of the mouse genes

To determine whether the rat transgenes were being expressed in the mouse placenta, we isolated total RNA from placental tissues of each of the transgenic animals and representative non-transgenic littermates and carried out RT-PCR assays. In all cases the primers were designed using the rat sequence and primer pairs were selected that would amplify both the mouse and rat rPLII (IIF1-RNA/IIR2-RNA) and rPLP-B (BF1-RNA/BR9-RNA) cDNAs. They were also designed to cross intron/exon boundaries and to produce amplified products that contained unique restriction sites for the either the rat or mouse fragments. Primer sequences are given in **Methods, Table 2.1**. The presence of both mouse and rat PCR products could be easily visualized after agarose gel electrophoresis.

To compare the level of expression of the rPLII and rPLP-B genes to the endogenous mouse genes, the forward primer for PLII and the reverse primer for PLP-B were end-labelled and then the amplification products were digested with Pvu II (PLII) or Pst I (PLP-B) to distinguish between the mouse and rat PCR products. The scheme is shown in **Figures 4.5** and **4.6**. I quantified the signal intensities by densitometry for each labeled rat fragment and compared them to the signal from the labeled mouse fragment in the same sample, which was given a value of 1.

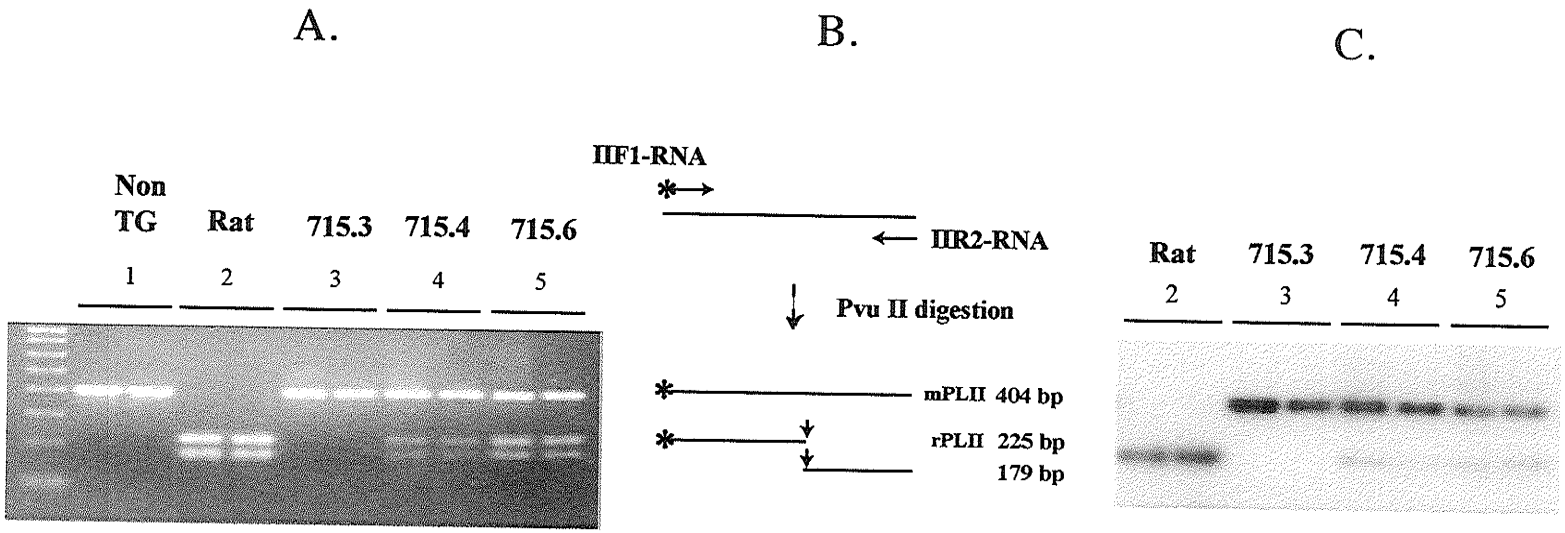


Figure 4.5: rPLII mRNA expression levels in the placentas of P1 12830 transgenic mice.

Total RNA was isolated from placentas of transgenic animals 715.4, 715.6, a non-transgenic littermate, 715.3, and day 16 rat placenta. RT-PCR was carried out using the primer pair IIF1-RNA and IIR2-RNA which amplified both mouse and rat PLII cDNA, giving the same product size, which could be distinguished by a Pvu II digest as shown in B. The ethidium bromide stained gel showing the digested PCR products is shown in (A). The mouse PCR product is 404 bp; the digested rat PCR products seen in lanes 2, 4 and 5 are 225 and 179 bp. To compare endogenous mouse PLII and rat transgene expression levels, primer IIF1-RNA was end - labelled with $\gamma 32P$ - ATP. After autoradiography only 404 bp mouse and 225 bp rat fragments were detected, as shown in (C). The signal intensity of each band was quantified by densitometry after suitable exposure times and the signal intensity of the rat band was compared to the mouse band in the same lane, which was given a value of 1. Only TG715.4 and TG715.6 expressed rPLII mRNA, at a very low level compared to the endogenous mouse PLII gene.

Placentas of both the 715-4 and 715-6 fetuses expressed the rPLII mRNA as indicated by the appearance of two fragments after Pvu II digestion, which cleaved the rat RT-PCR product (**Figure 4.5A**, lane 4 and 5). These same placentas also expressed rPLP-B transcripts as determined by digestion with Pst I, which cleaved the mouse PCR product (**Figure 4.6A**, lanes 1, 2 and 3). However, the 715-3 placenta did not express either rPLII or rPLP-B.

Expression levels of the rPLII gene in the 715.4 and 715.6 placentas were less than one percent of the mPLII expression levels when corrected for copy number, although the control day 16 rat placental cDNA itself was strongly amplified as seen in **Figure 4.5**, lane 2, indicating that normal expression levels are high at this time. Expression of the rPLP-B gene, however, was very similar to that of the mouse gene in both conceptuses (**Figure 4.6**). Rat PLP-B expression levels were approximately forty percent (715-4), and seventy percent (715-6) of the mPLP-B RNA level when corrected for copy number, suggesting that P1 12830 may contain a more complete complement of rPLP-B than rPLII regulatory elements. Although it appeared to contain an intact copy of the P1 transgene by Southern blot and PCR analysis, the 715.3 fetus did not express either of the rat genes.

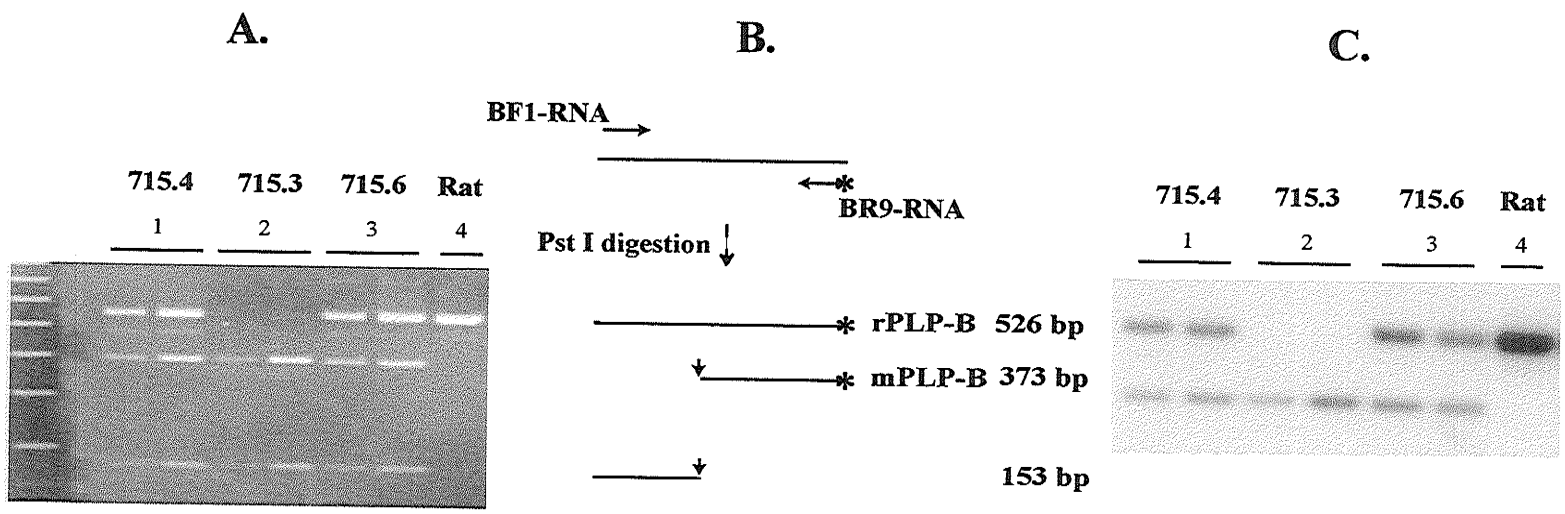


Figure 4.6: rPLP-B mRNA expression levels in the placentas of P1 12830 transgenic mice.

Total RNA was isolated from the placentas of two transgenic animals, 715.4 and 715.6, a non-transgenic littermate and day 16 rat placenta. RT-PCR was carried out using the primer pair BF1-RNA/BR9-RNA. Both the rat and mouse PLP-B were amplified to produce the same size 526 bp fragment; a Pst I digestion distinguished the mouse PLP-B product, producing 373 and 153 bp fragments. An ethidium bromide stained gel showing the products of the different lines is seen in (**A**). To compare expression levels, primer BR9-RNA was end-labelled as shown in (**B**). After auto-radiography only the 526 bp rat and 373 bp mouse fragments were seen (**C**). The signal intensities were quantitiated by densitometry; the intensity of the rat fragment was compared to that of the mouse fragment in the same lane, which was given a value of 1. Levels of expression of the rat and mouse PLP-B fragments for both TG715.4 and 715.6 were very similar to those of the mouse.

