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DISSERTATION

TRANSCRIPTIONAL REGULATION OF THE OVINE PLACENTAL LACTOGEN GENE

Submitted by

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Department of Physiology

In partial fulfillment of the requirements

for the Degree of Doctorate of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2000

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY SEAN W. LIMESAND ENTITLED "TRANSCRIPTIONAL REGULATION OF THE OVINE PLACENTAL LACTOGEN GENE" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTORATE OF PHILOSOPHY

Committee on Graduate Work

Advisor

Department Head

ASTRACT OF DISSERTATION

TRANSCRIPTIONAL REGULATION OF THE OVINE PLACENTAL LACTOGEN GENE

Prenatal growth and development influences the quality of life one has after birth. The placenta is an essential organ during prenatal development and has endocrine functions that regulate maternal intermediary metabolism to provide nutrients for fetal growth. A member of the growth hormone / prolactin gene family produced exclusively by the placenta, placental lactogen (PL) has been described as a key hormone in nutrient regulation in the mother and fetus. However, conclusive data on PL's biological action has not been forthcoming and a reduction/ablation of PL will be required for true analysis. Therefore, transcriptional regulation of ovine (o) PL was examined to identify important *cis*-acting elements that interact with nuclear proteins to regulate its production, which in turn could lead to methodologies to reduce oPL expression *in vivo*. The oPL gene has been structurally characterized and the *in vitro* transcriptional regulation was localized to the proximal 383 bp of the oPL gene promoter. Trophoblast-specific transactivation in rat (Rcho-1) and human (BeWo) choriocarcinoma cell lines was demonstrated within the -124/+16 bp region (minimal promoter) of the oPL gene, but full *in vitro* activity of the promoter required a region within –383/-217 bp. DNase I footprinting assays with chorionic binucleate cell (BNC) nuclear proteins identified potential protein-DNA interactions that may function to enhance transcription of the oPL gene. An initiator element encompassing the transcriptional start site (-12/+7) of the oPL gene is thought to be required for transcription because a functional TATA box was not

identified. Footprint 2 (FP2) was confirmed to interact with AP-2α. Immunohistochemical analysis of AP-2α expression in the ovine placentome localized AP-2α in the BNC, but expression was not restricted to BNC and was identified in other cells in the chorionic epithelium. Three AP-2α splice variants were purified from a placental cDNA library. Two of the AP-2α splice variants (AP-2αv6 and AP-2αv7) stimulated transactivation through the oPL gene AP-2 element in HepG2 cells, which lack endogenous AP-2 expression. A third AP-2α was specific to sheep, but was not capable of enhancing transcription. These data indicate that regulation of AP-2α expression during trophoblast differentiation may effect oPL gene transactivation. Two GATA elements in the oPL gene at –67 bp (FP2) and –102 bp (FP3) are functional and interact with GATA-2. A novel *cis*-acting element (-109/-102) was identified in FP3 by mutational analysis in human (BeWo) choriocarcinoma cell transient transfections. A heat stable nuclear protein with an apparent M_r of 41,000 was found to interact with a CCACGA element in BeWo and BNC nuclear extracts. Screening a placental cDNA expression library by Southwestern techniques identified Purα, a single-strand DNA binding protein. Purα from BeWo and BNC nuclear extracts was found to interact with the novel element by electrophoretic mobility shift assays (EMSA) and supershift assays. Additionally, co-transfection of a Purα expression vector with the oPL minimal promoter stimulated transactivation in BeWo cells. A fourth footprint (-173/-137) protected a canonical E-box at -163 bp, and EMSA demonstrated a specific protein-DNA interaction at this site. However, the functionality of this element was not evident by deletion analysis in choriocarcinoma cell lines, but appears to play a context dependent role in choriocarcinoma cells. Two DNase I protected sites (FP5 and FP6) reside within a region

of the oPL promoter that is required for full transactivation of the promoter in choriocarcinoma cell lines. The *cis*-acting element in FP5 was identified as –260 CANAGGCT –253, and mutating the element reduced transactivation in BeWo and Rcho-1 cells. Three nuclear proteins specifically interact with FP5 in Southwestern analysis of BeWo nuclear extracts and two proteins were identified in Rcho-1 nuclear extracts, which may indicate a family of transcription factors or a hierarchy of regulation at this element. Footprint-6 was examined with EMSA and the *cis*-acting element, -337 AGGAGGGCATGGCAAC –322, was identified to interact with a BNC nuclear protein with an apparent M_r of ~140,000 by Southwestern analysis. A triplicate concatamer of FP6 enhanced trophoblast-specific transactivation in an orientation dependent, minimal promoter independent fashion, implicating this *cis*-acting element as a trophoblast-specific enhancer element for the oPL gene. These data indicate that oPL gene expression in BNC of ovine placenta is mediated by two known placental elements, AP-2 and GATA, but three novel *cis*-acting elements are important in its regulation. One of these elements functions by interacting with Purα, a single-strand DNA binding protein, which has not been previously shown to enhance transcription of placental genes.

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TRANSCRIPTIONAL REGULATION OF THE OVINE PLACENTAL LACTOGEN GENE

LIST OF ILLUSTRATIONS

Chapter I.

INTRODUCTION

Growth retardation or abnormal development *in utero* can impose hefty side effects on the quality of life one has postnatally. From extensive epidemiological studies, Professor David Barker has proposed that a predisposition for adult diseases can originate during fetal life. Many of his studies were performed in localized regions where an increase in the incidence of an adult disease was found. One such study, which Barker out lined in his book, "*Mothers, Babies and Health in Later Life*," was the rapid rise in coronary heart disease in Western countries at the beginning of the century. The reported incidences were so rapid that genetic changes could not account for the increase. Studies on social or cultural effects of adult lifestyles, like smoking or obesity, were not able to fully explain the rise of coronary heart disease. Geographically, in Britain, Rose and Marmot (1981) noticed poorer regions exhibited higher rates of coronary heart disease. This seemed paradoxical to biological indicators of coronary heart disease, like increased cholesterol and hypertension, which are associated with more affluent areas. Further analysis of these data implicated fetal development or the lack of normal fetal growth as a prominent factor. Barker hypothesized that human fetuses adapt to a limited nutrient supply, which permanently changes fetal physiology, and these events lead to a modified

adult status. Barker's hypothesis originated with observations that babies with low birthweights had a greater incidence of coronary heart disease. Additionally, he confirmed the affiliation of low birthweight to coronary heart disease among women in the United States, and for both men and women in southern India with similar epidemiological studies. These later studies support the association between birthweight and the incidence of coronary heart disease, and further suggest that the correlation is not bound by gender or lifestyle differences. Barker and colleagues have also provided evidence for predisposition of other adult disease states due to low birthweight, including: hypertension, non-insulin dependent diabetes mellitus, cholesterol metabolism, and blood coagulation.

The idea of abnormal development in the womb influencing the status of adult life is a staggering concept to grasp, but evidence exists that there are cross-generational effects associated with these events. The Pedersen (1977) hypothesis supports Barker's findings with a slightly different twist. The Barker hypothesis looked at maternal nutrition as a primary cause, whereas Pedersen studied mother's that possess a malady during pregnancy. Poorly managed cases of diabetics during pregnancy result in maternal hyperglycemia, which causes fetal hyperglycemia and hyperinsulinemia. This state *in utero* increases neonatal morbidities and neonatal macrosomia. Postnatally, humans and experimental animals, which exhibit an increased fetal growth rate, tend to be obese during adult life. Oh *et al.* (1991) designed a set of experiments to study generational effects of neonatal macrosomia in rats. These studies confirmed that an insult causing an increased rate of macrosomia during the first generation, persisted to the second and third generations. The observations indicated an increase in glucose

intolerance due to insulin insensitivity, and the authors conclude that the intrauterine metabolic environment plays a crucial role in perpetuating obesity and glucose tolerance across generations.

Associations between fetal growth and development and the outcome of adult life are very intriguing and warrant further study. The observations presented above indicate the ability of one's life and the life of their children to be changed while they are growing and developing *in utero*. One aspect pertaining to this dissertation is the effect of placental regulation mediating fetal growth and development. Barker discussed adaptations by the placenta to compensate for undernutrition of the mother. An enlarged placenta imposes a long-term penalty on blood pressure, which increased as placental weight increased, when people from Preston, England, were studied (reviewed by Barker 1998). Additionally impaired glucose tolerance, blood coagulation disorders and coronary heart disease have been consequences correlated with an enlarged placenta. These seem to mimic the results of low birthweights described earlier. Therefore, placenta alterations may lay the foundation for programming the fetus for an altered adult status.

The placenta is the fundamental structure involved in regulating fetal growth and development for eutherian animals, but is unique because of its transient nature. The fascination with this organ dates back to ancient times as seen in cultural beliefs, traditions and practices carried out with placentas after birth. Studies on placental structure were described by Aristotle (384-322 B.C.), and followed up by Galan (200 A.D.) who taught the anatomy of the pregnant uterus to his pupils (cited by Stevens, 1975). Galan was the first to describe that umbilical vessel ligation or compression

resulted in asphyxiation of the fetus. His descriptions marked the foundation of placental physiology and vascularization, which were not updated until the Renaissance period. Adelmann (1942) summarized the work of Hieranymous Fabricius of Aquapendente, who was the first to accurately detail the anatomy of placentas from several species and classify the various structures he observed. These early descriptions may seem crude and inadequate to the standards of present studies. However, the observations and conclusions on placental morphology and physiology made by these individuals remain sound even today.

Placental functions fill the *in utero* void of many adult organs that are developing during gestation. One of the most obvious functions of the placenta is oxygen and nutrient transport. Gas exchange between the mother and fetus occurs in the placenta instead of the lungs. Nutrients are acquired through the placenta from the maternal vasculature, rather than from the gut. The placenta also forms a conduit for fetal waste and metabolic by-products, which in the adult is the function of the renal system. In addition to the metabolic responsibilities of the placenta, it protects the developing fetus from the mother's immune system as well as pathogens. In the womb the fetus may seem isolated and unable to fend for itself, but the placenta acts as an endocrine organ, which enables the fetus to interact and alter both maternal and fetal physiology. When considering all the responsibilities the placenta must fulfill, it may seem like an overwhelming task for one organ.

Studies presented in this dissertation will focus on the placenta functioning as an endocrine organ. A placental hormone, placental lactogen, is thought to be the "fetal growth hormone" because of its structure and implicated action in the fetus.

Additionally, this hormone is thought to repartition metabolites on the maternal side during gestation, to accommodate the fetus. Placental lactogen is expressed exclusively in differentiated trophoblast cells that line the maternal-fetal barrier. Transcriptional regulation of this hormone is of interest because of its timing of expression, the exclusive nature of its expression and the location of expression. Background on placental lactogen and its actions on maternal and fetal tissues will be presented. A discussion on the transcription of other placental hormone genes will be evaluated in hopes to identify *cis*-acting elements and their *trans*-acting factors responsible for trophoblast-specific production. Finally, I will present data for the transcriptional regulation of the ovine placental lactogen gene and compare and contrast the current literature.

Chapter II

LITERATURE REVIEW

One of the many responsibilities of the placenta is to modulate the maternal environment during gestation. An important requirement for fetal growth and development is repartitioning metabolic substrates from the mother to provide the developing fetus. Architectural characteristics and endocrine actions of the placenta provide a site for nutrient uptake to make available an adequate nutrient supply for fetal growth (Talamantes and Ogren 1988; Handwerger 1991; Anthony *et al.* 1995). Placental morphology exhibits considerable diversity between species and can be classified by its gross morphological structure and the tissue layers separating the maternal and fetal blood. The endocrine activity of the placenta produces an enormous repertoire of hormones, growth factors and cytokines. The placenta produces some of these hormones exclusively (Ogren and Talamantes 1994). These true placental hormones include the conceptus interferons, chorionic gonadotropins (hCG), and members of the growth hormone (GH) – prolactin (PRL) – placental lactogen (PL) gene family (Talamantes and Ogren 1988; Roberts *et al.* 1992). Fully differentiated trophoblast cells at the maternal-fetal interface secrete PL into both maternal and fetal vasculature. The inferred actions of PL on the maternal and fetal tissues are paradoxical and have been hypothesized to

establish a nutrient gradient between the mother and fetus, which facilitates the transfer of nutrients across the placenta to supply the fetus (Handwerger 1991). However, these data are implied and a true test of the necessity of PL remains to be determined, although classical ablation-replacement experiments are not feasible due to the requirement of the placenta during pregnancy (Browne and Thornburn 1989). Understanding the transcriptional regulation of the PL gene may allow us to modulate hormone production and secretion, thereby permitting researchers the opportunity to fully ascertain the endocrine actions of PL on maternal and fetal tissues.

Placental Types

The placenta is an organ that has a limited lifespan, exhibits varying degrees of association between maternal and fetal tissues, and in comparison to other conventional organs exhibits a great deal of diversity between species. Chorio-allantoic placentas represent the definitive placenta for a majority of mammals. The chorion is the outer membrane, and a vascular bridge is formed between the chorion and embryo/fetus by its fusion with the outer mesoderm of the allantois (Amoroso 1952; Steven 1975). This gives rise to the placenta, which attaches to the uterus to bring the fetal and maternal vasculature into close proximity, allowing for nutrient exchange. A transient structure resulting from the apposition of the chorion and yolk sac, the chorio-vitelline placenta, is formed early in gestation and is responsible for temporary support of the embryo while the chorio-allantoic placenta forms (Steven 1975).

The morphological shapes of the chorio-allantoic placenta fall into four broad classifications, which were described by Fabricius in 1604 (reviewed by Steven 1975).

These placental shapes include: diffuse, cotyledonary, zonary, and discoid. The chorion of the diffuse placenta (mare and pig) is mostly covered with small villi or folds that are in contact with the sulci of the uterine epithelium. The cotyledonary placenta (ruminants) restricts the villous outgrowth to isolated regions within the uterus called caruncles, and these areas are separated by smooth chorion. The placentome is the unit encompassing both the fetal and maternal tissues (Amoroso 1952). The number of placentomes can vary greatly from 4-6 in the deer to more than 160 in the goat and giraffe (Amoroso 1952; Steven 1975), and the structure of placentomes formed can be concave (sheep) or convex (cow). The zonary placenta is found in carnivores like the dog and cat, and forms a chorionic villous region that encircles the equatorial area of the chorion. The placenta found in man and rodents has a discoid appearance, in which the villi are arranged as a circular plate (Steven 1975).