4.3.3. Conclusions

Expression analysis of the P1 transgene in the placentas of transgenic mice suggested that more than 4 Kb of 5' flanking DNA was required to produce complete expression of rPLII gene in placenta. Although no evidence was found for expression in the general head, thorax or abdominal regions of the fetus itself, expression levels in placenta were low as compared to the endogenous mouse gene. The fact that the level of the rPLP-B transgene expression was essentially the same as that of the endogenous mouse gene did suggest, however, that regulatory elements proximal to this gene, as opposed to distant regulatory regions, were sufficient to determine near normal expression levels.

4.4. DNase I Hypersensitivity Assays To Identify Further rPLII *Cis*-Regulatory Element(s)

Complete transcriptional regulation of eukaryotic genes during development requires the interaction of many nuclear proteins with many different *cis*-regulatory DNA elements that include promoters, enhancers, silencers and insulators (Novina and Roy, 1996; Smith and Hager, 1997; Blackwood and Kadonaga, 1998; Dillon and Sabbattini, 2000; Dean A., 2004; Szutorisz *et al.*, 2005). The specific recognition of these DNA control elements by regulatory protein factors requires accessibility at the chromatin level; these accessible regions are nucleosome free regions (Elgin, 1988) and are defined to possess hypersensitivity to DNAse I digestion as compared to surrounding chromatin.

This hypersensitivity has been used to locate regulatory regions within chromatin in potentially active or actively transcribed genes that are occupied by protein factors.

Transient transfection assays and *in vitro* protein transcription-translation systems were useful in identifying an enhancer element within the proximal 5' flanking region of the rPLII gene. Experiments with transgenic mice suggested, however, that important additional elements are required to regulate the full developmental expression of rPLII. In order to try to identify further *cis*-regulatory elements in the proximal and more distal regions surrounding the rPLII gene I undertook a DNAse I hypersensitive site analysis in rPLII expressing and non-expressing cells.

4.4.1 A DNAse I hypersensitive site at 2 Kb 5' of the rPLII transcription start site in rat placental tissue

DNAse I hypersensitivity experiments were carried out to identify transcription factor binding sites within a 20 Kb flanking region immediately 5' and 3' of the rPLII gene. Rcho cells cultured for 14 days and day 17-18 rat placental tissue were analyzed as a source of expressing cells. The C6 rat glioma cell line, which does not express rPLII, was used as a control in parallel with the placental cells. Freshly isolated nuclei from differentiated Rcho giant cells, rat placental tissues and C6 cells were exposed to DNAse I digestion using increasing enzyme concentrations (Nickel and Cattini, 1996). The experimental procedure is outlined in **Figure 4.7**. Optimal conditions for DNase I digestion were established for each cell line. Following DNAse I treatment and genomic

DNA isolation, samples were digested with specific restriction enzymes. The restriction enzyme digestion sites were mapped using the rat genome sequence that had become available. Samples were run on 0.8-1% agarose gels and transferred to nitrocellulose membranes for Southern blot analysis. A typical experiment is shown in **Figure 4.8**.

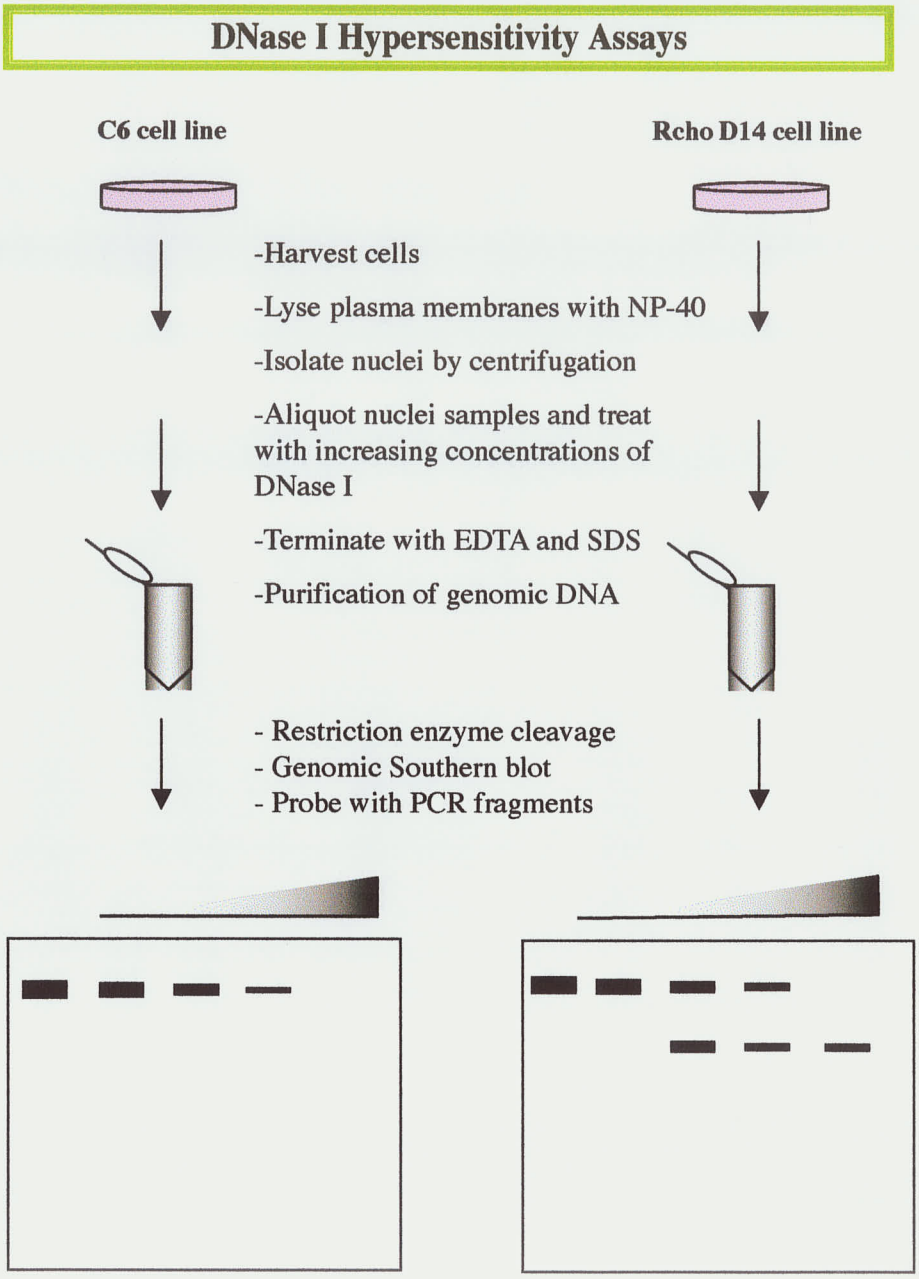


Figure 4.7: Outline of hypersensitive site (HSS) analysis used to assess the chromatin state around the rPLII gene in Rcho cells.