Placenta classifications have also been based on the number of tissues layers intervening maternal blood and fetal blood. These tissue layers include: fetal endothelium, fetal connective tissue, fetal epithelium, maternal epithelium, maternal connective tissue and maternal endothelium. Grosser's (1909) broad classification of placenta types are: 1) epitheliochorial, with all six layers present; 2) syndesmochorial, describes a discontinuous uterine epithelium; 3) endotheliochorial, with the three fetal layers and the endothelial layer of the mother intact; and 4) hemochorial, with only the three fetal layers intact, and the maternal blood bathing the fetal epithelial layer (reviewed by Amoroso 1952; Steven 1975). The morphological shape and tissue structures of the placental are used to define the type of placenta formed in each species.

Human and rodent placenta fall into the discoid-hemochorial classification, where the placenta has a discoid shape and the fetal epithelium is in direct contact with the mother's blood. Ruminant placenta was initially classified as a cotyledonary-syndesmochorial type. However, classification of ruminant placenta has gone through modifications since Grosser's initial classification. Early studies indicated the maternal connective tissue was adjacent to the fetal epithelium (Steven 1975), and the term syndesmochorial placenta was established, meaning chorion adjacent to connective tissue. Recent evidence indicates that uterine epithelium persists in a syncytial form and chorionic epithelial cells, specifically the binucleate cells, migrate to the uterine epithelium and fuse with the syncytial layer (Amoroso 1952; Ramsey 1982; Steven 1983; Wooding 1992). The fusion of these two tissue layers does not completely negate the maternal epithelium, but it is no longer a finite layer. Therefore, the term synepitheliochorial placenta was adopted for the classification of ruminant placenta, and it is now classified as a cotyledonary-synepitheliochorial type (Wooding 1992). These morphological differences indicate a great deal of evolutionary pressures on mammalian placental structure. Nevertheless, placenta function appears quite similar with respect to the duties it is required to carry out for mammals.

Placenta Cellular Components

Placenta cellular architecture for the human and rat differ slightly in their organization within the mature placenta. Nevertheless, certain types of differentiated trophoblastic cells that express PL appear to be analogous in function and location. Invasion of the human blastocyst occurs at the polar trophectoderm, which forms

progenitor trophoblast cells that are classified in two categories by the number of nuclei; syncytiotrophoblast cells (multinuclear) and cytotrophoblast cells (mononuclear; Aplin 1991; Ringler and Strauss 1990). The syncytiotrophoblast cells form the interface with an underlying layer of cytotrophoblast cells. Cytotrophoblast cells migrate deeply into the uterine stroma and reach the myometrium. These cells erode the endothelial layers of the maternal vessels and replace the cellular layer, which places them in direct contact with maternal blood (Aplin 1991; Ohlsson *et al.* 1993). The cytotrophoblast cells are the progenitor cells for the endocrine producing syncytiotrophoblast cells that are responsible for the placenta's endocrine activity (Talamantes and Ogren 1988; Page 1993). Cytotrophoblasts proliferate and increase the placental size, and during this growth they fuse with the overlying syncytium to expand the surface area in contact with the maternal blood supply (Pierce and Midley 1963).

Decidual cells actually surround the rodent blastocyst during attachment. The decidua basalis is adjacent to the embryo, whereas the decidua capsularis is the portion of the uterus closest to the lumen surrounding the embryo (Soares *et al.* 1991). A single layer of differentiated trophoblast cells, trophoblastic giant cells, fuses with the endoderm of the yolk sac to form the chorio-vitelline placenta, which has physiological significance from implantation to mid-gestation (day 14) and then disappears (Soares *et al.* 1996). After this the chorio-allantoic placenta assumes responsibility for the developing fetus and develops at the decidua basalis forming two distinct zones, the junctional zone and the labyrinth zone (Soares 1987; Soares *et al.*1991; Soares *et al.* 1996; Soares *et al.*1998). Four distinct trophoblast cell phenotypes are found in the rodent placenta. Trophoblast giant cells (TGC) arise by endoreduplication (Ilgren 1983; Varmuza *et al.* 1988), and

possess invasive and endocrine activity. In the junctional and labyrinth zones, TGC are situated at the maternal-placental border (Soares *et al.* 1998). Glycogen cells and spongiotrophoblast cells are cellular components of the junctional zone underlying the TGC (Soares 1997; Soares *et al.* 1998). The function of glycogen cells is thought to be a potential energy reservoir (Davies and Glasser 1968). Spongiotrophoblast cells possess endocrine activity by secreting other PRL family members and are thought to arise from trophoblastic stem cells, but are morphologically distinct from TGC (Soares *et al.* 1996). Syncytiotrophoblast cells of the rodent placenta reside in the labyrinth zone at the placental-fetal interface and appear to be involved in nutrient and waste exchange (Sibley 1994; Soares 1997; Soares *et al.* 1998). The recruitment of stem cell proliferation or differentiation into one of the four trophoblastic phenotypes has not been defined (Soares *et al.* 1996).

Ruminant placentation varies slightly from the previous description presented for human and rodents due to the lack of trophectoderm invasion and uterine response. The ovine blastocyst hatches at day 8 and elongates from day 12-14, by which time it is 100 mm in length (Rowson and Moor 1966). In sheep, contacts begin to form at the 15th day, and by the 20th day post coitus apposition and attachment over the entire uterine surface can be demonstrated, and is described to be similar to diffuse placenta (Davies and Wimsatt 1966). By the 4th week, the chorio-allantoic placenta develops cotyledons at the uterine caruncular epithelium to form the placentomes (Boshier 1968; King *et al.* 1982). Binucleate cells are found in the chorion of the ruminant conceptus by day 16 in sheep (Boshier 1969) and day 19 in cattle (Wathes and Wooding 1980). These cells have been shown to migrate into the maternal uterine epithelium and fuse with maternal epithelial

cells and/or uterine syncytium (Wooding 1982; King *et al.* 1982). Binucleate cell migration and fusion results in the displacement of a finite maternal epithelium layer. In the cow, continued growth of uterine epithelial cells and cessation of multinucleate cell formation with uterine epithelium at day 28, reestablishes a distinct maternal columnar epithelial layer (Wathes and Wooding 1980). In sheep isolated regions or plaques of maternal-fetal syncytium persist throughout gestation (Wooding 1992). Binucleate cells migrate out of the chorion at a greater rate in the sheep throughout gestation, thus accounting for the persistence of the maternal-fetal syncytial plaques (Wimsatt 1951). The migrating population of binucleate cells in the ruminant placenta accounts for 15-20% of the total binucleate cell population, which resides in the chorionic epithelium. This epithelial layer covers the villous surface of the ruminant placenta and the binucleate cells comprises approximately one-fifth of the chorionic epithelium (Wooding 1983; Wooding *et al.* 1986; Wooding 1992). Binucleate cells are derived from differentiating cytotrophoblast cells, which perform two important functions. First as an important player of placentation and secondly the major endocrine cell secreting protein and steroid hormones (King *et al.* 1982; Wooding 1992).

Placental classification and cell types have been described in regards to location and function for the differentiated trophoblast cells. An understanding of placental structure, morphological changes and the development of trophoblast cells in each of these placental types will aid in further discussion of hormone secretion and transcriptional regulation. Placental lactogen expression and secretion in the human, rodent, and ovine placenta occurs in the fully differentiated trophoblast cells that form at the maternal-fetal interface. These differentiated trophoblast cells have similar function in that they secrete

hormones, are involved in placenta attachment, and transport nutrients between the mother and fetus. Therefore, it is thought that these cells are analogs of one another.

• Structure Of Placental Lactogen

Between species the chemical and biological properties of placental lactogen vary significantly, but structurally they can be categorized into two groups. The first group is GH-like and this group consists of PLs from primates that share greater homology to GH (Anthony *et al.* 1995b). Human (h) PL has been the most extensively studied, since it was initially purified (Ito and Higashi 1961; Josimovich and McLaren 1962). Following hPL purification, PLs have been described for the baboon (Josimovich *et al.* 1973), rhesus monkey (Shome and Friesen 1971; Vinik *et al.* 1973), chimpanzee and orangutan (Forsyth 1986). Human PL is a single chain, nonglycosylated, 191 amino acid polypeptide, which has a molecular weight of 22,279 (Talamantes and Ogren 1988). Comparison of amino acid sequences for hPL and hGH reveal 87% identity (Bewley *et al.* 1972a), with only 23% amino acid sequence identity to hPRL (Shome and Parlow 1977). Two disulfide bridges form between Cys^{53} and Cys^{164} and between Cys^{181} and Cys^{188}. The formation of these bonds are hypothesized to stabilize the secondary and tertiary structure of hPL and hGH. They have been shown to be required for full immunological activity, but not for lactogenic activity (Beck and Catt 1971; Handwerger *et al.* 1972). Inference from studies on hGH structure insinuate that the growth-promoting activity of hGH requires these disulfide bridges to inhibit protein degradation in rat tibia tests (Bewley *et al.* 1969). Another report indicated that cleavage of one disulfide bridge decreased growth-promoting activity by 50-80% (Mills and Wilhelmi

1968). In either case the lactogenic activity of hGH was unaffected (Mills and Wilhelmi 1968; Bewley *et al.* 1969). Studies on the chemical nature of hGH and hPL revealed very strong secondary and tertiary interactions, due to their notable resistance to acid, alkali or urea denaturation (Aloj *et al.* 1972). The disruption of the disulfide bonds in hPL altered the secondary and tertiary structure of the protein, which affected the immunological properties of the protein, but growth promoting activity was not assessed (Aloj *et al.* 1972). These data coincide with the conclusion that hGH has a greater conformational stability than hPL (Handwerger *et al.* 1972; Aloj *et al.* 1972). A single tryptophan residue buried in the middle of the polypeptide was found to be responsible for maintaining hPL's somatogenic and lactogenic activity, but less of an affect was observed with alteration of the hGH tryptophan residue (Bewley *et al.* 1972b; Handwerger *et al.* 1972; Neri *et al.* 1973; Russell *et al.* 1981). These data suggest that although little diversity exists between the amino acid residues of hGH and hPL, physicochemical properties differ between the two hormones.

The second category of PL is the PRL-like group, which comprise the rodent and ruminant PLs. Rodents have two distinct PLs, PL-I and PL-II, which are temporally secreted at the utero-placental border. Mouse PL-I (Colosi *et al.* 1987a) exists as acidic N-linked glycoprotein in two molecular-weight forms (M_r 29-32k and 36.5-42k). Rat PL-I was partially purified and described to be high-molecular-weight molecules, as well ranging between 40-50,000 (Robertson *et al.* 1982). For both mPL-I (Colosi *et al.* 1987b) and rPL-I (Robertson *et al.* 1990) a 30 amino acid signal peptide sequence must be cleaved off to give mature proteins of 194 and 200 amino acid residues, respectively, which contain two N-linked glycosylation sites. In the mouse the amino acid sequence

identity between mPL-I and mPL-II is 44%, 33% for mPRL and 21% for mGH (Colosi *et al.* 1987b). Placental lactogen-II has been identified in the mouse (Jackson et al. 1986), rat (Duckworth et al. 1986), and hamster (Southard et al. 1986), and the mature forms are nonglycosylated 191 amino acid polypeptides with an apparent M_r between 20-25,000. Recently a third PL was found in the rat placenta (Robertson *et al.* 1991; Deb *et al.* 1991). Rat PL-I variant (PL-Iv) shares 85% amino acid sequence identity to rPL-I, and has a M_r of 33,000. Temporal and cellular expression patterns, distinct from PL-I, may infer distinct actions for PL-Iv (Deb *et al.* 1991).

Ruminant PLs have a similar relationship to PRL, but unlike the rodents only a single polypeptide has been identified in each species (Anthony *et al.* 1995b). Placental lactogens have been identified in cattle (Murthy *et al.* 1982), sheep (Martal and Djiane 1975), and goats (Currie *et al.* 1990). The amino acid sequences of cattle (Schuler *et al.* 1988; Yamakawa *et al.* 1990), sheep (Colosi *et al.* 1989; Warren *et al.* 1990) and goat (Sakal *et al.* 1998) PLs have been deduced from their cDNA sequences. Ruminant PL share between 47-50% amino acid sequence identity to PRL, but only 22-25% to GH. The most interesting comparison of PL is between species. Bovine (b) PL shares 67% identity with ovine (o) PL and caprine (c) PL, but the sequence identity between ovine and caprine is only 86%. This suggests a close relationship between species, but when compared with other family members, like GH or PRL, which have ~99% interspecific identity, questions are raised about the divergence of the genes encoding these proteins and the evolutionary pressures that influenced them (for review Wallis 1993; Anthony *et al.* 1995a; Anthony *et al.* 1998). Ovine PL and cPL are nonglycosylated polypeptides with an apparent M_r of ≅22,000 and ≅23,000, respectively. Bovine PL was shown to be

glycosylated by both N- and O-linked oligosaccharides, and the polypeptide exists in multiple isoforms with a M_r between 32-34,000 (Murthy *et al.* 1982; Colosi *et al.* 1989; Warren *et al.* 1990; Sakal *et al.* 1998). Chemical and physical analysis of the structure of oPL has revealed that it has three disulfide bonds, forming three loops in the protein, indicating the secondary structure of oPL is similar to that of oPRL (Hurley *et al.* 1977). Modification of Trp^{150} diminished the binding activity of oPL to rat somatogenic (28.7%) and lactogenic receptors (22.6%) similar to what was described for oPRL (Kawauchi *et al.* 1973; Cymes *et al.* 1993). A tyrosine residue (Tyr^{46}) has been shown to be involved in the binding capacity of the hormone to lactogenic and somatogenic receptors in rat liver (Cymes and Wolfenstein-Todel 1996). A three-dimensional structure of oPL bound to the rat PRL receptor extracellular domain identified 25 residues of oPL that comprise binding site I, and 24 residues that comprise site II (Christinger *et al.* 1998). Additionally, interaction of ruminant PLs at site I with the extracellular domain of hGH receptor was confirmed by mutational analysis which changed Thr^{185} to Phe in recombinant oPL, and The^{188} to Asp in recombinant bPL (Herman *et al.* 1999). Site II mutations in oPL (Gly^{130} to Arg) and bPL (Gly^{133} to Arg) resulted in a 1:1 hGH receptor:ruminant PL complex (Herman *et al.* 1999). However, the validity of these data become questionable when one considers the evolutionary divergence between ruminant PLs, and the fact that these studies were performed in a heterologous system. However, similar amino acid residues for hPL, the ruminant PLs and other family members were demonstrated to be important for receptor binding by direct interaction or structural conformation of the protein. These data may link the physiochemical properties of these

hormones with their homologous receptors, but subsequent analysis is needed with their specific receptors.