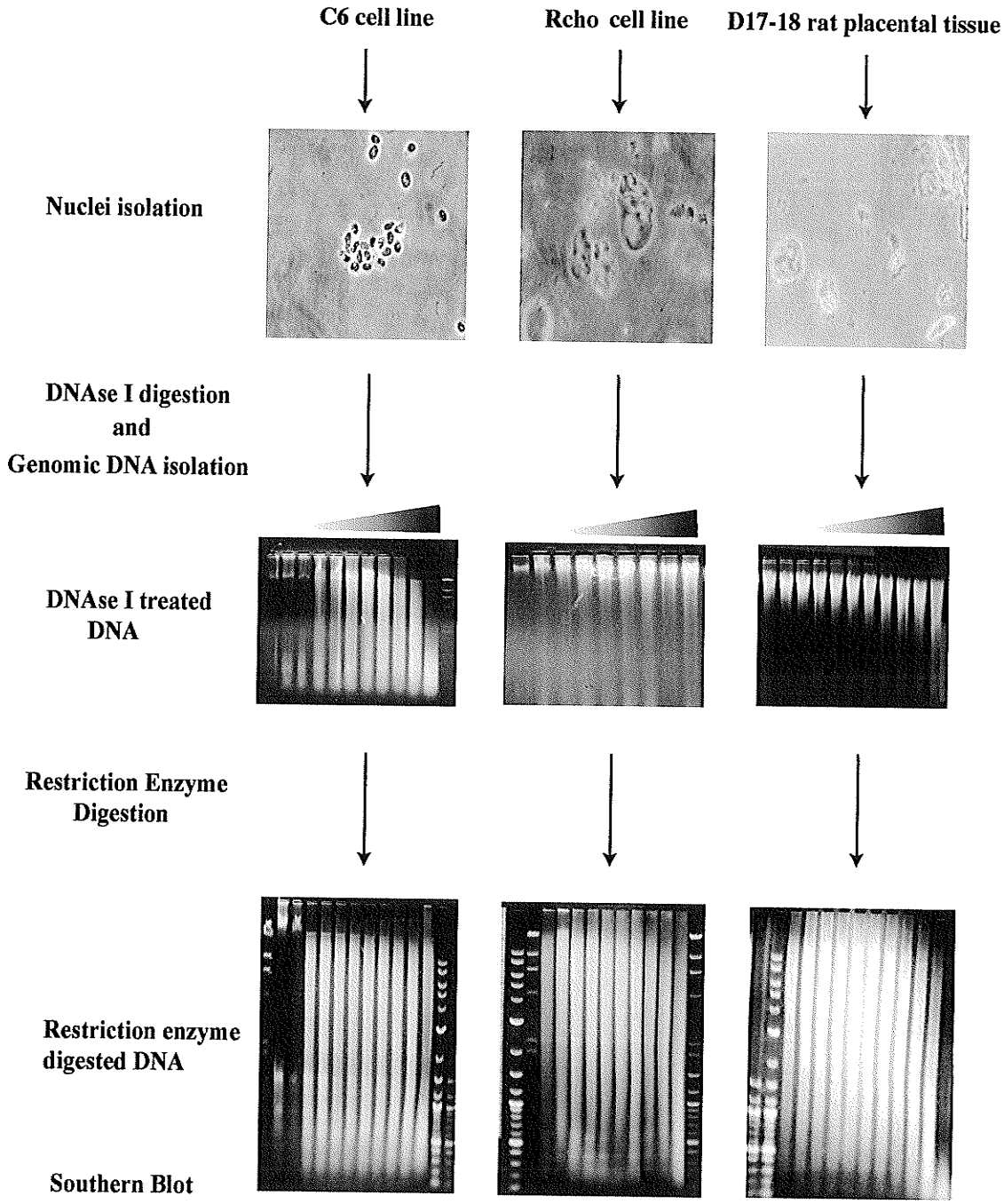

C6 cell line
Rcho cell line
D17-18 rat placental tissue
Nuclei isolation
DNAse I digestion
and
Genomic DNA isolation
DNAse I treated
DNA
Restriction Enzyme
Digestion
Restriction enzyme
digested DNA
Southern Blot

Figure 4.8: DNAse I hypersensitive analysis: nuclei isolation, DNAse I digestion and separation of genomic DNA for Southern Blot analysis.

Nuclei were isolated from C6 rat glial and Rcho trophoblast cell lines and D17-18 rat placental tissue. Nuclei samples were treated with increasing concentrations of DNAse I from 0 to 40U. DNA was isolated and samples were run on agarose gel to assess digestion. 15-20 μg of isolated DNA was digested with Pst I restriction enzyme, electrophoresed on a 0.8 and 1% agarose gel and Southern blotted onto a membrane for hybridization.

To examine hypersensitive sites in the region of the rPLII gene, an indirect end-labeling procedure was used. Sequences at known locations relative to the transcription start site were amplified by PCR and used as targeting probes. Primers were based on rat genomic sequence (**Methods, Table 2.1**). Probe 1, which was at the 5' end of a Pst I fragment, hybridized to a region approximately 10 Kb 5' of the transcription start site (**Figure 4.9**). Probe 2 was at the 5' end of an approximately 17 Kb Dra III fragment covering both 5' and 3' regions of the rPLII gene (**Figure 4.10**).

Using Probe 1 with Pst I-digested DNA, a potential hypersensitive site was seen only in the nuclei of rat placental tissue. A band was seen at approximately 8 Kb, after digestion with the highest amounts of DNAse I (**Figure 4.9**). This would indicate a HSS in a region approximately 2 Kb 5' of the rPLII transcription start site. No hypersensitive sites were seen in the in Rcho DNA, including around the transcription start site. As with the control C6 cells, the 10 Kb band disappeared without the appearance of a lower band. The HSS fragment in rat placental tissue appears to overlap the region of the rPLII enhancer sequence. The appearance of an enhancer associated hypersensitive site would support structural changes in chromatin and its association with gene activation by the rPLII enhancer.

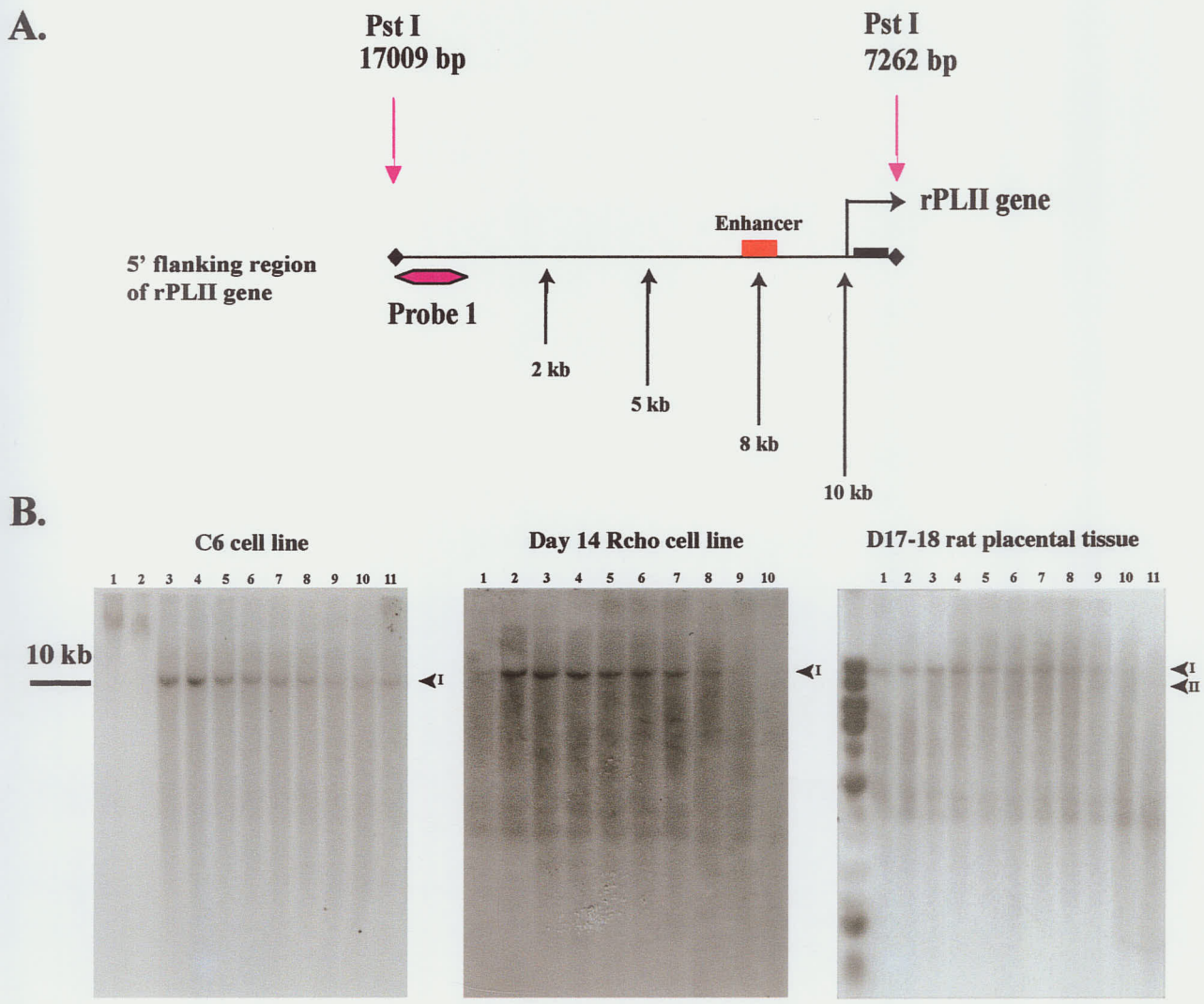

A.
Pst I
17009 bp
Pst I
7262 bp
rPLII gene
Enhancer
5' flanking region
of rPLII gene
Probe 1
2 kb
5 kb
8 kb
10 kb
B.
C6 cell line
Day 14 Rcho cell line
D17-18 rat placental tissue
1 2 3 4 5 6 7 8 9 10 11
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10 11
10 kb
I
I
I
II

Figure 4.9: Southern blots showing detection of a HSS site approximately 2 Kb 5' of the rPLII transcription start site only in rat placental tissue.

Southern-blotted membranes of DNAse I / Pst I digested DNA were hybridized with DNA probe 1, which was amplified from rat genomic DNA. This probe was localized approximately 8 Kb upstream of the enhancer element as shown in (A). Map information of the location of DNA probe 1 and its sequence was determined from the rat genome database (Genbank AABR01038619). Results for non - expressing C6 cells, expressing Rcho cells and rat placenta are shown in (B). The 10 Kb Pst I, 5' fragment is indicated by the arrow head marked "I". A HSS site in the rat placental genomic DNA is indicated by an arrow head marked "II". This HSS is in the approximate region of the rPLII enhancer.