Synthesis And Secretion

Differentiated trophoblast cells that line the maternal-fetal interface secrete PL. In human placenta, syncytiotrophoblast cells arise from cytotrophoblast (Langhan's cell) migrating into the syncytium. Ruminant chorionic binucleate cells express PL, and arise from cytotrophoblast cells. Giant trophoblast cells of the rodent placenta express PL-I and PL-II, which differentiate by endoreduplication from stem cells (Soares *et al.* 1996). The expression of PL from these analogous cell types appears to be associated with the development and differentiation of trophoblast cell types.

Human

The messenger (m) RNA for human PL has been localized to syncytiotrophoblast cells after week 6, and cellular concentration does not appear to change from week 6 to term (McWilliams and Boime 1980; Boime *et al.* 1982; Hoshina *et al.* 1982; Maruo *et al.* 1992). Cellular localization of PL before the sixth week of gestation identified PL synthesis in the cytotrophoblast or undifferentiated stem cells, suggesting a developmental switch in hPL production in the early placenta (Maruo *et al.* 1992). The hormone was detectable in the placenta by day 18 of pregnancy (McWilliams and Boime 1980). Detection of hPL in maternal serum by the third week is followed quickly by increases during the first trimester, and continued gradual increases until term, with a maximal concentration of 5-10 μg/ml (Beck 1970; Biswas *et al.* 1972; Tyson 1972;

Braunstein *et al.* 1980). By the end of pregnancy, about 5% of the total placental mRNA is hPL (Chen et al. 1989), which can produce 0.5-1 g of protein per day (Beck and Daughaday 1967; Kaplan *et al.* 1968; Tyson, 1972). Pregnancies with placental insufficiencies or reduced placental weight have reduced hPL maternal serum concentrations (Saxena *et al.* 1969). Additionally, a significant correlation between placental weight and hPL concentrations was observed when blood samples were taken before labor (reviewed by Tyson 1972). The increase in hPL during pregnancy seems to be correlated with placental weight, which may be accounted for by the increase in syncytiotrophoblast cells as gestation progresses (Pierce and Midley 1963). The only instances where a decline in maternal hPL concentration was described were associated with post-maturity and ante-partum fetal distress (Saxena *et al.* 1969). Collectively these data suggest the hPL is constitutively produced, and the gestational increase is associated with an increase in syncytiotrophoblast cells not a stimulation of hPL transcription or secretion.

Placental lactogen is also secreted into fetal blood, but the concentrations are considerably lower. Fetal serum concentrations range from 4-500 ng/ml between weeks 12-20 of gestation, and 20-30 ng/ml at term (Crosignani *et al.* 1972; Hill *et al.* 1988). PL does not cross the placental barrier (Kaplan *et al.* 1968) and must be secreted into both maternal and fetal vasculature from the syncytiotrophoblast cells.

Rodent

In rodents, PL-I and PL-II are temporally secreted from the trophoblastic giant cells in the chorio-vitelline and chorio-allantioc placentas (Robertson *et al.* 1982; Soares *et al.*

1983; Colosi *et al.* 1987a). Mouse PL-I is present at day six of gestation at low concentration (20 ng/ml), and rapidly increases until mid-gestation, peaking at day 9 with a concentration of 8 μg/ml in the maternal serum (Colosi *et al.* 1986). Mouse PL-I declines to undetectable levels by day 13 and remains undetectable through the remainder of pregnancy (Colosi *et al.* 1986). Mouse PL-II is present at day 9 at a concentration of 1 ng/ml, and gradually increase to parturition with maternal serum levels from 135 ng/ml to greater than 250 ng/ml depending on the strain of mouse (Soares and Talamantes 1982; Soares and Talamantes 1983). Genetic affects on the secretion of mPL-II were observed and crossbreeding experiments confirmed these results. When males of one mouse strain were mated with females of a different strain, the concentration of mPL-II differed (usually higher) from the profiles of the non-out bred female stain, indicating that mPL-II secretion was influenced more by the fetal-placental unit than the maternal environment (Soares and Talamantes 1982; Soares and Talamantes 1983). Changes in placental morphology or PL clearance rates were two hypothesizes given for the increased hormone concentrations in the hybrid mice (McLaren 1975; Sinha *et al.* 1979; Soares and Talamantes 1983). The number of conceptuses was also found to be associated with the concentration of mPL-II in maternal serum (Soares and Talamantes 1983). Mouse PL-II is also secreted into the fetal compartment, with detectable levels observed in fetal blood and amniotic fluid. In the amniotic fluid, mPL-II was found from day 10 to parturition, peaking at day 14, whereas in fetal serum mPL-II was not detectable until day 15 (Talamantes and Ogren 1988).

The rat placenta also produces and secretes two PLs from the trophoblastic giant cells into maternal blood, in the same temporal fashion as seen in the mouse. Rat (r) PL-I

was detected in maternal serum by day 8, peaks at day 12 (~3 μg/ml) and has disappeared by day 15 (Robertson *et al.* 1982). A second hormone, rPL-II was present at day 14 and increased to a concentration of ~500 ng/ml a day prior to parturition (Klindt *et al.* 1981; Robertson *et al.* 1982). The rPL-I variant (rPL-Iv) was identified late in gestation, between day 15 and 20, and *in situ* hybridizations reveal that it is a product of the cytotrophoblast cells, but also was identified in giant trophoblast cells of the junctional zone (Robertson *et al.* 1991). Serum profiles for rPL-Iv in pregnant rats detect the hormone at day 14, which exponentially increases until day 18 peaking at 2 μg/ml and dropping off slightly by term (Robertson *et al.* 1996).

In contrast to human and ruminant PL's, pituitary and ovarian hormones effect the secretion of PL-II. Hypophysectomies in mice and rats at mid-gestation increase the concentrations of PL-II, and indicate some pituitary control of the hormone in rodents (Blank and Dufau 1983; Voogt *et al.* 1985; Day *et al.* 1986). The effects on mPL-II did not alter the placental mass, and the concentration of the hormone in placental homogenates remained unchanged, suggesting that synthesis and secretion are unaltered (Day *et al.* 1986). Clearance of mPL-II is diminished with the removal of the hypothalamus, thus increasing the mPL-II concentrations in maternal circulation (Pinon *et al.* 1988). In contrast to these data, the effects of the ovary on PL-II secretion appear to be inhibitory, since bilateral ovariectomy results in an increase in PL-II (Robertson *et al.* 1984a; Robertson *et al.* 1984b; Soares and Talamantes 1985). In the mouse the addition of progesterone to placental explants inhibits the secretion of mPL-II into the medium (Soares and Talamantes 1985). In the rat, the affects of the fetus appear to be stimulatory since fetectomy suppress rPL-II, but removing both the fetus and the

mothers' ovaries increased PL-II. Replacement therapy with estradiol, to these ovariectomized-fetectomized rats, decreased the PL-II concentration similar to the fetectomized rats (Robertson *et al.* 1984b). These experiments suggest that pituitary factors do not regulate PL-II secretion, but influences from the ovary and the fetus can affect PL-II production.

Ruminant

Ruminant PL is produced in chorionic binucleate cells of the placenta in both cattle and sheep (Wooding and Beckers 1987; Milosavljevic *et al.* 1989; Kappes *et al.* 1992). The hormone has been localized in the trophectoderm at day 17 in cattle (Kessler *et al.* 1991a) and day 16 in sheep (Martal and Djiane 1977; Carnegie *et al.* 1982). Discrepancy in the location of trophoblast cell expression exists in the literature, with PL identified in the mononucleate cells of the extraembryonic tissue (Carnegie *et al.* 1982; Kessler *et al.* 1991a), but by day 22 in cattle and day 28 in sheep the binucleate cells were the only trophoblast cell expressing PL (Carnegie *et al.* 1982; Wooding and Beckers 1987). However, these reports do not coincide with Boshier (1969), which identified binucleate cells in the ovine trophectoderm at day 16. Therefore, the identification of PL in these early mononucleate cells of the ruminant placenta may be an artifact of the antiserum used to localize PL. The expression of PL in later gestation (after day 50) is restricted to the BNC for the duration of gestation (Martal and Djiane 1977; Milosavljevic *et al.* 1989; Kappes *et al.* 1992). A change in cytotrophoblast to binucleate cell expression, which was observed in the human trophectoderm is not thought to occur in the ruminant placenta.

The chorionic binucleate cells migrate out of the chorionic epithelium and fuse with cells in the uterine epithelium to form trinucleate cells or a syncytium (Wooding 1992). The migration of these cells into the maternal tissue may represent the method of delivering PL into the maternal vasculature. Maternal oPL serum concentration is associated with the number of BNC, increasing from 7.1 ng/ml at day 60 to ≥1 μg/ml at day 135 of gestation (Taylor *et al.* 1980; Kappes *et al.* 1992). Maternal serum concentration of bPL is much lower and may coincide with the decreased migration of binucleate cell out of the chorionic epithelium. Bovine PL can be detected by the fourth month, peaks at ≤ 3 ng/ml and remains constant throughout gestation (Beckers *et al.* 1982; Wallace 1993). In the fetus, oPL serum concentrations increase from day 60 (11 ng/ml) to day 90 (29 ng/ml) and remain constant throughout the remainder of pregnancy (Kappes *et al.* 1992). Similarly, bPL fetal serum concentrations are detectable by the second month and peak at 20-30 ng/ml by day 110 (Beckers *et al.* 1982).

Maternal and fetal hypothalamic, pituitary and adrenal hormone fluctuations do not appear to affect oPL secretion. Fetal adrenalectomy (Wintour *et al.* 1982), fetal hypophysectomy (Taylor *et al.* 1983), or acephali (Nie and Chan 1990) do not alter normal fetal or maternal oPL concentrations. Additionally the infusion of somatostatin, TRH, or dopamine agonists and antagonists had no effect on oPL concentrations in maternal or fetal plasma (Taylor *et al.* 1983). A live fetus seems to be the only requirement to maintain oPL synthesis and secretion, suggesting that there may be a positive control signal from the fetus for oPL production (Taylor *et al.* 1983). However, fetal signaling may be required to maintain the placenta, thereby inhibiting the demise of the binucleate cells. The increase of oPL in maternal serum during gestation is correlated

to the increase in binucleate cell number (Kappes *et al.* 1992), and production/secretion of oPL was indicated to be constitutive.

Mechanism of Action

Activity and influences of a hormone on a specific tissue must be mediated though a receptor to elicit a cellular response. Placental lactogen's ability to stimulate fetal growth and modulate the maternal system has not been directly linked to a specific receptor. The difficulty associated with identifying a specific PL receptor was attributed to the binding affinities that PL shares with the GH and PRL receptors (as reviewed by Anthony *et al.* 1997). Therefore, the mechanism of action of PL may involve a variety of binding sites, which include a specific PL receptor (PLR; unidentified), the GH receptor (GHR), and/or the PRL receptor (PRLR). The affinity PL shares for the GH/PRL receptor family may indicate that PL acts through one of these receptors or a similar receptor type.

The GHR/PRLR receptors are members of the cytokine receptor super family, which are single transmembrane-domain tyrosine kinase-associated receptors that activate the Janus Kinase (JAK) family of tyrosine kinases (Leung *et al.* 1987; Cunningham *et al.* 1991; Darnell *et al.* 1994; Edens and Talamantes 1998). JAK 2 is phosphorylated by ligand binding to GHR/PRLR, and modulates the actions of signal transduction activators of transcription (STAT) proteins (GHR; STAT-1,3,5 and PRLR; STAT-5) which are phosphorylated, dimerize, and move into the nucleus to initiate transcription of genes (Dusanter-Fourt *et al.* 1994; Wakao *et al.* 1994; Han *et al.* 1996). Recent signal transduction studies of hPL on the hGHR and hPRLR demonstrate the phosphorylation of JAK 1 and JAK 2 (Takeda *et al.* 1997). STAT-3 in these cells was activated, and

suggests a JAK-STAT signal transduction mechanism (Takeda *et al.* 1997). In addition to JAK and STAT proteins, two other proteins were phosphorylated that are smaller than STAT-3, but their identity is unknown (Takeda *et al.* 1997). These data implicate an alternative transduction scheme by hPL, through PRLR and GHR, which were both present on the cells and phosphorylated following hPL binding (Takeda *et al.* 1997).

In the human fetus, distinct binding sites for hPL were demonstrated in the liver and skeletal muscle (Hill *et al.* 1988). The affinity for hPL to the hepatic binding site was greater than that for hGH and hPRL, but GH and PRL binding sites were observed for the hepatic microsomes (Hill *et al.* 1988). However, unlabeled competition of hPL or hGH in the presence of radiolabeled hPL or hGH were not equivalent, thereby suggesting that the respective binding site for these hormones were different (Hill *et al.* 1988). On the other hand, skeletal muscle only exhibited binding sites for hPL and had no affinity for hGH or hPRL (Hill *et al.* 1988). Confirmation of these results was obtained by immunostaining for GHR in fetal tissue. The GHR was localized to the fetal liver, pancreas, kidney, skin, and cerebral cortex, but not in fetal skeletal muscle, cardiac muscle, adrenal gland, intestine, lung or epiphyseal growth plate (Kawauchi *et al.* 1973). In postnatal tissues GH binding greatly exceeds that described in fetal tissues, and the maternal hGHR binding protein (extracellular domain) has a 2,300-fold lower affinity for hPL than it does for hGH (Lowman *et al.* 1992). The hPRL receptor binding protein interacts with both hGH and hPL with similar affinity, albeit lower than its affinity for hPRL (Lowman *et al.* 1992).

Rodent PRL, PL-I and PL-II are analogs of one another and possess the actions of pituitary PRL (Kelly *et al.* 1975; Colosi *et al.* 1982; Soares and Talamantes 1985; Soares

et al. 1983; Colosi *et al.* 1987a). The target tissues of these hormones in rodents are the mammary gland, ovary (corpus luteum), uterus, liver, immune system, extraembryonic and embryonic tissues (Soares *et al.* 1991). Rodent PL's bind only to the PRLR and do not have specific affinity for the GHR (Talamantes and Ogren 1988). The interaction of fetal GH within the system is unclear and the GHR is hypothesized not to be present until late in gestation. This was deduced by the presence of a GHR splice-variant alteration in the 5' untranslated region (UTR) expressed in fetal tissue. The fetal GHR 5'-UTR has a high GC content, and includes a short open reading frame from a preferred translational initiation site (Pekhletsky *et al.* 1992; Anthony *et al.* 1997; Edens and Talamantes 1998; Moffat *et al.* 1999). These attributes may inhibit translation of GHR by increased secondary structure of mRNA or translating the short open reading frame instead of the mGHR (Anthony *et al.* 1997). Prolactin receptors, with various affinities for the PRL family of placental hormones, have been postulated to invoke the responses of the PL's (Glaser *et al.* 1984).

Ovine PL binding studies, using maternal and fetal liver microsomes, have demonstrated a specific high affinity interaction (K_d=0.12-0.27 nM) that is distinct from the GHR and PRLR (Freemark *et al.* 1986; Pratt *et al.* 1995b). The receptor concentration expressed per mg of protein remains relatively constant across gestation, but significantly increases within one week following birth (Freemark *et al.* 1986; Pratt *et al.* 1995b). This increase was not due to the lack of endogenously bound oPL, but may result from decreased receptor internalization due to the loss of ligand (Freemark *et al.* 1986; Pratt *et al.* 1995b). Fasting decreases the number of oPL receptors in maternal and fetal liver microsomes, and binding sites are reestablished by refeeding (Freemark *et al.*

1990). Purification of the PLR from fetal liver microsomes identified a binding protein with the apparent M_r of ≅44,000, which interacted specifically with oPL (K_d 0.5 nM), and the potency for GH and PRL was 30-50 and 500-1000 times less, respectively (Freemark and Comer 1989). Fowlkes and Freemark (Fowlkes and Freemark 1992) demonstrated oPL stimulated amino acid transport, DNA synthesis and IGF-1 production in fetal fibroblast cells, but GH had no affect. Binding studies reveal a specific oPL interaction with a K_d of 0.2 nM, and crosslinking studies identified an interaction with a protein with an apparent M_r of ≅108,000 (Fowlkes and Freemark 1992). Combined these data may indicate heterogeneous PLR, proteolysis of PLR during preparation to form the M_r of 44,000 binding protein, or a complex formation which generated a M_r of 108,000 (Fowlkes and Freemark 1992). Recently, Gertler and Freemark have suggested that the 44,000 PLR was an artifact, which in retrospect seems plausible because of the change in affinity. Further analysis of the larger PLR isoform has not been reported to date, and definitive conclusions on PLR in the fetus remain to be demonstrated. However, fetal oPL concentration late in gestation is 29 ng/mL or 1.3 nM (Kappes *et al.* 1992) and under physiological conditions the fetal PLR with a K_d 0.2 nM would be occupied by oPL (Fowlkes and Freemark 1992; Anthony *et al.* 1997). Furthermore, saturation analysis using radiolabeled GH or PRL on sheep fetal liver membranes did not identify specific binding, whereas saturable binding was obtained with oPL (Pratt *et al.* 1995b). These data provide evidence for a specific PLR in the fetus, but not a GHR or PRLR.

Transcripts encoding GHR have been demonstrated in fetal liver, but were ~300 bp larger than the maternal liver GHR mRNA (Pratt and Anthony 1995a; Anthony *et al.* 1997). The larger oGHR mRNA possesses a GC-rich segment with a short open reading

frame in the 5' untranslated region, which is thought to impede translation of the oGHR (Anthony *et al.* 1997). This transcript resembles the splice variant described in mouse fetal tissues. Therefore, inhibiting GHR translation would decrease the presence of the receptor on the membrane, and possibly explain the lack of specific oGH binding to ovine fetal liver.

Converse biological effects of PL on fetal and maternal tissues may implicate two independent receptors between maternal and fetal tissues. Placental lactogen may exhibit its action through the maternal GHR and/or PRLR and activate an alternative signal transduction pathway to regulate maternal metabolism. The somatogenic effects of PL in the fetus indicate action though a unique receptor, PLR, which mediates a signal transduction system regulating fetal growth. Placental lactogen's response via a specific receptor in ruminants' remains unresolved due to the inability of purifying PLR, but its actions in the maternal system have been studied using the GH and PRL receptors.

The actions of PL in the mother may be working through the GHR and/or PRLR (Hill and Hogg 1989; Wells 1994; Anthony *et al.* 1995a; Anthony *et al.* 1995b). Peak concentration of PL in maternal sera of humans (5-7 μg/mL; Handwerger, 1991), sheep (902 ng/ml; Kappes *et al.* 1992), and mice (mPL-I 8 μg/ml; Colosi *et al.* 1986; mPL-II 100 ng/mL Soares and Talamantes 1982) indicate excessive ligand relative to receptor affinity, even with lower binding affinities (Anthony *et al.* 1997). Adding PL to the maternal circulation has resulted in inconsistent results, and rightly so as the receptors are already likely saturated (reviewed in Anthony *et al.* 1997). Human PL phosphorylated both the GHR and PRLR *in vitro* (Takeda *et al.* 1997), but potentially different intracellular proteins were phosphorylated suggesting a different signal transduction

cascade. Therefore, the action of these hormones on maternal tissues may be via the GHR and PRLR and not a separate or new receptor.

Binding studies with the extra cellular domains (ECD) of human, rabbit and ovine GHR to oPL have given rise to some questions on the ability of oPL to generate a 2:1 complex with the oGHR (Herman *et al.* 1999). Ovine PL was capable of binding human and rabbit (rb) GHR-ECD in an appropriate fashion, but oPL and oGHR-ECD only bound 1:1, whereas oGH bound 1:2 with oGHR-ECD. Additionally, mutation of oPL in site I (T185D) blocked formation of a ligand-receptor complex, but a mutation in site II (G139R) exihibited a 1:1 stoichiometry with h/rb/oGHR. Therefore, the binding of oPL to oGHR-ECD requires site I (Herman *et al.* 1999). The functionality of these hormones was tested in stable and transiently transfected cells with the full length human, rabbit, or ovine GHR. Biological activity of oPL was conveyed through the rabbit and human GHR, but studies with the oGHR revealed that oPL was an antagonist, which was hypothesized to be the result of forming a 1:1 complex (Herman *et al.* 1999). The oPL G130R recombinant protein had antagonistic action with all the species GHR, which supports the hypothesis that receptor dimerization is required for biological activity (Herman *et al.* 1999). The authors indicate that this was not specific for sheep, and similar results were seen with bPL and cPL, possibly indicating that all ruminant PLs are antagonists of GHR action (Herman *et al.* 1999). Additionally, Herman *et al.* (1999) suggest that heterodimerization needs to be reexamined because of the inability of site II to specifically interact with oGHR. The results of Herman *et al.* (1999) must be interpreted cautiously because: 1) generation of stable GHR cell lines for which cell proliferation was assessed was not performed with the oGHR; 2) transiently transfected

cells were analyzed with a STAT 5 responsive element, and did not consider STAT 1 or 3; 3) the cell lines transfected are heterologous carriers of the receptors and may not have the necessary signal transduction pathway to mediate the responses of oGHR:oPL binding; and 4) they did not test if the oGHR was phosphorylated by oPL, which would indicate that the signal transduction pathway is different, as suggested by Takeda *et al.* (1997). Therefore, the inhibitory action of PL on GHR may be an artifact of the cell system or the lack of analyzing all possible transduction schemes, and the enhancement of PL on h/rbGHR may be the result of species difference.

However, this report (Herman *et al.* 1999) did identify a critical factor that supports further examination of the possibility of heterodimerization of receptors. The point was that oPL T185D stimulated Nb2 proliferation via the rPRLR, whereas G130R was a weak promoter of proliferation of Nb2 cells (Herman *et al.* 1999). These data coincide with the evidence provided for the structure of PLs, which suggested that lactogenic and somatogenic effects of PL is regulated through different epitopes of the protein. A subsequent study revealed that 293-HEK cells, co-transfected with the oGHR, oPRLR, and a STAT 5 response element *tk*-luciferase reporter vector, exhibited hormone-induced activity with oPL or bPL, but mutant analogs oPL and bPL were inactive (Herman *et al.* 2000). Additionally, chimeric receptors with the cytoplasmic domains of GHR or PRLR and α or β extra cellular domain (ECD) of the human granulocyte and macrophage-colony stimulating factor (GM-CSF) receptor were generated. GM-CSF binds α and β ECD to form a heterodimer and stimulate a signal transduction domain that leads to enhanced reporter activity. Transient transfection into CHO cells with both α and β ECD:GHR chimeras or both α and β ECD:PRLR chimeras result in an enhancement of

activity with GM-CSF, whereas singlely the receptors were not influenced with GM-CSF (Herman *et al.* 2000). Co-transfection of α ECD:GHR and β ECD:PRLR or the reverse α ECD:PRLR and β ECD:GHR stimulated promoter activity with GM-CSF in CHO cells. These data suggest that cytoplasmic receptor heterodimerization of GHR and PRLR stimulates transactivation of a reporter gene, and may indicate the functions of PL in the maternal system. The functions of PRLR have been suggested for more than 85 distinct actions in the vertebrate subphylum and are widely distributed among tissues. Interestingly, GHR has been identified in a variety of tissues some of which overlap the PRLR (Kelly *et al.* 1991). Therefore, the conclusions that GHR and PRLR heterodimerize with PL, suggests tissue-specific action. However, in the fetal liver no GHR or PRLR have been identified with binding studies (Freemark *et al.* 1987; Pratt *et al.* 1995b), and the presence of a specific PL receptor, which remains to be identified appears to be the functional mediator of PL action in the fetus.

In summary, the placenta of each species is capable of modulating the maternal and fetal environments to promote growth and development of the fetus. Placental lactogen has been implicated as an important factor, which modulates both the maternal and fetal systems. The mechanism of action in both systems appears to vary and the response elicited on intermediary metabolism is paradoxical. Therefore, the identification of a specific PLR in the fetus and the implication of using GHR and/or PRLR in the maternal systems may be a plausible assumption. Additionally, the molarity of ligand and the affinity for the suggested receptors for maternal and fetal tissues indicates that the hormone would be present at a concentration that would elicit a physiological response.

Biological Action

Understanding the physiological roles for PL has been hampered by an inability to conduct ablation-replacement experiments, as well as the high endogenous concentrations of PL in maternal vasculature. In the maternal system, PL is purposed to repartition nutrients for the fetus by regulating intermediary metabolism. Maternal glucose and amino acid sparing are required for fetal growth and appears to be modulated by PL (Handwerger 1991; Anthony *et al.* 1995; Anthony *et al.* 1998). Furthermore, PL has been proposed to stimulate steroidogenesis in luteal tissue and augment mammogenesis for some species (Talamantes and Ogren 1988; Anthony *et al.* 1995; Anthony *et al.* 1998). Placental lactogen plays a role as an anabolic hormone in fetal tissues and stimulates amino acid uptake, IGF production, glycogen synthesis, DNA synthesis and ornithine decarboxylase activity (Handwerger 1991; Anthony *et al.* 1998). The inferred biological functions of PL in both the maternal and fetal systems appear to be paradoxical in regards to metabolism. The interpretation of PL's biological activity have been generated from: 1) *in vitro* experiments, usually with heterologous receptors or tissues; 2) *in vivo* analysis in a system that is already saturated with PL; 3) non-pregnant animals; or 4) clinical reports identifying a reduction in PL. Therefore, unequivocal evidence for PL's biological actions in the maternal and fetal systems remains to be obtained. Diversity in PL structure, secretion and mechanism of action may indicate that biological activity of PL will vary between species.

Maternal System

The structural similarity of hPL to GH and PRL has focused examination on biological activities that are influenced by the latter two family members. Growth promoting actions of hPL similar to GH have been described with postnatal tissues. In hypopituitary individuals an increase in circulating free fatty acids (FFA) was obtained with an injection of hPL or hGH (Grumbach *et al.* 1966). The FFA levels rose to levels comparable to those in women in the early third trimester, which result from mobilization of fat stores (Grumbach *et al.* 1966; Grumbach *et al.* 1968). Grumbach and colleagues (1966) hypothesized that mobilizing maternal fat stores may act as a fuel source for the mother, and conserve glucose and nitrogen for the developing fetus. Several lines of evidence have come to support or refute this hypothesis. Subsequent, human studies have indicated a lack of lipolytic stimulation with PL (Beck and Daughaday 1967; Berle *et al.* 1974). Adipocytes isolated from fasted rats exhibit increased lipolysis in the presence of hPL, whereas cells collected from fed rats underwent lipogenesis in the presence of hPL (Felber *et al.* 1972). Intravenous infusion of oPL in non-pregnant, non-lactating animals resulted in a reduction in FFA (Thordarson et al. 1987), whereas an increase in FFA with the infusion of oPL has also been reported (Regnault, 1997, reviewed by Anthony *et al.* 1998). However, pregnant ewes immunoneutralized with an oPL antiserum had a trend for decreasing FFA (Waters *et al.* 1985). In the rodent, two phases of maternal metabolism are observed, during early pregnancy the fetal needs are low, but late in pregnancy the fetus has a much greater nutrient demand and an increase in insulin antagonism and lipolysis occurs (Knopp *et al.* 1973; Flint *et al.* 1979). In the mouse, PL-II has no affect on the mobilization of fatty acids *in vitro* (Fielder and

Talamantes 1987). However, rodent PLs major target organs include the liver and uterus (Robertson *et al.* 1982; Soares *et al.* 1983; Harigaya *et al.* 1988a; Harigaya *et al.* 1988b). Indirect actions of PL were suggested because an increase of GH binding proteins from the liver, increase GH serum levels in the mother by increasing its half-life (Soares and Talamantes 1984; Smith and Talamantes 1988; Baumbach *et al.* 1989; Smith *et al.* 1989). Mouse GH has been shown to stimulate lipolysis within adipocytes *in vitro* (Fielder and Talamantes 1987). Although, *in vivo* and *in vitro* studies with PL are not homogeneous or able to fully recapitulate the animal model, non-pregnant or normal glycemic subjects at the onset are less susceptible to lipolysis.

In addition to increasing FFA available for β-oxidation, PL may increase basal and glucose stimulated insulin secretion, increase insulin resistance in some tissues, increase glucose tolerance and promote nitrogen retention (Grumbach *et al.* 1968; McGarry and Beck 1972). These actions influence the amount of nutrient substrates that are available to cross the placenta (Grumbach *et al.* 1968; McGarry and Beck 1972). Fasted pregnant and non-pregnant sheep infused with placental extracts containing PL had lower glucose and amino-acid nitrogen concentrations in maternal plasma, while insulin plasma concentrations were greater (Handwerger *et al.* 1976). However, non-pregnant, non-lactating ewes administered partially purified PL for 36 hours, had greater plasma glucose and urea nitrogen concentration but insulin concentration was unchanged (Thordarson *et al.* 1987). Immunoneutralization of PL for 12 hours late in gestation significantly effected glucose metabolism and increased plasma insulin (Waters *et al.* 1985). In non-lactating cows, that were pregnant or non-pregnant, recombinant bPL did not alter glucose or insulin concentrations, but bGH did increase these parameters (Byatt

et al. 1992). *In vitro* studies with rat pancreatic islets have shown stimulation of insulin with hPL (Martin and Friesen 1969; Laube *et al.* 1972; Nielsen 1982; Brelje *et al.* 1993), whereas *in vivo* hPL glucose-stimulated insulin secretion has been variable (Josimovich and Mintz 1968; Samaan *et al.* 1968; Kalkhoff *et al.* 1969). Conclusions on PL's actions are difficult to decipher, since the variability in PL dose, pregnancy status, gestational age, and nutritional state of the individuals are not consistent between studies. The nutritional status has been shown to effect oPL concentrations (Brinsmead *et al.* 1981) and the number oPL binding sites (Freemark *et al.* 1992). Species differences between PL may be attributed to some of the actions observed in heterologous animals.