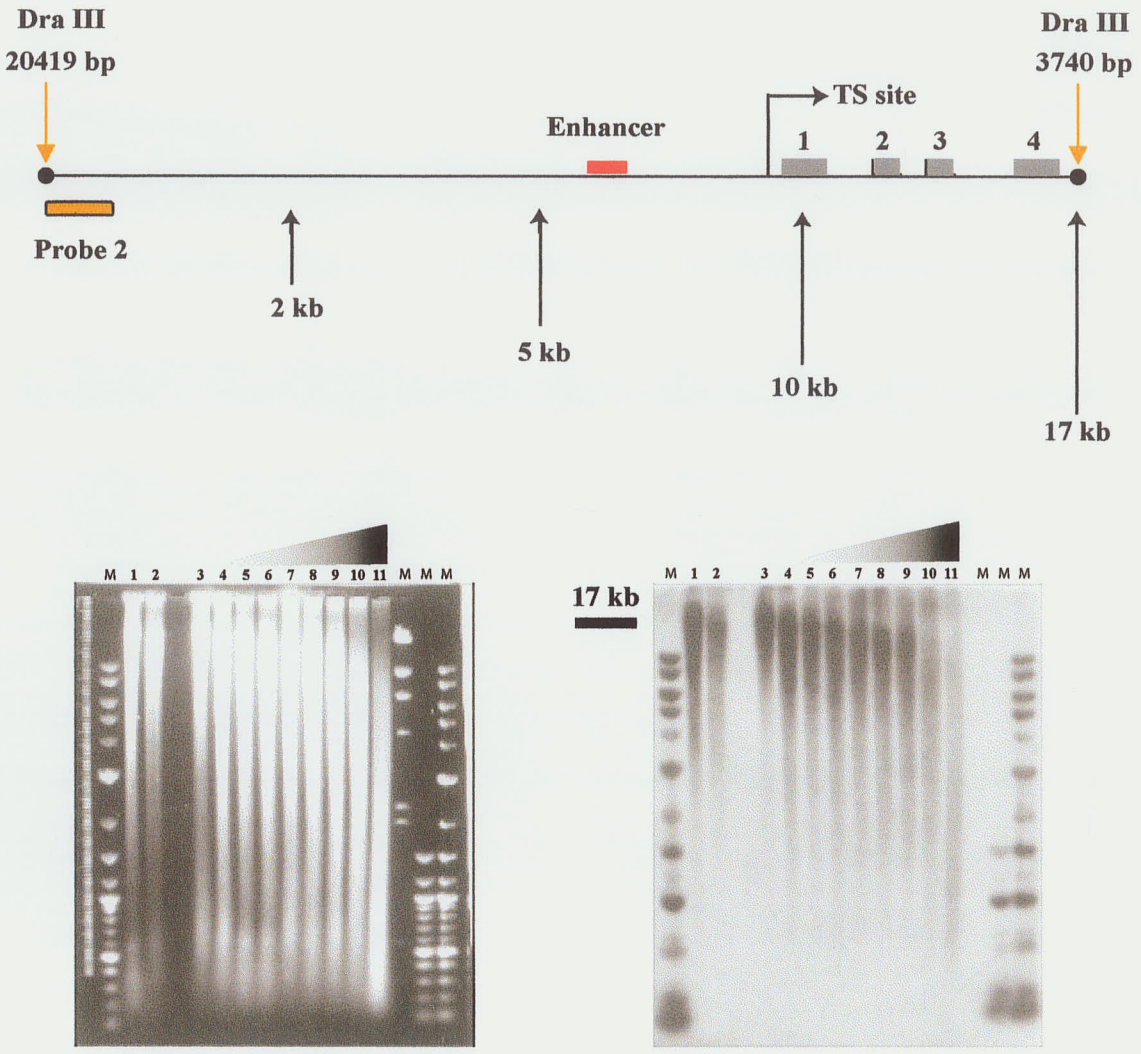


Figure 4.10: DNAse I treated Rcho genomic samples displayed resistance to Dra III restriction enzyme digestion.

The Southern - blotted membrane of Dra III digested DNA from DNAse I treated Rcho nuclei was hybridized with genomic DNA probe2, which was isolated from rat genomic DNA corresponding to the rPLII gene, using PCR amplification.

Using probe 2 with Dra III-digested DNA (**Figure 4.10**) revealed only a high molecular weight smear (>15 Kb) in Rcho cells, which disappeared with increasing DNAse I concentrations. C6 DNA also appeared resistant to degradation, perhaps even more so than the Rcho DNA. Dra III is a methylation sensitive enzyme, suggesting that this region may be a potential target for differential methylation in Rcho cells. Unfortunately, Dra III digestion was not applied with the rat placental nuclei so the two rPLII expressing nuclei cannot be compared. Digestion with other methylation sensitive restriction enzymes would help to confirm this result. Assessing the potential role of methylation in rPLII expression would require additional experiments as outlined in **Future Directions**.

These results are preliminary, but indicate that for additional HSS analyses rat placental tissue may be a better source of nuclei than the highly polyploid, differentiated Rcho giant cells. Trophoblast giant cells are polyploid as a result of endoreduplication in which DNA replication occurs without mitosis. The nuclei in the differentiated Rcho cells are very large, and it is not yet known whether all rPLII gene copies are expressed in each nucleus. Although giant cells in placenta are also polyploid, rPLII mRNA levels are considerably higher in placenta than in Rcho cells, suggesting that there may be differences between the chromatin states of the rPLII gene in these two nuclei.

CHAPTER 5

DISCUSSION

The rodent PRL family members possess high-level tissue-specific, cell type-specific and temporal expression patterns (Dustin *et al.*, 2003; Soares *et al.*, 2004). With the availability of genome sequence data it has now been demonstrated that there are 26 PRL family members in the mouse and rat; these genes are organized in a single gene locus in each species (Weimers et al., 2003; Alam *et al.*, 2006). Regulation of the pituitary-associated PRL gene expression has been well studied (Keech and Gutierrez-Hartmann, A. 1989; Gutierrez-Hartmann *et al.*, 1987; Iverson *et al.*, 1990; Jackson *et al.*, 1992; Liang, *et al.*, 1992; Peers *et al.*, 1992; Cohen *et al.*, 1996; Goffin *et al.*, 1999; Quentien *et al.*, 2006) but there is only limited information on the molecular mechanisms that regulate the majority of the members of the rodent PRL gene family that are expressed in placenta and decidual cells during pregnancy. The question we have been addressing is how expression of the individual genes in the same locus is modulated during development in temporal and cell specific fashion. The rat placental lactogen II gene (rPLII) was among the first of these pregnancy-specific genes, and the first PRL-related placental lactogen to be identified (Duckworth *et al.*, 1986). It is highly expressed only in trophoblast giant cells of the placenta from midpregnancy to term, and has served us as a model to examine the regulatory factors, which define placental cell type specific gene regulation within the PRL locus.

In previous studies in our laboratory, a genomic 3 Kb 5' flanking fragment of the rPLII gene was shown to be sufficient to drive placental expression in transgenic studies *in vivo* (Shah *et al.*, 1989). In addition, a 65 bp length fragment within this flanking region was shown by mutagenesis and reporter transfection studies in Rcho cells to contain an enhancing element (Sun and Duckworth, 1999). Two DNAse I protected regions were identified which affected enhancing activity when mutated. When the same mutations were used in the 3 Kb fragment to drive reporter gene expression in transgenic mice, expression was not lost, but instead showed extensive ectopic expression, suggesting that there were additional regulatory domains required to control the high level, placental-restricted rPLII gene expression (Cattini and Duckworth, 2001). The general aim of my thesis work was to understand in more detail the factors that control the developmental expression of rPLII gene in placenta. My work focused on two areas. One was to identify the regulatory binding factors associated with the 65 bp rPLII enhancer element characterized in the earlier studies (Sun and Duckworth, 1999). The other was to identify and characterize additional placental-specific *cis*-acting transcriptional control elements, which were involved in the developmental expression of the rPLII gene during pregnancy.

5.1 Evidence of Ets factor interactions with FP1 region of rPLII enhancer element

Although the FP1 region of the rPLII enhancer appeared to contain a GGAA sequence that makes up the core recognition of members of the Ets family of

transcription factors, binding of an Ets factor had never been formally shown. I was able to demonstrate by EMSA studies with wild type and mutant oligonucleotides that a number of *in vitro* synthesized Ets factors, known to be expressed in placenta, showed a specific interaction with the GGA sequence in the FP1 region of the rPLII enhancer element. I demonstrated interactions between the 43 bp enhancer oligonucleotide and *in vitro* synthesized MEF (Elf-4), ELF-1 and NERF-2 (Elf-2), all members of the ELF subfamily of Ets transcription factors, but not with Ets-1 and Ets-2, which belong to a different subfamily. These data were the first evidence that Ets family members can in fact bind directly to the enhancer element of rPLII. I was unable to identify the specific family member that binds the FP1 region of the enhancer *in vivo*. A lack of specific antibodies that are able to supershift DNA-protein complexes is a difficulty. It is also possible that more than one of the Ets factors that are present in trophoblast giant cell nuclear extracts could shift the 43 bp oligonucleotide. One factor that would be interesting to pursue further is Elf-5 (ESE-2). Null mutations of Elf-5 (ESE-2) in mice indicate that it is essential for chorion development and therefore placental development; Elf-5 (ESE-2) mRNA is also present in later mouse placenta (Zhou *et al.*, 1998) and rat placenta (M.L. Duckworth, unpublished data). The consensus binding site for this factor, (T/a)A(T/a)AAGGAAGT(A/t)(A/t), is essentially the same as that of our putative Ets binding site ANCAGGAAGTAN (Oettgen *et al.*, 1999; Choi and Sinha, 2006). Elf-5 antiserum did form higher molecular weight complexes in EMSA reactions with Rcho nuclear extract and WTFP1-2 (**Figure 3.9** and **3.10**), but I could not detect a loss of any complex associated with FP1 (**Figure 3.11**). This antiserum also was reported to have some cross-reactivity with other closely related Ets factors (such as ESE-1/ESX, ESE-3a

and –3b) so although these data are interesting they are not conclusive. Our putative Ets binding site is also very similar to the rat PRL basal transcription element (BSE), to which the GABPα/ GABPβ1 Ets factor was shown to bind and regulate rPRL gene expression (Schweppe and Gutierrez-Hartmann, 2001; Schweppe *et al.*, 2003). GABPα/ GABPβ1 expression in rat placenta has not been studied but a functional role in placental gene regulation could be considered (Sadasivan *et al.*, 1994).

Our expectation was that Ets-factor binding to the rPLII enhancer would have an activator role, given the loss of enhancing activity with mutation of the GGA site. Based only on our binding experiments we cannot exclude that there might be a repressor activity as has been shown with some other Ets family members (ERF, YAN, NET and TEL). Potentially one family member may directly compete with another member, which has been also shown with TEL and ERF factors (Sementchenko and Watson, 2000); this may be more of a problem when only a small region is being studied. Analysis of the functional significance of Elf proteins binding to the rPLII enhancer element needs to be investigated further in future studies.

Ets family members contain a common DNA-binding domain, the Ets domain, which is conserved in all family members; Ets domain-containing transcription factors can, however, achieve sequence-specific DNA recognition and do show differential DNA binding specificities. There is considerable evidence that amino acid residues in the Ets binding domain can dictate the DNA recognition specificity within Ets core binding motifs (Shore *et al.*,1996; Sementchenko and Watson, 2000; Yan *et al.*, 2000; Sharrocks,

2002; Oikawa and Yamada, 2003). It has also been demonstrated that Ets members show preference outside the GGAA/T motif, recognizing DNA sequences in a span of over 15 nucleotides. For example, in footprint assays an Ets-1 binding site shows the GGA centered over a 20 bp region (Ghysdael and Boureux, 1997; Graves and Peterson, 1998); the rPLII FP1 region covers approximately 14 bp. The core GGAA sequence in the rPLII enhancer is, however, located close to the DNAse I protected FP2 region. In our EMSA studies, we observed that there was a dramatic loss of only complex 4, when the mutated GGA oligonucleotide was used as probe (**Figure 3.18**) suggesting that this complex was also associated with Rcho factor binding to the FP1 site. In competitor assays, there was no loss of complex 3 when a mutated GGA enhancer oligonucleotide was used as a competitor of the wild type enhancer element (**Figure 3.8** and **Figure 3.17B**). These data may suggest that not only core nucleotide-associated interactions but also protein-protein interactions are involved in the specificity of the Ets factor binding. In future studies, the role of specific nucleotides inside and outside the GGA core element in the FP1 region could be tested by footprinting analysis and EMSA studies using placental cell and GC cell nuclear extracts as a source of rPLII expressing and non-expressing cells.

Some Ets factors can interact with other distinct types of transcription factors such as AP-1, Pax-5, NF-κB, Stat-5, c-Myb, Sp-1 and these interactions are involved in the regulation of target genes in specific cell types (Li *et al.*, 2000; Oikawa and Yamada, 2003). Different Ets factors contain other protein-binding domains such as the pointed domain (PNT) that are involved in specific protein-protein interactions (Kim *et al.*, 2001). However, since we have not able to identify the specific Ets factor that binds the

FP1 region of the enhancer, even by our mass spectrometry analysis, it is difficult to speculate on what interactions might be possible. It may be that there is a low binding efficiency under *in vitro* conditions or low abundance of the factor. This possibility is supported by the weaker complex formation that is associated with the FP1 site in EMSA studies. Ets factor binding might be stabilized in the context of chromatin by association with AP2γ that we now know binds the FP2 region of the enhancer or by other coactivator proteins (Jayaraman *et al.*, 1999; Kihara-Negishi *et al.*, 2001).

5.2 Characterization of FP2 region of rPLII enhancer element

When I began my studies on the rPLII enhancer element there was good evidence implicating AP-1 as the factor that bound to the FP2 region of the enhancer. A computational database search suggested an AP-1 consensus element [TGA(C/G)TAC] as the best candidate. EMSA and enhancer mutation studies using a 65 bp cloned enhancer fragment confirmed that AP-1 (*c-fos/c-jun*) could bind the enhancer element (Sun and Duckworth, 1999). In support of these data, AP-1 had been determined to be important in human and rodent placental development as well as in the regulation of the placental and decidual expression of a number of genes including mouse placental lactogen I, mouse proliferin, matrix metalloprotease-9, placental-specific expression of human gonadotropin releasing hormone receptor and decidual-specific expression of human PRL (Shida and Linzer, 1993; Carney *et al.*, 1993; Groskopf and Linzer, 1994; Alexander *et al.*, 1996; Cheng and Leung, 2001, Watanabe and Brar, 2001). *Jun*-B deficient mice showed severe defects in decidual and extra-embryonic tissue

vascularization including defective neovascularization of the placental labyrinth region (Schorpp-Kistner *et al.*, 1999).

It was only when I synthesized a defined synthetic oligonucleotides to study the factor that bound to the FP1 region of the enhancer that these results became suspect. When the 43 bp WTFP1-2 oligonucleotide was tested for enhancer activity in reporter assays it showed similar enhancing activity to the larger cloned fragment, suggesting that complete functional sequence was present (**Figure 3.2**). Further, when I made mutations in the FP1 and FP2 regions, which were altered in the GGA-core (m6 mutation) and CAAG (M4 mutation) motifs respectively, reporter expression was reduced (**Figure 3.2**). These results were also similar to those obtained with the larger fragment. When WT33/AP-1 binding was tested in EMSA studies, however, no shift was seen (**Figure 3.13**). The *in vitro* synthesized c-*fos*/c-*jun* dimers did however bind a consensus AP-1 binding site and could be shifted by the specific antiserum. Complexes formed between WTFP1-2 and Rcho nuclear extracts were not supershifted by c-*jun* antiserum, nor did a known AP-1 binding site compete any complex. Taken all together, the smaller enhancer oligonucleotide functioned just as the cloned 65 bp fragment, except for the ability to bind AP-1, suggesting that the binding to the larger fragment might be due to sequences outside the enhancer region itself. No other AP-1 binding site had been identified on this cloned fragment, but it also contained a small amount of polylinker left over from the cloning procedure and this extra sequence may have contributed to a cryptic AP-1 site. No reporter studies had been previously carried out using the construct with a mutated FP2 region, which would have addressed this question directly. Even though the factor

that bound the FP2 region did not appear to be AP-1, the fact that overlapping mutations in the 65 bp fragment and the 43 bp oligonucleotide both resulted in the loss of at least 40% of the enhancing activity suggested that this region was important for binding an enhancer protein. Identifying this protein now became a part of my thesis project.

My first approach was to reexamine the 43 bp enhancer sequence for similarities to other transcription factors binding sites by database searches. The consensus DNA binding motif for C/EBP proteins [T(T/G)NNGNAA(T/G)] was found to overlap with the gGTTAtttg sequence within the FP2 region (data not shown). C/EBP α and β factors are reported to be expressed in human villous syncytiotrophoblast cells, which are a major source of placental hormones, and to be involved in the regulation of placental genes in human and rat (Toda and Shzuta, 1996; Chen and Chou, 1995, Chen and Liu, 2000; Bamberger *et al.*, 2004) suggesting that these factors were worth exploring further. I examined the ability of C/EBP α to bind the 43 bp rPLII enhancer element using nuclear extracts from Rcho cells (**Figure 3.15**). The results demonstrated that C/EBP α is unlikely to be associated with the enhancer element *in vitro*. None of the complexes formed between WTFP1-2 and Rcho nuclear extracts were shifted by a C/EBP-specific antiserum; neither a C/EBP consensus oligonucleotide or another previously identified C/EBP binding element competed enhancer binding with Rcho nuclear factors (data not shown).

A further step to identify the FP2 binding factor was to try to define more accurately the nucleotides that were important for factor binding to this region. Mutations

that altered the TGCT, CAAG and GGTA motifs (M3, M4 and M5) across that region resulted in the elimination of specific Rcho/DNA protein complexes (**Figure 3.18**), suggesting that these interactions were specific for binding nucleotides within this 12 bp region. Although this approach was useful in defining the FP2 binding site more completely, further examination of transcription factor databases came up with no more likely candidates.

The last and finally successful method that was used to identify FP2 binding factor was a proteomics approach using mass spectrometry to identify proteins that bound to the enhancer element. This approach had the additional advantage that it could also potentially identify the factor bound to the FP1 sequence and co-activator proteins that must also be playing a role at the enhancer. Mass spectrometry is becoming a standard procedure for identifying proteins (Shevchenco *et al.*, 1996; Woo *et al.*, 2002; Thang *et al.*, 2005); the approach we chose, because of access to instrumentation and collaborators, was to use MALDI - TOF MS. An innovation was to use off-line HPLC to separate the tryptic peptides into less complex pools. Each pool was spotted onto the target directly. The standard way of analyzing complex mixtures of proteins has been to run the mixtures on single or two-dimensional polyacrylamide gels to separate the proteins, cut out the gels slices and digest these proteins with trypsin before spotting onto the matrix. This requires much larger amounts of protein than our method, which allowed the use of quite small amounts of nuclear extract in our binding reactions. This direct binding method finally made it possible to identify AP2γ as a promising FP2 binding factor. Unfortunately it did not provide any further information on the possible Ets factor that

bound the FP1 sequence. This may partly be due to the state of the searchable rat protein and genome databases. These are improving rapidly, but it was actually by comparison to the mouse and human databases that the first identification of AP2γ was made. A further possibility is that the binding to the FP1 site may be weaker *in vitro* than binding to FP2; EMSA studies showed the complexes associated with FP1 as much fainter than those associated with FP2.