A direct luteotropic action of mPL (Galosy and Talamantes 1995) and bPL (Lucy *et al.* 1994) has been demonstrated in the mouse and cow, but evidence for PL action on the ovary of sheep or humans is lacking. Luteal phase duration and progesterone secretion were unchanged by the administration of hPL (Stock *et al.* 1971; Goldsmith *et al.* 1978; Ramasharma and Li 1987), and administration or neutralization of oPL (Schramm *et al.* 1984; Waters *et al.* 1985). The need of PL to modulate luteal functions may be a result of the necessity of the corpus luteum during pregnancy (mouse and bovine), or the lack of corpus luteum requirements later in pregnancy (human and sheep).

A conceptus-derived agent in cattle has been shown to mediate mammogenesis, the development of the mammary gland (Schams *et al.* 1984). Bovine PL and hPL augment DNA synthesis in mammary tissue explanted into athymic nude mice (Welsch *et al.* 1978; Vega *et al.* 1989). Heifers induced to lactate with estradiol and progesterone, subsequently treated with bPL exhibited a mitogenic effect on the mammary tissue and increased α-lactalbumin concentration in blood sera (Byatt *et al.* 1994). A similar study

by Kann *et al.* (1999) in sheep reported a slight increase in DNA content of the mammary gland, a significant increase in β-casein, and an increase in blood sera IGF-I. However, pituitary PRL was not diminished in this study, which may alter the effects of PL administration and result in a lack of a significant mitogenic effect as observed in heifers (Kann *et al.* 1999). The efficiency of oPL to stimulate β-casein mRNA expression in mammary gland explants is less than PRL and it does not compete for PRL binding sites in mammary tissues (Servely *et al.* 1983; Emane *et al.* 1986). Furthermore, significant enhancement of milk yield was observed in the steroid induced lactating sheep with oPL, but milk production in heifers was not significantly increased with bPL administration (Byatt *et al.* 1997; Kann *et al.* 1999). Post parturition treatment of ewes with oPL did not increase milk production or mammary gland weight, whereas administration of bGH augmented both parameters (Min *et al.* 1997). Therefore, PL may have a more profound effect on mammogenesis than lactogenesis, and its somatogenic effects may be mediated by IGF-I.

Fetal System

The actions of PL to promote growth in the ovine fetus have been described indirectly. An increase in ornithine decarboxylase (ODC; (Butler et al. 1978)), amino acid transport into fetal muscle (Daughaday and Kapadia 1978; Freemark and Handwerger 1982; Freemark and Handwerger 1983), DNA synthesis, and IGF-I production (Adams *et al.* 1983) are examples of somatotrophic influences by PL. Ovine PL stimulates glucose incorporation and glycogen synthesis in a dose-dependent fashion, thereby increasing total cellular glycogen content in fetal sheep and rat hepatocytes

(Freemark and Handwerger 1984a; Freemark and Handwerger 1984b; Freemark and Handwerger 1985; Freemark and Handwerger 1986). In postnatal tissues GH regulates these events, and acts in the absence of PL. Therefore, fetal growth and postnatal growth of the animal appears to be stimulated by the same actions, but by different hormonal stimuli.

It has been postulated that many of PL's somatogenic effects are mediated through insulin-like growth factors (IGF) (Handwerger 1991; Owens 1991). This hypothesis is supported by several lines of evidence: 1) when oPL was infused into the vasculature of fetal sheep for 14 days, fetal serum IGF-I concentration increased from 30.3 ± 5.7 ng/ml to 43.1 ± 1.7 ng/ml ($P<0.05$), but serum IGFBP-2 concentrations were not effected (Schoknecht *et al.* 1996); 2) IGF-II concentrations from rat fetal fibroblasts increase with *in vitro* oPL treatment (Adams *et al.* 1983); 3) hypophysectomy of the fetus does not change IGF-II levels (Mesiano *et al.* 1989) and has no major detrimental effect on overall fetal growth; therefore, IGF-II production appears unlikely to be affected by oGH; 4) IGF-I concentration in human maternal circulation increases dramatically during the third trimester during which the greatest fetal growth occurs (Wilson *et al.* 1982; Mesiano *et al.* 1989; Gargosky *et al.* 1990); 5) oPL stimulated IGF-I in hypophysectomized immature rats and pregnant sheep (Handwerger 1991); and 6) IGF-I had anabolic effects on carbohydrate metabolism in rat fetal hepatocytes (Freemark *et al.* 1985). These results support the role of oPL in promoting fetal growth, possibly by stimulating IGF production. However, short-term infusion into the fetus (24 h) with recombinant oPL did not result in an increase in IGF-I, but it did lower fetal amino nitrogen in the blood while other metabolic parameters were unchanged (Oliver *et al.* 1995). Interestingly, IGF-

binding protein-3 in the fetus declined 30%, but the in the maternal vasculature it was increased (Oliver *et al.* 1995). Recently, IGF-II analysis in fetal tissues indicates that IGF-II concentrations are unchanged during late gestation and mRNA concentration are also unaffected (Mesiano *et al.* 1989; O'Mahoney *et al.* 1991). Therefore, the action of PL may indirectly alter IGF's or the binding proteins to elicit an action on fetal tissues.

Gene ablation of mouse IGF-I and IGF-II result in fetal and placental growth retardation. Removal of the paternal IGF-II allele resulted in fetal growth rates that were 60% of normal (DeChiara *et al.* 1990; DeChiara *et al.* 1991). Additionally, the paternal IGF-II allele was required for fetal expression and suggested imprinting of the mouse IGF-II gene. Investigations of IGF actions in fetal development were assessed with IGF-I, IGF type 1 receptor (IGF-IR), and IGF-II (p-) gene ablations, and a combination of double mutants (Baker *et al.* 1993). Deletions of IGF-I or IGF-IR yield birth weights 60% or 45% of normal, respectively. Deletion of both IGF-I and IGF-IR resulted in the same decrease in birth weight as IGF-IR mutants. However, when IGF-II and IGF-IR genes were ablated simultaneously, fetal growth was exacerbated further (30% of normal birth weight; Baker *et al.* 1993). These results indicate that IGF-II was acting through more than the type-1 receptor. Follow up experiments examined the effects of IGF-1 and -II, and IGF-IR gene ablation, at various days of gestation (Baker *et al.* 1993). Results from those experiments suggested that IGF-II was working via the IGF-IR from day 10.5 to day 12.5 of gestation. However, after day 12.5 the growth rates diverge and the double mutants (IGF-II $^{(p-)}$:IGF-IR $^{(-/-)}$) become more growth retarded, indicating the involvement of another IGF-II receptor. The IGF type-2 / mannose-6-PO_4 receptor was examined to determine its role in fetal growth. A strain of mice ($T^{hp/+}$) lacking a portion of

chromosome 17 containing the IGF type-2 receptor gene were mated to IGF-IR$^{(-/-)}$ mice, and little to no effect on fetal growth was reported (Baker *et al.* 1993). These data suggest the existence of an additional receptor for IGF-II during later gestation in mice.

Growth hormone in postnatal life has been shown to stimulate cell differentiation and proliferation indirectly by mediating IGF-I, and can directly effect cellular metabolism (Isaksson *et al.* 1987). However, during prenatal growth the effects of GH are minor and GH deficiency produced by anencephaly or removal of the pituitary has no effect on fetal growth (Browne and Thornburn 1989). Supplementation of GH to the fetus stimulates IGF-I from the liver (Strain *et al.* 1987) and pancreatic β-cells (Sandler *et al.* 1987), thereby suggesting that GH and PL have overlapping effects in prenatal life. Nevertheless, the requirement for GH during pregnancy does not appear to be necessary.

Clinical reports on human pregnancies lacking hPL and/or hGH-V during gestation due to a deletion in both chromosomal alleles have been described. Rygaard and colleagues (Rygaard *et al.* 1998) reported on such a pregnancy, in which the baby was severely growth retarded (10th percentile) but other wise normal. At week 33 no detectable hPL was found in maternal serum, IGF-I concentrations were below normal and GH was within the normal range (Rygaard *et al.* 1998). Additionally, no mRNA capable of translating hPL was identified in placental tissue, but low levels of an hPL-1 gene, which is a pseudogene was found (Walker *et al.* 1991). The pregnancy was complicated with a single umbilical artery and mild pre-eclampsia, which may attribute to the reduced birth weight (Rygaard *et al.* 1998). Postnatally the baby boy grew normally and no malformations were indicated (Rygaard *et al.* 1998). These data suggest that hPL expression during gestation has a significant effect on fetal growth and

development. However, other clinical reports on genetic alteration within the human GH/PL locus have been identified, with no alteration in fetal growth being observed (Wurzel *et al.* 1983; Goossens *et al.* 1986; Simon *et al.* 1987). These data suggest a great deal of ambiguity in the functions of PL during pregnancy. They also indicate a need to study an *in vivo* model that lacks PL to clearly identify the hormones action on fetal growth and development.

Transcriptional Regulation of Placental Genes

Genes encoding the GH/PRL gene family are thought to have diverged from a single precursor 350-400 million years ago (Barsh *et al.* 1983; Miller and Eberhardt 1983). A subsequent separation from either GH or PRL precursor gene has given rise to the PL genes, and these genes have a similar chromosomal structure of 5 exons separated with 4 introns (Miller and Eberhardt 1983). The divergence of the PL genes likely occurred after the main order of mammals separated. Since the primate and non-primate PL genes are derived from the different precursor genes, either PRL or GH. The separation was estimated to have occurred 80 million years ago, giving rise to the PL genes in mammals today (Walker et al. 1991; Wallis 1993).

Transcription of eukaryotic genes requires a transcriptional initiation site which is thought to be the primary control point for regulation of gene expression (Eloranta and Goodbourn 1996). Eukaryotic genes have a core promoter region, which is located immediately upstream and/or overlapping the transcriptional initiation site and are composed of *cis*-acting elements, such as a TATA box and/or initiator element (Eloranta and Goodbourn 1996; Struhl 1999). The function of these elements is to nucleate the

preinitiation complex (recruit RNA polymerase II holoenzyme) and identify the exact start site by binding TATA binding protein (TBP), initiator proteins or TBP associated factors (TAFs; Eloranta and Goodbourn 1996; Lin *et al.* 1997; Tansey and Herr 1997). Assembly of basal transcriptional machinery at the core promoter region is able to stimulate basal levels of transcription. Additional recognition sites within the gene (i.e. promoter) can interact with regulatory factors that either enhance or antagonize transactivation (Eloranta and Goodbourn 1996). Transacting factors have been suggested to influence the rate of initiation by interacting with the basal machinery to regulate transcription (Colgan and Manley 1992; Klien and Struhl 1994; Klages and Strubn 1995; Sauer *et al.* 1995). These regulatory factors are modular, and have DNA-binding domains that are separate from the domains that mediate activation. Transactivator activation domains have been classified by the predominance of amino acid residue, and include acidic, glutamine-rich, proline-rich regions (Eloranta and Goodbourn 1996). Tissue-specific regulation of genes has been shown to require a single or multiple regulatory factors. Trophoblast-specific regulation of placental genes has been described for human, rodent, and ovine PL genes as well as a couple of other placentally expressed genes. Regulation of these genes may indicate important *cis*-acting elements that are conserved between species and modulate transactivation in the placenta.

Human

Placental Lactogen

Nucleotide sequence homologies, assessed by cDNA clones, revealed a 93.5% identity between hGH and hPL, and a 42% identity to hPRL, respectively (Hirt *et al.*

1987). The hGH/hPL locus is on chromosome 17, whereas the hPRL gene is located on chromosome 6 in the human genome (Barsh *et al.* 1983). Five genes encoding hGH and hPL are grouped within a 66 kilobase (kb) region, and the final arrangement is thought to have arisen within the last 10-15 million years by gene duplication and recombination events at *Alu* elements. The order of the hGH/hPL locus (5' to 3') is: 1) hGH-N (pituitary hGH); 2) hPL-1; 3) hPL-4; 4) hGH-V (placental hGH variant); and 5) hPL-3 (Kidd and Saunders 1982; Hirt *et al.* 1987; Chen *et al.* 1989). Human PL-4 and hPL-3 encode identical mature proteins with only one amino acid alteration in the leader sequence, whereas hPL-1 appears to be a psuedogene due to a transversion in the 5'-splice site of the second intron (Hirt *et al.* 1987; Walker *et al.* 1991). Human GH-V is the major GH in circulation during late gestation (Handwerger 1991; Walker *et al.* 1991). In addition to the high homology within the coding region, 500 bp of the 5'-flanking sequence for all five genes share greater than 90% sequence identity (Walker *et al.* 1991).

Selby *et al.* (1984) analyzed mRNA expression from the hPL-3 and hPL-4 genes and determined that transcription of the hPL-4 gene accounted for 60% of the hPL mRNA present in placental tissue. The transcriptional start site for hPL is located 30 bp downstream of a consensus TATA box, which is involved in the initiation of transcription (Selby *et al.* 1984; Selvanayagam *et al.* 1984). *In vitro* transcription analysis revealed that all of the hPL genes were transcribed by RNA polymerase II, via α-amanitin sensitivity testing (Selvanayagam *et al.* 1984). The similarity between promoter regions of the placentally expressed genes and the pituitary expressed gene indicated that the transcriptional regulatory factors are different to enhance cell-specific expression. The difference in *cis*-acting elements may be due to subtle differences within the promoter

region (500 bp) of the genes, or the tissue-specific regulatory elements are located at a greater distance upstream or downstream (Selby *et al.* 1984).