5.3 Regulatory function of transcription factor AP2γ

Given the state of the rat genome/proteome databases at the time this work was done we were very fortunate that the first AP2 peptide identified was common to several species and had a specific sequence that was found only in AP2γ (**Figure 3.21-23**). There have been five AP2 family members identified, including α, β, γ, δ and ε (Mitchell *et al.*, 1987; Williams Tjian, 1991; Moser *et al.*, 1995; Bosher *et al.*, 1996; Oulad-Abdelghani *et al.*, 1996; Zhao *et al.*, 2001; Feng and Williams 2003; Tummala *et al.*, 2003); expression patterns show some tissue specificity, although there is also some overlap. AP2γ is a key transcription factor in the development of the rodent placenta (Aumann *et al.*, 2002; Werling and Schorle, 2002); after implantation its expression is restricted to all cell types in the trophoblast lineage. Loss of mAP2γ leads to a decrease in the number of trophoblast giant cells, disorganization of the extraembryonic ectoderm, reduced size of the ectoplacental cone and disrupted contacts with the maternal decidua. As a consequence of these many changes, the labyrinth layer, which is derived from the fusion of the chorion and allantois, fails to form. AP2γ null mice are severely growth retarded

and die at days 7–9 of embryonic development. In human placenta both AP2α and AP2γ appear to have a role in placental gene expression, depending on the state of differentiation of placental cell types (Richardson *et al.*, 2000; Richardson *et al.*, 2001). Both factors have been shown to control expression of many placental genes including human chorionic gonadotrophin α and β (hCGα and β), human chorionic somatomammotrophin (hCS), ovine P450 side chain cleavage enzyme (CYP11A1), leucine aminopeptidase, adenosine deaminase (ADA), 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) suggesting that AP2 might function as a key transcriptional regulator in placental cells (Delegeane *et al.*, 1987; Steger *et al.*, 1993; Yamada *et al.*, 1995;Johnson *et al.*, 1997; Piao *et al.*, 1997; Shi and Kellems, 1998; LiCalsi *et al.*, 2000; Richardson *et al.*, 2000; Peng and Payne, 2002). In support of our MS data, ChIP assays showed that AP2γ was associated with the rPLII enhancer element in Rcho cells *in vivo*. Functionally, exogenous AP2γ activated the WTFP1-2 rPLII enhancer element in reporter gene transfection studies in GC cells, which contain no endogenous AP2γ, but constructs containing the M3 and M4 mutations were inactive (**Figure 3.33**). A further study which we did not do, but which might address the specific role of AP2γ in the expression of the rPLII gene in the context of chromatin, would be to reduce or eliminate endogenous AP2γ in Rcho cells by siRNAs. If AP2γ is required for rPLII expression in this cell type we would expect to see dramatic effects on the expression of endogenous rPLII or on an rPLII enhancer-reporter gene construct. A conditional mutation of AP2γ in mice, so that AP2γ activity was lost only after placental development, would also address this question.

Since AP2γ appears to be such an important regulatory factor for placental gene expression, why didn't we recognize this earlier? This factor was considered during previous work (Sun and Duckworth, 1999), but the sequence similarity was not thought to be very high, and another candidate, AP-1 appeared to have been confirmed as the binding factor. After the MS results we looked more carefully at the 12 bp sequence that had been defined by EMSA and functional studies as important for enhancer activity and found that is was related to an AP2 consensus motif $^{C}/_{G}CCN_3GG^{G}/_{C}N$ (Williams and Tjian, 1991). The primary difference was the presence of a T in the rPLII FP2 sequence (GCTCAAGGGT) where there was an invariant C in the consensus sequence. Although this is not a unique change, it may have been sufficient to give it a lower probability in database searches. This C to T change has also been identified in a trophoblast specific element (TSE) in the hCG α and β genes (LiCalsi *et al.*, 2000, Delegeane *et al.*, 1987, Jameson *et al.*, 1988). When such a C to T change was made in the IL-2 promoter and human estrogen receptor α promoter there was a decreased affinity for this sequence in gel shift studies. More recent analyses indicate that the AP2 binding site is more flexible than was indicated by the original consensus sequence. Using a PCR selection-based SELEX analysis Mohibullar *et al.* (1999) identified a range of functional binding sequences including $GCCN_3GGC$, $GCCN_4GGC$ and $GCCN_{3/4}GGG$. None of the selected sequences contained the C to T change seen in the rPLII enhancer, perhaps because this change affects affinity. Yet another group using Cyclic Amplification and Selection of Targets (CAST'ing) with random oligonucleotide sequences and recombinant human AP-2 protein, identified 17 novel AP-2 binding sites and came up with the broader consensus sequence TAGAAAGNYCYNG (Gee *et al.*, 1998). Other work has suggested that the

nucleotide at position 9 following the 3'GGC/GGG should be a T, as it is in the rPLII enhancer FP2 element (McPherson and Weigel, 1999).

These studies all suggest that the AP2 binding site may be highly flexible and that whether an AP2 protein binds to the site *in vivo* may depend on which of the several AP2 family members is present and/or on interactions with other proteins. In the case of the rPLII enhancer, the relatively weak AP2 binding site may be strengthened by interactions with the protein that binds to the FP1 sequence. The observation that not all reporter activity is lost unless both DNAse I protected regions of the rPLII enhancer are mutated suggests that this possible interaction would be a stabilizing one rather than a required or co-operative interaction that must occur before binding can take place. Given what now appears to be a highly variable AP2 binding site it may be that protein/protein interactions are very important in the selection and specificity of the site and suggests that many of these sites would not be identified by database searches alone.

Both AP2α and AP2γ are expressed in human and rodent placenta (Morasso *et. al.*, 1999; Shi and Kellems, 1998; LiCalsi and Mellon, 2000; Knofler and Husslein, 2000; Roberson *et. al.*, 2001). The gene mutation studies in mice support the idea that AP2γ is the key regulator of placental gene expression in rodents, although a conditional AP2γ mutation would address this more specifically in the case of PLII expression. In human placenta there appears to be a switch between expression of AP2α and AP2γ depending on the cell type. AP2γ is the dominant AP2 family member present in the cytotrophoblasts; on differentiation to syncytiocytotrophoblasts, AP2γ levels decline and

AP2α levels increase (Richardson *et al.*, 2001). Although there may be some species differences as to which AP2 family members are expressed during placental development, and when and where they are expressed, there is a great deal of data, including ours, that support the idea that AP2 is a key regulator of placental-specific gene expression (Shi and Kellems, 1998). Our data do not allow us to determine the specific role of AP2γ binding to the rPLII enhancer element although they suggest that its role may be in determining placental specific expression. There is no information on whether AP2 proteins are able to open and/or reposition chromatin through histone modifications in placental tissue. Since it is present in the native chromatin of trophoectoderm cells before differentiation, AP2γ protein may bind the rPLII enhancer element prior to rPLII transcriptional activation in differentiated trophoblast giant cells. This early binding could promote genetic potentiation, whereby AP2γ binding may facilitate the binding of additional factors that subsequently activate rPLII transcription in the later placental developmental stage. We have evidence from EMSA studies showing that there is essentially no difference in protein complex 1 and 2 formation between the undifferentiated and differentiated state of Rcho trophoblast cells (**Figure 3.16**). It would be interesting to assess the AP2γ/chromatin association in this model system undergoing differentiation using ChIP to compare AP2γ binding to the rPLII enhancer in undifferentiated versus differentiated trophoblast cells.

In theory, the application of mass spectrometry should be a first step in the identification of all the endogenous protein factors that form the various specific DNA-protein complexes that are seen in gel shift assays. MS does not require any

labeling procedure, or tissue or cell type specific library screening as do yeast one or two hybrid systems. The protein selection procedure is relatively straightforward. It does rely on being able to identify the proteins by mass matching or sequence, which depends on extensive protein and genomic databases and sophisticated search and analysis programs. This method of protein identification is also probably very dependent on the binding affinity of the protein-DNA and protein-protein complexes. If these are weak, the proteins will probably not be retained during the wash procedures to remove non-specifically bound proteins. Using mass matching alone I was not able to identify more than one peptide for any other transcription factor; none of the factors that did have a potential match appeared at all relevant based on known binding sites or expression patterns. It is also generally recognized that at least three peptides should be identified from any specific protein to be considered for further testing. In particular, no Ets factor peptides were ever among the potential matches. One protein for which several peptides were identified by mass matching and by sequencing using tandem MS was PARP-1.

5.4 Identification of PARP-1 association with the rPLII enhancer element

The identification of PARP-1 is intriguing. PARP-1 is a highly abundant nuclear protein; until recently its best known role was to catalyze the transfer of ADP-ribose chains onto glutamic acid, aspartic acid and lysine residues of various nuclear acceptor proteins, including histone H1 (Amé et al., 2004; Kraus and Lis, 2003). Our identification of both PARP-1 and five different isoforms of histone H1 could simply be the result of the large quantities of these interacting proteins present in nuclear extracts, and binding to

the rPLII enhancer oligonucleotide as an artifact of our selection method. It is now recognized, however, that PARP-1 can take part not only in local chromatin remodeling, but also by directly altering the activity of enhancers and promoters. PARP-1 has been shown to modify the formation of transcription regulatory complexes at the promoters of target genes by promoting or repressing the binding of transcription factors to target *cis*-elements or by interacting directly with sequence-specific elements (Akiyama *et. al*, 2001; Nirodi *et. al.*, 2001; Butler and Ordahl, 1999; Kraus and Lis, 2003). Of particular interest for our studies, PARP-1 has been shown to bind specifically to AP2α (Griesenbeck et al., 1997; Kannan et al., 1999; Li *et al.*, 2004), suggesting that our MS identification of PARP-1 peptides may indicate a functional significance for this protein in rPLII enhancer activity. Kannan et al (1994) suggested that the overexpression of AP2 resulted in self-interference or sequestering of cofactors, one of which appeared to be PARP-1. This is an effect that I saw when I tried to carry out AP2γ co-transfection studies in Rcho cells, which already expressed high levels of AP2 γ. Li *et al* (2004) have recently shown that, depending on which region of the PARP-1 protein interacts with AP2α, AP2 transcriptional activity can be either inhibited or enhanced. Interaction with the PARP-1 C-terminal activity domain poly (ADP-ribosyl)ates AP2α and reduces transcriptional activation by preventing DNA binding. Interaction with the PARP-1 central automodification/BRCT domain appears to significantly enhance AP2 activity. These data suggest that PARP-1 can have a dual regulatory effect on AP2 activity leading to opposing effects on transcription, a particularly desirable property for controlling the expression of a developmentally expressed gene such as rPLII. I did one EMSA supershift experiment to determine if PARP-1 was part of one of the rPLII enhancer

DNA-protein complexes but saw no change. The antibody used in this experiment had not been proved to be useful for supershifts, however, making a negative result inconclusive. If a useful antibody could be found, ChIP assays could be used to determine if PARP-1 is part of the complex at the enhancer element; immunoprecipitation/pull-down assays using extracts from Rcho and placental cells could also be used. Whether PARP-1 enzyme activity is required could be assessed using inhibitors of PARP-1 such as 3-aminobenzamide (Chiba-Falek *et al*, 2005).

There is also genetic evidence that PARP-1, like AP2γ, may play a direct role in the development of trophectoderm cell lineages. PARP-1 null mice survive, but show altered placental development, which includes reduced numbers of spongiotrophoblasts and increased numbers of giant cells (Hemberger *et al.*, 2003). Mutant PARP-1 embryonic stem (ES) cells also show an increased ability to differentiate into trophoblast giant cells in culture and in teratocarcinoma-like tumours formed in nude mice, as compared to wild type ES cells (Masutani *et al.*, 2001). How the different null phenotypes for AP2γ and PARP-1 are related is not clear at this point.

5.5 Chromatin state analysis of rPLII gene locus

AP2γ is the first trophoblast-specific transcription factor that has been shown to be involved in the expression of a member of the rodent PRL gene family. Since this transcription factor is expressed in all cells of the trophoblast lineage (Aumann *et al.*, 2002; Werling and Schorle, 2002) it is unlikely to be the only factor that determines the

complete developmental expression of rPLII and possibly other members of this family, although it may contribute to placental-specific expression. Our P1 transgenic mouse data provide evidence that further DNA sequences are required for complete rPLII expression. This P1 clone contained slightly more 5' flanking sequence than had been previously assessed in transgenic mice (Shah *et al.*, 1998) and although rPLII was expressed in placenta the expression level was very low compared to the endogenous mouse gene. We were unable to determine placental cell-specific expression because of the close similarities of the two proteins, making immunohistochemistry impossible. It may have been that the P1 12830 clone would have been expressed in more than one placental cell type. In mouse development, knock-out experiments have identified transcription factors that are important for determining different trophoblast cell lineages. In the mouse Mash 2 is important for determining spongiotrophoblast cells and Hand-1 specifies the trophoblast giant cells type (Riley *et al.*, 1998; Scott *et al.*, 2000). Other regulatory elements are likely to be responsible for the more cell restricted, high level expression that characterizes rPLII expression *in vivo*.

One question that we had hoped to answer with the transgenic mice was whether sequences within the surrounding area of the rPLII gene, as compared to very distant sequences, would be enough to facilitate high expression. Since the rat PRL gene family is found in a large locus on chromosome 17, a locus control region may be necessary for the regulation of family members. Although we were unable to answer this question for rPLII, the P1 transgenics did suggest that for the rPLP-B gene, the sequences around the gene were sufficient to at least regulate the level of expression, since this gene was

expressed similarly to the mouse PLP-B gene. Again this gene was expressed only in placenta, but cell type specificity was not determined. Perhaps AP2γ could have a role in the expression of this gene as well. Our transgenic experiments did not clarify a possible role for an LCR. Given that the order of the genes in the locus seems to be closely related to the gene sequences and numbers of exons and introns rather than cell type or temporal expression, the form and function of a potential LCR would probably be different from either the β-globin or hGH loci.

Since our transgenic results clearly indicated a role for further regulatory elements I undertook DNAse I hypersensitivity studies to try to identify other *cis*-elements surrounding the rPLII gene. This is a partially *in vivo* approach to identify elements that bind various regulatory elements since it examines chromatin for sites with a more open configuration. I used mainly Rcho cells for these experiments since they were the model system used for my other studies, and the differentiated cultures contained large numbers of rPLII-expressing giant cells. I had worked out a successful protocol for nuclei preparation when I made nuclear extracts for EMSA studies. Unfortunately I never obtained any clear HSS with these nuclei after numerous attempts and using probes that tested different flanking regions. I was somewhat more successful in these studies using nuclei from day 17-18 placenta; even with these nuclei I saw only one potential site at approximately 2 Kb 5' of the transcription start site. This site is, however, in the general location of the rPLII enhancer and these data support the importance of that site.

The Rcho cell line is the only cell culture model currently available for studying regulation of members of the rodent PRL family. Although this line has proved to be very useful for many studies, it does have limitations. Rcho cultures do not express rPLII nearly as strongly as the late term placenta, suggesting that some factors are missing, or that the chromatin associated with the PRL locus is not in the same state as *in vivo*. It would be interesting to compare chromatin methylation and acetylation states of the rPLII gene in Rcho cells and placenta. The original Rcho cultures in the laboratory expressed rPLI as soon as giant cells appeared, but did not express rPLII until they had been grown for more than ten days in culture. This temporal expression pattern has been lost and could not be regained, suggesting that changes have occurred during culturing. Trophoblast giant cells undergo endoreduplication rather than cell division so that they have very large nuclei containing many copies of the genome. This is particularly true of the Rcho cells and may have contributed to the difficulty of carrying out DNAse I hypersensitive studies. DNAse I hypersensitive studies are still one of the best ways of identifying regions of regulatory importance in large fragments of DNA and may still give important information for the PRL gene locus. For future experiments nuclei from whole placenta would seem to be a better choice than Rcho cells, even though multiple cell types would be present. It would be interesting and perhaps more informative to also use this tissue for further ChIP assays.

CHAPTER 6

FUTURE DIRECTIONS

6.1 Characterization of the functional effect of AP2γ activity on the endogenous rPLII enhancer element in rat placental trophoblast cells

Although AP2γ was identified as the factor that bound the FP2 region of the rPLII enhancer, I did not do experiments to show the effect of the loss of endogenous AP2γ on rPLII expression in rat placental cells. To assess AP2γ's role in rPLII gene expression in trophoblast giant cells, I would deplete AP2γ via RNA interference (RNAi) in rPLII expressing Rcho cells. If the knock down of AP2γ by siRNA in these cells was successful, I would assess the expression of rPLII mRNA and protein under these conditions and then transiently transfect AP2γ back into these cells to determine if the rPLII expression is restored by ectopic AP2γ expression. Since these cells also express many other members of the rat PRL gene family, I would also test for effects on expression of those members expressed in Rcho giant cells as well. These experiments would address the hypothesis put forward in our 2006 Endocrinology paper that AP2γ may be a common regulatory factor for PRL family members.

6.2 Identification of the factor that binds the FP1 region of rPLII enhancer element in rat placental trophoblast cells

My hypothesis is that a member of the Elf family binds the rPLII enhancer element. Both my studies, using the defined enhancer oligonucleotide, and earlier studies using the cloned enhancer element indicated that mutation of an Ets core-related GGA sequence in the FP1 region had a marked effect on enhancer activity. In addition, I was able to show that members of the Ets family of transcription factors, in particular Elf subfamily members bound FP1 region in EMSA studies.

ESE-2/Elf-5 has been shown by targeted mutagenesis in mice to be involved in polar trophoectoderm/extraembryonic ectoderm lineage maintainance and essential for the development of the mouse placenta. These data indicated the significant involvement of an Elf subfamily member in a specific placental phenotype. The rPLII enhancer FP1 sequence is also very similar to the ESE-2/Elf-5 binding site identified using the CASTing procedure (cyclic amplification and selection of targets) (Sementhchenko and Watson, 2000; Choi and Sinha, 2006).

In my EMSA supershift experiments using placental nuclear extracts with an ESE-2/Elf-5 specific antiserum, I found evidence that Elf-5 was associated with a nuclear protein complex. Unfortunately there was no loss of the FP1 associated complexes under these conditions, and the same antiserum also showed a supershift when a GGA-mutated probe was used. I considered that there might be some protein-protein assisted

interactions between the factors bound to the FP1 and FP2 regions, even though functional studies suggested that the factors bound independently; perhaps a supershifted complex was seen because the FP2 site and its binding factor AP2γ were still present. The GGA core flanking nucleotides may also have been effectively involved in specific protein-DNA interactions.

I did not pursue these EMSA studies further and instead chose the mass spectrometry (MS) approach to try to identify proteins directly. Although this approach led to the identification of AP2γ, no known Ets protein was identified. If there is a weak association of the Ets/Elf factor with the FP1 region, or the protein is present at much lower levels in the nuclear extracts, as perhaps is indicated by the weakness of the complex(es) associated with FP1, this might affect its isolation following the multiple washes in our binding protocol. If this were the case, the weak interactions of the FP1-binding factor could be stabilized using a cross-linking procedure.

We could go back to more standard methods and "pull-down" nuclear complexes using an AP2γ antiserum, or transfect Rcho cells with a tagged AP2γ and use an antiserum to the tag, and carry out Western blots to determine if Elf members (or other Ets proteins) are found in the complex. If there are indications of a specific Ets factor association, we can then directly test its functional role *ex vivo* by co-expressing the factor cDNA with the rPLII enhancer-reporter construct. To demonstrate the significance of the candidate Ets transcription factor in rPLII gene expression, I would first consider decreasing the expression of the factor in Rcho trophoblast giant cells using RNA

interference and look for differential changes in expression of the endogenous rPLII mRNA and protein. If changes in rPLII expression are seen, I would transfect these cells with an rPLII reporter gene, either with the 43 bp rPLII enhancer element or the complete 5' flanking genomic region ligated to a luciferase reporter gene to define the potential changes in expression caused by the loss of specific endogenous Ets factor. These experiments would directly address the role of the candidate Ets factor through the rPLII enhancer. If there are additional elements working at a long distance to regulate rPLII expression the reporter assays using RNA interference studies may not be the best method to assess the significance of the candidate Ets factor.