The latter conclusion was supported with the identification of an enhancer region located within a 1.0 kb region about 2.2 kb 3' of the hPL-3 gene (Rogers *et al.* 1986). Transient transfection studies indicated that the enhancer region was not entirely cell-type specific because it functioned in both human choriocarcinoma (JEG-3) and pituitary lactotroph (18-54,SF cells) cell lines, but the level of transcription was greatly increased in the placental cells (Rogers *et al.* 1986). Furthermore, the enhancer region stimulated activity in two other human choriocarcinoma cell lines (BeWo and JAR), but did not function in a rat pituitary cell line (GC) or non-pituitary cell lines, such as Hela, HepG2, U-373MG (Walker *et al.* 1990; Jiang and Eberhardt 1994). The enhancer region also contained three important characteristics: 1) it functions independent of orientation; 2) it functions independent of position; and 3) it has the ability to activate heterologous promoters (Rogers *et al.* 1986). Continued deletion analysis further localized a tissue-specific element to a 138 bp fragment, between nucleotides 103 and 241 of the 1022 bp region (Walker *et al.* 1990). DNase I protection assays of this fragment revealed a GT-IIC or TEF-1 site (126/133 bp), and transient transfection with the 55 bp region encompassing this site was able to stimulate activity in a tissue-specific fashion, albeit reduced from the maximal enhancer region (1022 bp) stimulated activity (Walker *et al.* 1990). However, Hela cell nuclear extracts were able to protect the GT-IIC sequence in DNase I analysis as well (Walker *et al.* 1990). Walker *et al.* (1990) implicated differences in the footprinting pattern of the GT-IIC site as an indication for tissue-

specificity, but DNase I protection analysis is not sensitive enough to determine these minute changes.

Subsequent studies on the GT-IIC site recognized that additional elements are required in the first 241 bp of the enhancer region to achieve full transcriptional activity, (Jiang and Eberhardt 1994; Lytras and Cattini 1994). DNA-binding studies. DNase I protection assays and electrophoretic mobility shift assays (EMSA), are inconsistent between various reports describing protein-DNA interactions within the enhancer region (Jacquemin *et al.* 1994; Jiang and Eberhardt 1994; Lytras and Cattini 1994). Lytras and Cattini (1994) identified three sites, RF-1 (13/36 bp), DF-1 (54/74 bp) and a GT-IIC site (117/139 bp). A mutation in the DF-1 site impaired transactivation, whereas a mutation in the RF-1 site stimulated transcription in JEG-3 placental cells (Lytras and Cattini 1994). Furthermore, when the RF-1 element was placed adjacent to the GT-IIC element or SV40 enhancer regions, it was found to reduce transactivation in choriocarcinoma cells (Lytras and Cattini 1994). Lytras and Cattini (1994) indicate similarity between the DF-1 element and an Ets element, which may interact with a trophoblast-Ets family member (Asano *et al.* 1990).

Jacquemin *et al.* (1994) reported four binding sites by DNase I protection assays, which were named distal footprints (DF) and positioned relative to the hPL-3 transcriptional start site: DF-1 (3745 bp), DF-2 (3802 bp), DF-3 (3928 bp), and DF-4 (3983 bp). DF-1 and 2 were reported to have no effect on transcription in transiently transfected human choriocarcinoma cells, and are located upstream of the Lytras and Cattini (1994) enhancer region. DF-3 and DF-4 protected the region Lytras and Cattini (1994) identified as DF-1 and GT-IIC elements, whereas Jacquemin and colleagues

(1994) identified no protected region that encompassed the RF-1 element. Deletion of DF-3 resulted in a decrease in transactivation, and multiple copies of DF-3 or DF-4 stimulated transactivation synergistically in JEG-3 cells (Jacquemin *et al.* 1994). Similarity, between the TEF-1 binding sites (TEA/ATTS DNA binding sites) in the human papillomavirus type 16 E6 and E7 oncogene enhancers and the cardiac troponin T gene promoter, was used to identify a consensus binding site of N(A/G)NNATG(C/T)N, which was shown to reside in each of these protected regions (Jacquemin *et al.* 1994).

Jiang and Eberhardt (1994) also provided experimental data suggesting the 241 bp region could activate heterologous promoters, and multiple copies acted synergistically. However, deletion and mutation analysis indicated that the entire enhancer region was required for a full response, which does not coincide with Walker *et al.* (1990). DNase I protection assays reported by Jiang and Eberhardt (1994) identified five footprints (FP) and the numbering coincides with Lytras and Cattini (1994): FP-1 (5 to 27 bp; matching to RF-1), FP-2 (68 to 83 bp; matching to DF-1), FP-3 (115 to 140 bp; matching to GT-IIC), FP-4 (145 to 165 bp), and FP-5 (199 to 240 bp). Mutational analysis of predicted TEF-1 binding sites in the protected regions indicates that multiple *cis*-acting elements are required for full activity, and mutations in the GT-IIC element appeared to have the greatest reduction in enhancer activity. The mutation at 24/31 bp (FP-1) did not stimulate transactivation as previously reported by Lytras and Cattini with a mutation at 24/28 bp (RF-1). Jiang and Eberhardt (1994) named this region the chorionic somatomammotropin gene enhancer (CSEn; Jiang and Eberhardt 1994).

Transacting enhancing factor-1 (TEF-1) was suggested to mediate trophoblast-specific transcription by interacting with CSEn at the GT-IIC element. However, several

questions arise on its diverse tissue distribution, ability to activate various genes, and functional role in placenta transactivation (Martin *et al.* 1988; Jiang and Eberhardt 1994; Azakie *et al.* 1996). A novel protein CSEF-1 was discovered in BeWo and COS cells which bound GT-IIC with the same affinity, but had distinct characteristics including: 1) CSEF-1 had an apparent Mr of ≅30,000, whereas TEF-1 was 55,000; 2) CSEF-1 had a higher thermal stability than TEF-1; 3) CSEF-1 exhibited greater affinity to heparin sepharose; and 4) CSEF-1 was not immunoreactive with anti-TEF-1 (Jiang and Eberhardt 1995). Furthermore, multiple GT-IIC elements enhanced activity to a greater extent in COS-1 cells than BeWo cells, but were unable to stimulate activity in Hela cells (Jiang and Eberhardt 1995). These data suggest TEF-1 may be a repressor or lack enhancer functions, whereas CSEF-1 is an enhancing factor because TEF-1 was not identified in COS-1 cells and CSEF-1 was not present in Hela cells, whereas both factors are present in BeWo cells. Up- and down-regulation of TEF-1 in BeWo cells resulted in a detrimental or stimulatory effect, respectively, on CSEn or GT-IIC multimer enhancement of reporter activity (Jiang and Eberhardt 1995). Jiang and Eberhardt (1996) studied the TEF-1 transacting factor to identify its influence on the hPL gene promoter and viral gene promoter as a potential repressor of basal promoter formation. TEF-1 was found to interact with TBP, with or without forming a protein-DNA interaction (Jiang and Eberhardt 1996). Binding of TEF-1 to TBP was found to inhibit TBP binding to the TATA box, to decrease preinitiation complex formation (Jiang and Eberhardt 1996). This action of TEF-1 in placental cells may not exclude the function of this transacting factor as an enhancer in other cell types, but explains the activity TEF-1 exerts on the hPL promoter. The distinct transacting factor, CSEF-1, which is present in BeWo and

COS cells may be responsible for trophoblast-specific transactivation by interacting with the GT-IIC element.

Jiang and Eberhardt (1994) suggested that additional TEF-1 family members or TEF-1 related proteins might be in BeWo cells and influence transactivation through the CSEn. Three novel TEA/ATTS DNA binding proteins, which are similar to TEF-1, have been identified in the human and mouse, and TEF-5 was found to be predominantly expressed in the human placenta and JEG-3 choriocarcinoma cells (Jacquemin *et al.* 1996; Jacquemin *et al.* 1997). Furthermore, TEF-5 was expressed exclusively in syncytiotrophoblasts, and the expression of TEF-5 is up regulated during *in vitro* differentiation, whereas TEF-1 expression is down regulated (Jacquemin *et al.* 1998). Furthermore, TEF-1 transcripts were identified in the human placenta, but were identified as splice variants that when translated did not bind DNA, whereas two isoforms of TEF-5 were detected in human placenta RNA (Jacquemin *et al.* 1997). Human TEF-5 was found to interact with DF-4 (GT-IIC element) in the CSEn (Jacquemin *et al.* 1997). Interactions with the DF-3 element were also identified and 4 binding sites were recognized within this region. Disruptions in each of these elements characterized three elements that interacted with TEF-5 and were later shown to be functional elements in JEG-3 cells (Jacquemin *et al.* 1994; Jacquemin *et al.* 1997). Overexpression of TEF-5 in BeWo cells stimulated activity through multiple GT-IIC sites, CSEn or SV40 enhancers confirming its function as a transactivator of placental genes (Jiang *et al.* 1999). Additionally, CSEF-1 was immunologically related to TEF factors (TEA/ATTS DNA binding domain) and was described to be a proteolytic product of TEF-5 (Jacquemin *et al.* 1997). These studies confirm that TEF-5 is the transacting factor mediating hPL

enhancement through the CSEn, and it interacts with the GT-IIC element as well as other TEA elements.

To summarize the function of CSEn activity, multiple TEA binding elements are involved in mediating enhancer function by interacting with TEF-5. However, TEF-1 represses transactivation of the hPL promoter and may indicate its role in trophoblast-specific expression. Subsequently, TEF-1 repression has been shown to repress placental members of the GH/PL locus in pituitary cells by interacting with the GT-IIC element in similar CSEn regions (≅94%) 3' of hPL-1 (CSEn5, 3664/3972 bp), hPL-4 (CSEn2, 3729/4037 bp) and hPL-3 (CSEn1, 3724/4027 bp) genes, whereas stimulatory effects were observed in BeWo cells (Jiang and Eberhardt 1997). Additionally, TEF-5 was localized to the syncytium and is up regulated during differentiation of the trophoblast cell. Mouse TEF-5 has been cloned and was identified in extraembryonic tissue including mouse trophoblastic giant cells, which may infer its function with other placental genes (Jacquemin *et al.* 1998). These data confirm that TEF-5 mediates enhancer activity, possibly by stimulating basal machinery to nucleate at the hPL promoter.

The 5'-proximal promoter regions of the five genes within the hGH/hPL cluster are quite similar, but *cis*-elements were identified which appear to differentially stimulate transcription (Walker *et al.* 1991). Fitzpatrick *et al.* (1990) studied hPL promoter activity alone and found that the hPL promoter activates transcription 3- to 5-fold more than the hGH promoter in placental cells. The hPL promoter construct combined with the CSEn showed an 8-fold decrease with a deletion from -142 bp to -129 bp, which was recognized as a potential binding site for the transcription factor Sp-1 (Fitzpatrick *et al.*

1990). Gel mobility shift assays indicated that the hPL Sp-1 site specifically competed for protein binding, but was not a tissue-specific element. Therefore, Sp-1 was thought to be important for transcriptional activation of the hPL-3 enhancer. Furthermore, studies on the rhesus GH-V promoter region identified an identical element (-140/-131) and showed that it interacted with Sp-1 and Sp-3 transcription factors present in JEG-3 cells (Schanke *et al.* 1998). Jiang *et al.* (1995) followed up by mutational analysis, which identified the Sp-1 site as an important element in basal and enhancer mediated transactivation. However, a mutation of the Sp-1 site did not decrease transcriptional activity to the basal level. Therefore, other functional elements must reside within the proximal -129 bp. Deletion and site specific mutational analysis within the promoter region revealed additional *cis*-acting elements. The *cis*-acting elements regulating transcription were identified as the TATA box (-30 bp) and an initiator element (InrE) at -15 to +1 (Jiang *et al.* 1995). The InrE specifically bound a protein with the apparent M_r of 70,000, which was expressed in two human choriocarcinoma cell lines, BeWo and JEG-3 (Jiang *et al.* 1995). Questions arose on the ability of CSEn to stimulate placental-specific transcription though heterologous promoters, which do not contain the InrE. The CSEn is unable to initiate transcription on its own, and needs a promoter region to stimulate trophoblast-specific transactivation. Therefore, the CSEn must cooperate with various promoter elements or interact with the basal transcription factors to stimulate transactivation, and the InrE may facilitate preinitiation complex formation through CSEn (Jiang *et al.* 1994).

The hGH/hPL gene 5'-flanking sequences all contain a functional GHF-1/Pit-1 site in the 5'-flanking sequence (Nelson *et al.* 1986; Bodner and Karin 1987; Nelson *et al.*

1988; Castrillo *et al.* 1989; Lemaigre *et al.* 1989; Mangalam *et al.* 1989;). This conclusion resulted from GHF-1/Pit-1 binding to the 150 bp of promoter sequence and stimulation of reporter transcription in pituitary cell lines with -496 bp of either hPL-4 or hGH-N gene promoters (Lemaigre *et al.* 1989; Nachtigal *et al.* 1993). Therefore, how does the pituitary inhibit transcription of the placental-specific genes? An orientation dependent repressor, consisting of two PSF sequences, is located upstream (1.7 to 2.1 kb) of the genes expressed in the placenta (Nachtigal *et al.* 1993). PSF-A and PSF-B sites were protected by DNase I analysis with nuclear extracts from pituitary cells, but not placental cells, and the addition of these *cis*-elements to the 496 bp of 5'-flanking sequence inhibited transactivation in pituitary cell lines (Nachtigal *et al.* 1993). Therefore, placental genes are actively inhibited in the pituitary by an orientation dependent repressor. Therefore, complex regulation of the hPL gene is a combination of positive and negative *cis*-acting elements regulating tissue-specific transcription. The PSF elements actively inhibit transcription in the pituitary, while the CSEn and InrE may be modulating transcription in the syncytiotrophoblast cells both positively and negatively.

Chorionic Gonadotropin α- and β-subunit genes

Human chorionic gonadotropin (hCG) is a placentally expressed member of the heterodimeric glycoprotein hormone family, which include the pituitary-expressed members; luteinizing hormone (LH), follicle stimulating hormone (FSH) and thyroid-stimulating hormone (TSH). These hormones share a common α-subunit, but are distinguished by their unique β-subunit. Six hCGβ genes (number and order; 7, 8, 5, 1, 2,

3) and a single LHβ gene are located on chromosome 19 and share greater than 90% identity at the nucleotide level (Jameson and Lindell 1988; Jameson and Hollenberg 1993). These related members have been suggested to arise from the same precursor gene, but they are expressed in a cell-specific fashion (Jameson *et al.* 1986). LH and FSH are expressed in the gonadotropes, TSH is expressed by thyrotropes and the human trophoblast expresses hCG (Jameson and Hollenberg 1993). The α-subunit gene exists as a single copy gene and is expressed by all these cell types (Fiddes and Goodman 1981). In the first trimester placenta the predominant gene expressing hCGβ is the hCGβ5 gene (Jameson and Hollenberg 1993; Miller-Lindholm *et al.* 1997). The placental and pituitary α-subunit transcripts have identical start sites whereas the transcriptional start sites of the β-subunit of LH and hCG are located at different places, even though the sequences are quite similar (Jameson *et al.* 1986). The placenta-specific transcriptional regulatory elements of the hCG α- and β-subunit genes is distinct from the pituitary expression.

Placenta-specific transcriptional regulation of the α-subunit gene resides within the proximal 180 bp 5' of the transcriptional start site (Darnell and Boime 1985; Steger *et al.* 1991). Two juxtaposed cyclic AMP response elements (CRE, -146/-111 bp) and an upstream regulatory element (URE, -180/-151 bp) were demonstrated to mediate trophoblast expression *in vitro* (Delegeane *et al.* 1987; Jameson and Lindell 1988). A junctional response element (JRE) and CAAT box located downstream of the CRE elements are required for optimal expression (Andersen *et al.* 1990; Kennedy *et al.* 1990). The URE possess two binding sites, a trophoblast-specific element (TSE, -187//159) and a GATA (α-ACT, -165/-140 bp) (Pittman *et al.* 1994; Steger *et al.* 1994; Delegeane *et al.*

1987; Jameson and Lindell 1988). Functionality of these elements was described by mutational analysis in human choriocarcinoma cell lines and this region was able to convey expression in extraembryonic tissues of transgenic mice (Bokar *et al.* 1989; Steger *et al.* 1994). GATA-2 and GATA-3 factors were identified in JEG-3 cells, and shown to interact with the αGATA element (Steger *et al.* 1994). However, overexpression of the GATA binding protein in non-placental or JEG-3 cells did not augment expression, suggesting the necessity of other elements (Steger *et al.* 1994). The TSE element has been shown to interact with activator protein-2α (AP-2α). Furthermore, functional analysis of this region in JEG-3 cells supported the stimulatory effects of AP-2α (Johnson *et al.* 1997). Therefore, placental-specific regulation of the human α-subunit gene is mediated by two CRE elements, an AP-2 element and a GATA element.

The hCGβ gene transcriptional initiation site is located 366 bp 5' relative to the hLHβ transcriptional start site, implicating different transcriptional regulation. The LHβ gene contains a canonical TATA box, whereas the hCGβ genes do not contain a canonical TATA box with respect to their initiation sites. The transcriptional regulation of the hCGβ5 gene was localized to 311 bp of 5'-flanking sequence and the homologous region in the LHβ gene was not found to be important for expression (Jameson and Lindell 1988; Albanese *et al.* 1991). The cAMP responsiveness of the hCGβ gene was mapped between –305 and –249 bp, whereas basal activity required a region between -311 and –30 bp (Albanese *et al.* 1991; Steger *et al.* 1993). Four DNase I footprints (FP) were identified at –319/-303 bp (FP1), –301/-275 bp (FP2), -268/-259 bp (FP3) and -243/-199 bp (FP4; Steger *et al.* 1993). Footprints 1, 2, 4 were able to compete with the

AP-2 element identified in the URE of the α-subunit gene (Steger *et al.* 1993). Subsequently, AP-2α was demonstrated to interact with FP2 and FP4, and mutational analysis of these sites suggested that both elements are required for basal transactivation and cAMP responsiveness. Additionally, the AP-2 element (-292 bp) was overlapped by an Sp1 element (-302 bp) in FP2. The proximity of these two elements suggest that only a single protein can interact with either of these elements, and Sp1 was found to preferentially bind (Johnson *et al.* 1997; Johnson and Jameson 1999). An additional Sp1 site was identified between -200/-188 bp, which is downstream of the AP-2 element in FP4, and both elements were demonstrated to interact simultaneously in this footprint (Johnson and Jameson 1999). Functional analysis of the FP2 elements indicates that Sp1 interaction stimulates basal activity, but not cAMP enhancement (Johnson and Jameson 1999). On the other hand, both AP-2 elements are required for basal and cAMP responsiveness *in vitro* (Johnson and Jameson 1999). These results are interesting if one takes into account the two systems that have been tested, and the amount of AP-2 and Sp1 factors present. Johnson and colleagues (1997) identified AP-2α in both JEG-3 and human trophoblast nuclear extracts, but Sp1 was much greater in the JEG-3 nuclear extracts than in human trophoblast nuclear extracts. These data may indicate that *in vivo* AP-2 has more influence on transcriptional regulation of the hCGβ gene than Sp1.

Transgenic mice with a 36 kb cosmid containing the hCGβ gene cluster, exhibited gene expression in the mouse placenta and the hCGβ5 gene was the most prominent gene expressed (Strauss *et al.* 1994). However, the transgene did not convey temporal expression similar to that observed in the human (Jameson and Hollenberg 1993; Strauss *et al.* 1994). Furthermore, the placental expression of hCGβ in the transgenic mice was

not localized to trophoblast cells, but was identified in the parietal endoderm another component of the extraembryonic tissue (Strauss and Boime 2000). Therefore, it is difficult to assess the transcriptional control of these genes in transgenic mice.

Aromatase P450 (CYP19)

Aromatase P450, a product of the CYP19 gene, catalyzes the conversion of C_{19} steriods (androgens) to C_{18} estrogens and is the committed step in estrogen biosynthesis (Thompson and Siiteri 1974). In most vertebrates the expression of aromatase is restricted to the brain and gonads, but in the human, aromatase is expressed in syncytiotrophoblasts of the placenta, adipose tissue, and fetal liver (Fournet-Dulguerov *et al.* 1987; Simpson *et al.* 1994). CYP19 is a single copy gene that is composed of 10 exons. Exons II-X encode the mature aromatase enzyme in all tissues, but an alternative first exon containing 5' untranslated region is specific for CYP19 expression in various tissues (Means *et al.* 1989; Toda *et al.* 1990). The alternative first exons indicate that different promoter regions are responsible for tissue-specific expression. The placenta-specific exon I.1 resides ≅40 kb upstream of exon II, whereas the adipose exon I.4 is ≅15 kb upstream, and the ovarian exon I is adjacent to exon II (Means *et al.* 1996). The CYP19 exon I.1 5'-flanking sequence was analyzed in transgenic mice, which do not endogenously express aromatase in their placenta (Kamat *et al.* 1999). Two constructs were tested (-2400 bp to +103 and -501 bp to +103), and both expressed primarly in the placenta (Kamat *et al.* 1999). Transgene expression was localized to the mouse labyrinthine trophoblast layer, which is composed of the syncytial trophoblast cells, at day 10.5 and 17.5 post coitus. Expression was not identified in spongiotrophoblast or

trophoblastic giant cells. These data suggest the expression CYP19 in the placenta results from a species-independent *cis*-acting element, which is functional in mouse and human syncytiotrophoblast cells.

In vitro analysis of human primary trophoblast cells isolated at midgestation exhibited an increase in aromatase expression and activity, which peaked after 4 days in culture (Kamat *et al.* 1998). Cytotrophoblast cells, infected with a recombinant adenovirus containing –923 bp or –501 bp of 5'-flanking sequence of exon I.1, expressed a reporter gene in a similar manner. A three-fold stimulation was observed with a deletion from -501 bp to -246 bp, but a 50% and 80% reduction in expression was observed with deletions to –201 bp and –125 bp, respectively (Kamat *et al.* 1998). Cells infected with the adenovirus containing –42 bp of the I.1 5'-flanking sequence or 952 bp of ovary-specific promoter region did not express the reporter gene, and nontrophoblast cells were not able to elicit a response with any of the I.1 promoter regions (Kamat *et al.* 1998). A canonical Sp1 site was identified at -233 bp and a hexameric sequence (ATTCCAGAGGAGGTCATGC, Hex in bold) similar to the binding site for an orphan member of the nuclear receptor superfamily (Ad4BP/SF1) were identified within regions important for syncytiotrophoblast expression. Mutation of these two elements reduced expression, and binding studies confirmed the Sp1 interaction at -233 bp (Kamat *et al.* 1998).

The Hex site identified by Kamat *et al.* (1998) also over laps with an imperfect palindrome, AGGTCATGCCCC, identified by Sun *et al.* (1998). In JEG-3 cells mutation or deletion of this region resulted in the loss of basal- and retinoid-induced expression, and this element was shown to interact with the retinoid-X receptor and 1,25

D_3 receptor heterodimer (Sun *et al.* 1998). Another Hex overlapping site (ACCCTCATTCCA) was identified as TSE2 (Yamada *et al.* 2000). A cDNA encoding a mammalian homolog of Drosophila glia-cell-missing gene (GCM) was demonstrated to interact with the TSE2 site in a yeast one-hybrid system (Yamada *et al.* 2000). Furthermore, interaction with the hGCMa and TSE2 element was identified in a protected region important for placental expression of the leptin gene (PLE1; Bi *et al.* 1997; Yamada *et al.* 2000). Two AP-2 elements at -300/-274 bp and -177/-153 bp were also described as functional (Yamada *et al.* 2000). Therefore, stimulation of the CYP19 gene has similar factors mediating transactivation as does hCGβ, Sp1 and AP-2, but it also has novel factors (hGCMa and retinoid receptors) that are involved in mediating transcription in trophoblast cells.

Rodent

Rodents have two PL genes that are temporally expressed by the trophoblastic giant cells (Talamantes and Ogren 1988). Mouse PL genes are clustered with the mPRL gene on chromosome 13, whereas the mGH gene has been localized to chromosome 11 (Jackson-Grusby *et al.* 1988). Rat PL genes have been localized to chromosome 17 with the rPRL gene, and the rGH gene resides on chromosome 10 (Cooke *et al.* 1986). The similarities between rPL-I and rPL-II, with a consideration for conserved amino acids, to rPRL is 38% and 47%, respectively, and the mouse is similar (Soares *et al.* 1991). Transcriptional regulation of rodent PL genes has come under scrutiny to provide insight on the function of these genes *in vivo*.

Placental Lactogen

Mouse

Tissue-specific transcriptional regulation of the mPL-1gene has been localized to 274 bp immediately upstream of the gene's transcriptional start site (Shida *et al.* 1993). Deletion analysis of the mPL-1 promoter compared -2700 bp, -274 bp, and -64 bp of 5'-flanking sequence. The -274 bp construct yielded the highest reporter activity in trophoblast cells, whereas the -64 bp of promoter region was nonfunctional (Shida *et al.* 1993). Neither -2700 bp nor -274 bp of 5' -flanking sequence exhibited significant transactivation in nontrophoblast cell lines (Shida *et al.* 1993). Therefore, the placental-specific regulatory sites appear to be located with the -274 bp to -64 bp region of the mPL-I gene. Two AP-1 sites are located in this region at -246 bp and -80 bp and mutational analysis diminished transcriptional activity (Shida *et al.* 1993).

Further deletion analysis of the mPL-I promoter to -188 bp resulted in a partially active promoter, suggesting additional factors in mPL-1 trophoblast-specific transcription. DNase I footprint analysis revealed two GATA (GATCT; -224 bp and GATTT; -215) sequences within the -274 bp to -188 bp region, and one GATA at -93 bp in the reverse orientation (Ng *et al.* 1994). These sites have been shown to bind GATA-2 and -3 (Ko and Engel 1993), and mRNA transcripts for the two GATA factors were found in differentiated Rcho-1 cells (Ng *et al.* 1994). Co-transfection of GATA-2 or GATA-3 expression vectors with the mPL-I promoter vector in mouse L cells, which lack endogenous expression of these GATA factors, stimulated reporter activity (Ng *et al.* 1994). Mice with a disruption in the GATA-2 or GATA-3 genes had ≅50% reduction in mPL-I production with either GATA factor ablated (Ma *et al.* 1997). Therefore, GATA

and AP-1 factors appear to be mediating the trophoblast-specific activity of the mPL-I promoter. Kawana *et al.* (1995) provided evidence that GATA-2 and AP-1 (Jun and Fos) interact with each other to stimulate transcription of the endothelin-1 gene. However, these data question the tissue-specific control of GATA and AP-1 as the mPL-1 gene transcriptional regulators, because of their ability to regulate other genes. Therefore, additional *cis*-acting elements could be mediating transcription of the mPL-I gene. HAND1 (*hxt*, e-Hand, or Thing-1), a basic helix-loop-helix (bHLH) protein, was found to influence trophoblast differentiation and increase the number of differentiated Rcho-1 cells (Cross *et al.* 1995; Scott *et al.* 2000). Expression of this DNA binding protein resulted in a stimulation of mPL-1 promoter activity, with the -2700 bp 5'-flanking sequence (Cross *et al.* 1995). Therefore, Hand1 was suggested as a transacting factor involved in regulating the mPL-I gene, but direct binding to a *cis*-acting element in the mPL-I gene was not observed. However, Hand1 and E47 heterodimers have been described to interact with a Th1 box (NNTCTG) to stimulate transactivation in fibroblast cells, but Hand1 has also been shown to interact with itself and other bHLH proteins, such as Mash2 and ITF2 (Scott *et al.* 2000). These data may indicate a role for Hand1 to transactivate mPL-I directly, but may also suggest that Hand1 has indirect effects by stimulating other E-box transacting factors (Scott *et al.* 2000).

Little is known about the transcription of the mPL-II gene, and current data only implicates regions of the promoter. Shida *et al.* (1991) cloned and characterized the mPL-II gene, which consisted of 5 exons and 4 introns, and 2.7 kb of 5'-flanking sequence. Transgenic mice containing the 2.7 kb of mPL-II 5'-flanking sequence coupled to the SV40 T antigen promoter exhibited trophoblast giant cell-specific

expression, but no placental expression was observed in the placenta with a similar construct containing the proximal -569 bp (Shida *et al.* 1992). Further deletion analysis of the mPL-II promoter in founder transgenic mice at day 13.5 of gestation was used to localize the trophoblast-specific region of mPL-II (Lin and Linzer 1998). Transgenic mice generated with 1.3 kb of mPL-II 5'-flanking sequence had no expression in the placenta, but the addition of 680 bp of promoter (2.0 kb of promoter) resulted in significant activity in the placenta when compared to the fetus (Lin and Linzer 1998). Transient transfection assays in Rcho-1 cells revealed that –2.7 and –2.0 kb of promoter region had similar activity, but a reduction was observed with a deletion to –1.3 kb of 5'-flanking sequence (Lin and Linzer 1998). Furthermore, elements within the –1.3/–0.6 kb region were required for trophoblast-specific expression in Rcho-1 cells. Deletion analysis of the -2.0/-1.3 kb region identified a potential repressor element (-2.0/-1.78 bp), whereas the enhancer region resides within a 132 bp region between -1471/-1340 bp. Block mutations across this 132 bp region identified two functional regions at –1457/-1471 bp and –1353/-1393 bp (Lin and Linzer 1998). Binding studies with the –1457/-1471 bp region show a specific interaction with a protein that is present in Rcho-1 cells, but not in mouse fibroblast cells (L cells; Lin and Linzer 1998). The region between -1353/-1393 bp encompasses a GATA element, but competition with the mPL-I GATA element was not observed (Lin and Linzer 1998). These data indicate several regions are responsible for mPL-II promoter transactivation, but no specific transacting factors were identified.