An assessment of interactions between the endogenous rPLII enhancer and the candidate Ets factor would be very important to validate the role of that factor. *In vivo* interactions with the enhancer should also be examined using ChIP analysis with specific antibodies to confirm whether this candidate factor is associated with the rPLII enhancer element in the chromatin of rPLII-expressing trophoblast giant cells.

6.3 Identification of rPLII enhancer-associated protein partners

The identification of cofactors or other activators involved in the placental specific regulation of the rPLII gene, using the rPLII enhancer element, may provide additional information relevant for future studies designed to understand the complete regulation of rPLII gene expression *in vivo*. I identified one potential co-activating factor, PARP-1, during our MS studies but there are likely to be many more that form a complex

with the enhancer DNA binding proteins. I established ChIP analysis during our AP2γ studies and *in vivo* involvement of co-factor(s) like PARP-1 or the Cited 1-4 proteins, which are known to interact with AP2, could be identified by standard ChIP using AP2γ antibodies. The difference in the protocol would be in the isolation of protein samples versus the isolation of genomic DNA fragments. Both applications could be carried out in parallel to correlate protein-protein binding with specific protein-DNA enhancer interactions. The AP2γ pull-down samples could be analyzed using MS if enough protein could be obtained. Although the AP2γ antiserum used in my studies worked well in ChIP not all antisera are available or specific enough for this application. To overcome such a problem, we could tag our target AP2γ protein by a His-tag or endogenous biotin tag strategy, transfect cells and then use the tag-specific antiserum to pull down proteins for ChIP assay; samples could be examined using western blot or mass spectrometry analysis (Pandey and Mann, 2000; Aebersold and Mann, 2003; Grosveld *et al.*, 2005; Rodriguez *et al.*, 2006). If we wanted to examine specific interactions in placental tissue an endogenous tagging strategy could be carried out in transgenic mice and ChIP studies could be carried out to characterize binding partners (Grosveld *et al.*, 2005; Driegen *et al.*, 2005).

A number of applications have been developed to purify protein complexes for mass spectrometry analysis (Wilm *et al.*, 1996; Woo *et al.*, 2002; Bauer and Kuster, 2003; Link *et al.*, 2005). One method that has been used to identify proteins in complexes is tandem affinity purification tagging (TAP-tag). In this case the target protein, such as AP2γ, is tagged with both a Protein A moiety and a calmodulin binding site at either the

N- or C- terminal region, transfected into cells and complexes containing the target protein are gently affinity purified first on IgG-beads, followed by calmodulin-coupled beads (Rigaud *et al* 1997, Puig *et al.*, 2001; Drakas *et al.*, 2005). This method works best when the TAP-tagged protein replaces the endogenous protein, so might be tried in cells in which the AP2γ has been reduced by siRNA. Alternatively the endogenous gene could be replaced by a TAP-tagged gene, using a gene targeting approach (Zhou *et al.*, 2004).

6.4. Does PARP-1 have a role in rPLII gene expression?

Our MS studies showed that poly (ADP-ribose) polymerase 1 (PARP-1) may interact with the rPLII enhancer. Even though PARP-1 is a very abundant nuclear protein and may represent a non-specific interaction, it should be seriously considered as having a direct role in rPLII enhancer function since it has been demonstrated to interact with AP2 transcription factor and to produce both positive and negative effects on gene regulation. I tried to test, by EMSA, whether PARP-1 was associated with protein complexes formed with placental nuclear extracts and the rPLII enhancer element but antibody supershift studies were inconclusive. The PARP-1 antiserum I used had not been previously tested for this application, and when I later tested this same antiserum for use in ChIP assays, I found that it had very poor specificity. A search of the literature may identify a better antibody for these studies.

We could assess potential DNA assisted protein-protein interactions using pull-down assays with AP2γ-specific antiserum followed by western blot analysis using

PARP-1 specific antisera. If a suitable antibody cannot be identified, a tagged PARP-1 might also provide a useful tool in these studies, although the ratio of tagged to endogenous protein may be a problem given the large amount of PARP-1 expressed in cells.

If we found further evidence that PARP-1 was associated with the rPLII enhancer element we would wish to investigate further its role in rPLII expression. It has been shown that PARP-1 can be effectively down regulated using an siRNA strategy in human cell lines (Kameoka *et al.*, 2004). We could assess the possible modulatory effect of PARP-1 on rPLII enhancer function using PARP-1 siRNA knock down studies in Rcho cells transfected with the enhancer/reporter construct. PARP-1, however, can have an effect on transcription either through direct protein-protein interactions, or through its enzymatic activity, which catalyzes the covalent attachment of ADP-ribose units to several nuclear-acceptor proteins, including PARP-1 itself. The siRNA experiment would not clearly differentiate between these two effects. To determine if PARP-1 enzymatic activity was necessary for its effect, Rcho cells could be grown in the presence of a PARP-1 catalytic domain inhibitor, such as 3-aminobenzamide. Either endogenous rPLII expression or effects on the enhancer/reporter construct could be assessed in these experiments.

6.5. Regulation of the PRL locus

We have identified the trophoblast cell lineage-specific factor, AP2γ, to be associated with the function of the rPLII enhancer element. If a tagged AP2γ was "knocked-in" to the wild type allele in mice, an antiserum specific for the tag might be used in a global approach such as ChIP-on-chip assays to identify other genes regulated by AP2γ in the murine placenta, including other members of the PRL gene family (Jin *et al.*, 2004; Testa *et al.*, 2005; Wang *et al.*, 2005; Blais and Dynlacht, 2005; Negre *et al.*, 2006). Candidate placental genes containing the AP2γ binding sites could be identified by BLAST analysis of the rat genome database. Genomic fragments containing these sequences could be subcloned into reporter vectors and assessed for functional activity in Rcho cells or in GC cells using transient co-transfection studies with an AP2γ expression vector. These experiments would allow an overview of the potential role of AP2γ in the regulation of the PRL gene family as a whole.

It would be very useful if AP2 γ-specific binding could be used to identify other factors that are relevant to rPLII gene regulation and possibly other family members that are expressed in placenta. Several of the experiments I have proposed could lead to the identification of these other factors, which could then be tested for effects on rPLII and the other PRL-related genes. We could target these other elements using a pull-down approach with standard ChIP assay and assess the various proteins that are associated with the complexes.

A conditional AP2γ targeted mutation in the trophoblast giant cell lineage using the cre-lox deletion system in transgenic mice or lentivirus-assisted siRNA knock-down (Wiznerowicz *et al*, 2006) would address how rPLII gene expression was be affected by the loss of AP2γ function *in vivo*. These studies could potentially provide functional significance to the candidate AP2γ regulatory genomic elements identified by ChIP-on-chip studies.

6.6 Identification of long and short distance regulatory domains in rPLII gene locus

In previous studies in our laboratory, several F0 transgenic mice containing a 3Kb rPLII 5' flanking reporter construct showed variable luciferase expression levels in placenta and some ectopic expression in various compartments of the fetus, suggesting that the rPLII enhancer element within this region is not the only regulatory element to constrain the expression of rPLII to the placenta. Our P1 transgenic mice studies also revealed that there are additional regulatory elements involved in rPLII placental gene expression that are outside the regions on this clone. Perhaps shared long distance regulatory elements are involved in the spatial and temporal expression of individual members of the locus including the rPLII gene. Nothing is known about this possibility. To address this question, we could use recombineering technology to create tagged BAC genomic DNA clones that contain various members of the PRL gene family. Temporal and cell-specific expression could be followed by the tagged gene expression in transgenic mouse placenta, either RNA or protein. Deletions could be made within these

clones and changes in RNA expression levels, using real time PCR analysis, and variations in spatio-temporal expression of the genes, using immunohistochemistry against the tag, could be followed. This approach is very labour intensive and BAC are not large enough to contain the entire PRL locus but it could potentially identify regulatory domains to within a region of up to a distance of 100 Kb from specific genes. If the endogenous expression patterns of the individual genes could not be recreated with such a large BAC clone, it would suggest the requirement for even more distant elements in the expression of these genes.

Determining the chromatin structure of the functional rPLII gene might allow us to understand any reorganization or modification of the genomic DNA that takes place in rPLII-expressing rat placental cells *in vivo*. A primary approach would be the ChIP analysis of histone modifications, in particular the acetylation state within the rPLII genomic region and a correlation of this state with the expression level of the rPLII gene. We could also test the chromatin state within the promoter regions of neighbouring genes. In parallel, AP2γ assisted changes in the chromatin state could also be examined to determine whether AP2γ recruitment alone results in chromatin modifications and correlates with rPLII expression *in vivo*.

The rat PRL and PLP-P genes are located at either end of the PRL gene locus (Öztürk *et al.*, 2004; Alam *et al.*, 2006). It would be interesting to take both ends of the PRL locus, containing these genes and their surrounding DNA, and place the complete rPLII gene region within these boundaries to examine changes in the expression profile

of the rPLII gene in transgenic mice. This type of analysis may provide a tool to define the effects of boundary domains of the locus on rPLII gene activation. If there is no influence of these distant regions, it may mean that regulatory elements located within close proximity of the rPLII gene and/or within neighbouring genes are critical for spatio-temporal rPLII expression. The examination of expression profiles of rPLII and other PRL members on BAC clones containing overlapping regions of the entire PRL locus in transgenic mice, might also be useful in narrowing down the location of important regulatory regions. This analysis may show whether regulatory elements are located within the neighbouring genes, between these regions or potentially both. It would probably be necessary to use tagged genes in these experiments.

One final caveat. We have focused on positive regulatory elements within the rPLII gene and PRL gene locus; there are also likely to be equally important negative regulatory elements, which remain to be discovered.

CHAPTER 7

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