Rat

Much less is known about the transcriptional regulation of the rat PL-I and PL-II genes. Currently, only information on transcriptional regulation of the rPL-II gene has been reported. *In vitro* (Rcho-1 cells) and *in vivo* (transgenic mice) analysis of the rPL-II 5'-flanking sequence identified a region between –3000 and –765 bp, which mediated trophoblast transactivation (Shah *et al.* 1998). Subsequent transient transfections identified an orientation-independent trophoblast-specific enhancer region between -2838 and -1729 bp, and a region at -1435/-765 bp that provided minimal support (Sun and Duckworth 1998). A 65 bp region was found to possess the enhancer activity and DNase I footprint assays identified two adjacent *cis*-acting elements. An Ets element at -1743 bp and an AP-1 binding site at -1759 were previously defined elements within footprint 1 and 2 (Sun and Duckworth 1998). Mutation of these sites significantly reduced activity in transiently transfected Rcho-1 cells (Sun and Duckworth 1998). Binding studies confirmed a Fos/Jun heterodimer interaction with the AP-1 element, but specific binding with the 65 bp region to Ets1 or Ets2 was not demonstrated (Sun and Duckworth 1998). Co-transfection of Fos/Jun and/or Ets2 expression vectors stimulated transactivation of the 65 bp region in Rcho-1 cells (Sun and Duckworth 1998). These data confirm that the AP-1 element is functional and Fos/Jun heterodimers augment transactivation. The data on the Ets binding site is inconclusive because Ets1 and Ets2 did not bind to the element, but Ets2 was able to stimulate transactivation of the enhancer in Rcho-1 cells. This may suggest that Ets2 has an indirect effect on the 65 bp enhancer.

Mouse Adenosine Deaminase

Adenosine deaminase (ADA) is a purine catabolic enzyme, which catalyzes the irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinosine. Adenosine deaminase is enriched in trophoblast cells of the mouse chorio-allantoic placenta and is essential for proper development (Blackburn *et al.* 1995; Wakamiya *et al.* 1995). A 770 bp region located 5.4 kb upstream of the mouse ADA gene was able to convey placental expression that mimicked endogenous ADA gene expression (Shi *et al.* 1997). DNase I protection revealed three protein-DNA interactions in the ADA placental enhancer with placental nuclear extracts (Shi *et al.* 1997). One of these protected regions (FP1) at 241-270 bp resembled an AP-2 (TSE) element and the protein-DNA interaction was placental-specific in comparison to liver nuclear extracts. Deletion of FP1 resulted in a loss of placental activity in transgenic mice (Shi *et al.* 1997). Shi *et al.* (1997) identified two ubiquitous binding sites in both liver and placental nuclear extracts, FP2 (641/670 bp) and FP3 (731/750 bp). A deletion of a FP3 resulted in a loss of enhancer function in transgenic mice, but FP2 was not tested (Shi *et al.* 1997). Additionally, single bp mutations at two putative GATA elements or a deletion of the first 240 bp resulted in a loss of significant placental activity in transgenic mice (Shi *et al.* 1997). Although no protein-DNA interactions were observed in the mouse ADA gene, two GATA elements, 4 bHLH binding sites (E-boxes) and a region that share 77% identity to the hPL enhancer region (DF3 and DF4) were identified as potential transcriptional regulators in the ADA gene. Therefore, the functionality of these elements may coincide with previously identified elements for mPL-I, hCG, and hPL genes transcriptional regulatory regions.

Furthermore, the data suggest that multiple elements are required to convey placental-specific promoter activity of the ADA gene.

Shi and Kellems (1998) reported a detailed analysis on AP-2γ, a transacting factor that mediates placental-specific transactivation of the ADA gene. Binding studies confirmed that AP-2γ interacted with the FP1 sequence and a point mutation of the AP-2 element resulted in a loss of placental activity in transgenic mice (Shi and Kellems 1998). In the mouse, AP-2γ is predominantly expressed in the placenta, whereas AP-2α had greater expression in the embryo and AP-2β was barely detected in either the placenta or embryo by RNase protection assays. Activator protein-2γ was identified in all the trophoblast cell lineages throughout gestation, but was absent from primitive endoderm, extraembryonic mesoderm and endothelial cells of the fetal vasculature. These data implicate AP-2γ as a trophoblast-specific transacting factor in regulating ADA gene expression in the mouse placenta.

Ruminant

Placental Lactogen

Bovine and ovine PL genes have been characterized from the ruminant genome. The bovine PL gene is located on chromosome 23 along with the bPRL gene (Dietz *et al.* 1992). The bPL gene spans 12 kb and has the characteristic 5 exon and 4 intron structure (Kessler and Schuler 1991b). The oPL gene is located on chromosome 20 (Gootwine and Yossafi 1998). Liang (1995) sequenced 9,633 bp of the oPL gene, and by restriction mapping and PCR amplification found the gene encompasses 11.2 kb of the ovine genome. Structurally oPL shares similarity with other members of the GH/PRL gene

family (5 exons and 4 introns) and exists as a single copy gene (Liang 1995; Liang *et al.* 1999). The transcriptional start site for bPL and oPL genes were located at an identical nucleotide ahead of the translational start codon (Kessler and Schuler 1991b; Liang *et al.* 1999). Analysis of the bPL 5'-flanking sequence revealed putative regulatory elements, such as AP-1, AP-2, and thyroid hormone-responsive elements, but the functionality of these elements has not been tested (Kessler and Schuler 1991b). On the other hand the promoter region of the oPL gene (4.5 kb) has been analyzed and functional elements have been identified.

Initial studies on the transcriptional regulation of the oPL gene localized trophoblast specific elements within the proximal 1 kb of 5'-flanking sequence (Liang 1995). Deletion constructs of the oPL promoter were tested in transiently transfected choriocarcinoma (BeWo and Rcho-1) and non-choriocarcinoma (Hela and C127) cell lines to identify the trophoblast-specific domain (Limesand 1997; Liang *et al.* 1999). A construct containing 383 bp of oPL 5'-flanking sequence stimulated transactivation in the choriocarcinoma cell lines and was at or equivalent to maximal activity observed with constructs that had additional promoter sequence. Thereby signifying that elements responsible for full trophoblast-specific transactivation reside between –383/+16, relative to the transcriptional start site, in both BeWo and Rcho-1 cells (Limesand 1997; Liang *et al.* 1999). Additionally, a deletion to –124 bp (minimal promoter) exhibited trophoblast-specific activity albeit reduced from the maximal expression observed with -383 bp of oPL promoter (Limesand 1997; Liang *et al.* 1999). Some ambiguity in expression between BeWo and Rcho-1 cells was observed with the deletion constructs. Trophoblast-specific expression was lost in Rcho-1 cells with deletions to -607 bp and -217 bp, but

reinstated with subsequent deletions to –544 bp and –124 bp (Limesand 1997; Liang *et al.* 1999). While promoter activity in BeWo cells exhibited trophoblast-specific transactivation with all the deletion constructs tested (Limesand 1997; Liang *et al.* 1999).

Variations in oPL promoter activity for the two heterologous trophoblast cell lines may indicate alternative *cis*-acting elements stimulating transactivation. Therefore, protein-DNA interactions within the proximal 1 kb of promoter region were identified using ovine binucleate cell (BNC) nuclear extracts, which are the cell that endogenously express oPL in the ruminant placenta (Liang *et al.* 1999). Nineteen DNase I protected regions were identified within the proximal 1 kb promoter region (Liang *et al.* 1999). Ten GATA elements were identified in nine footprints (FP), and two GATA elements (FP2 and FP3) were identified within the minimal promoter (Limesand 1997; Liang *et al.* 1999). Six FP were found between -383/+16 bp, and in addition to the GATA elements, an AP-2 element (FP2) and an E-box (FP4) were located within this region. Furthermore, a direct repeat of GAGGAG in FP5 and FP6 was identified, and a potential initiator element encompassing the transcriptional start site was found (Limesand 1997; Liang *et al.* 1999 Limesand and Anthony 1999). The non-canonical TATA box was not protected from DNase I activity, which indicates the requirement of the initiator element.

Summary

Considerable diversity exists between species in PL structure, secretion profiles, ancestral precursor gene and transcriptional regulation, even between closely related species, like the cow and sheep. However, similarities between human and ovine PL have been noted. Resemblances in primary protein structure, inferred biological action

and secretion profiles were similar, even though the PL genes arose from different secondary precursor genes. Furthermore, the expression of these genes occurs in differentiated trophoblast cells that reside at the maternal-fetal interface and deposit the hormone into both blood supplies. The expression of these genes appears to be constitutive in both species with little or no influence on transcription by maternal hormones. In fact the most important event controlling PL expression appears to be the differentiation of the trophoblast cells.

Transcriptional regulation described for placental genes is insufficiently understood. Human PL transcriptional regulation has been described the most thoroughly. However, attempts to alter hPL expression *in vivo* to ascertain PL's "true" biological action would be fraught with ethical issues. On the other hand, rodent PL transcriptional regulation has been studied to some extent, but studying the affects of manipulations in PL expression during pregnancy may be difficult to assess due to fetal size constraints. Moreover, the actions of rodent PLs appear to be distinct from those described for the human and sheep, which may indicate that the mouse is only a model for the mouse. However, transcriptional regulation of placental genes appears to have the ability to cross species barriers, especially if one considers the regulation of the CYP19 (aromatase) gene, and its ability to express in rodent syncytiotrophoblast cells. Expression of hCGβ genes does not possess the same species independence. Therefore, transcriptional regulation of placental genes may require gene specific analysis to identify composite elements that mediate trophoblast-specific transactivation, but individual *cis*-acting elements may be similar or predominant in all placentally expressed genes. An AP-2 element was identified in genes expressed in the placenta for all the species described, but transactivation for these genes

was not exclusively mediated by AP-2 factors. Other species-independent factors identified include GATA elements and possibly TEA sites, but still unidentified *cis*-acting elements, which interact with know or unknown transacting factors may be necessary for placental expression.

The pregnant sheep, a long time model for the physiology of pregnancy, may be the most appropriate model to study PLs biological action. Pregnant sheep provide several attributes, such as they are large animals and fetal catheters can be sustained for long periods of time. The addition of PL to the maternal or fetal system seems fruitless, because of the high endogenous amount of PL already present. A reduction or ablation of PL expression in the pregnant model will prove to be the most conclusive analysis to identify the action of PL during pregnancy. Removal of the placenta and auto-immunization against PL have not been feasible approaches due to the adverse outcomes in the maintenance of pregnancy. Consequently, modifying the transcriptional regulation of oPL may be the only method to ascertain the biological action *in vivo*. Again the ability of trophoblast-specific transcription appears to be gene specific. Accordingly, the study of oPL transcriptional regulation must be assessed to define important regions, which could be used to alter endogenous PL expression *in vivo*.

Chapter III

TRANSCRIPTIONAL REGULATION OF THE OVINE PLACENTAL LACTOGEN GENE

Introduction

Placental lactogens (PL) are members of the growth hormone (GH)/prolactin (PRL) gene family and may regulate fetal growth by altering maternal and fetal metabolism. Fully differentiated trophoblast cells at the maternal-fetal interface secrete PL into both maternal and fetal vasculature. Ovine PL is constitutively produced by chorionic binucleate cells and is secreted as a non-glycosylated, 198 amino acid polypeptide, with an apparent M_r of 22,000 (Colosi *et al.* 1989; Warren *et al.* 1990; Kappes *et al.* 1992). As a member of the GH/PRL gene family, oPL shares more amino acid sequence identity with bovine PL (bPL; 67%) and ovine prolactin (oPRL; 49%) than it does with ovine growth hormone (oGH; 28%) (Colosi *et al.* 1989; Warren *et al.* 1990; Kessler and Schuler 1991). Although oPL shares significant sequence identity with bPL, the interspecific divergence is considerably greater than that for either GH or PRL between these two species, which share ≈99% amino acid sequence identity. This divergence may indicate a high degree of adaptive pressure on ruminant PL genes (Wallis 1993).

Interspecific divergence is also observed in their respective secretion profiles. The maternal sera concentration of bPL peaks at 2-3 ng/ml (Wallace 1993), whereas oPL in maternal vasculature reaches concentrations of ≈1,000 ng/ml during late gestation (Kappes *et al.* 1992). However, oPL exhibits similar secretion profiles and somatogenic actions with human PL (hPL), although primate PL genes evolved from the ancestral GH gene (Bewley *et al.* 1972; Walker *et al.* 1991; Anthony *et al.* 1995a; Anthony *et al.* 1998).

Maternal serum concentration of oPL or cotyledonary oPL mRNA concentration account for ≈80% of the variation observed in fetal weight during gestation (Taylor *et al.* 1980; Schoknecht *et al.* 1991; Kappes *et al.* 1992). *In vitro*, oPL, but not oGH, has been reported to stimulate glycogenesis in a dose-dependent fashion and to increase total cellular glycogen content in fetal sheep hepatocytes (Freemark and Handwerger 1986). Additionally, long-term infusion of oPL into prenatal lambs stimulated liver glycogen accretion, and increased circulating insulin-like growth factor I (IGF-I) in fetal vasculature (Schoknecht *et al.* 1996). Although no oPL deficient pregnancies have been reported, deletions within the hGH/PL gene cluster have resulted in severe growth retardation of the fetus (Rygaard *et al.* 1998), providing evidence of a role for hPL in fetal growth. However, there have been reports of hPL/hGH-V deletion that resulted in normal pregnancy outcome (Wurzel *et al.* 1983; Goossens *et al.* 1986; Simon *et al.* 1987), and these contrasting data underlie the need to examine the physiology of pregnancy in individuals or an animal model lacking PL, before its necessity can be ascertained.

Among the species (human and mouse) for which PL transcriptional regulation is best defined, considerable variation exists, and these species do not lend themselves readily to *in vivo* fetal physiology studies. Trophoblast-specific enhancement of hPL gene transcription requires a 138 bp enhancer located ≈2.2 kb 3' of the GH/PL locus (Rogers *et al.* 1986; Walker *et al.* 1991; Jiang and Eberhardt 1994). This region contains a GT-IIC site which is thought to interact with TEF-5, but multiple *cis*-acting elements are required for trophoblast-specific expression (Lytras and Cattini 1994; Jiang and Eberhardt 1994; Jiang and Eberhardt 1995a; Jacquemin *et al.* 1997). In addition to enhancers, two orientation-dependent pituitary repressors, PSF sequences, are found ≈2 kb upstream of the placentally expressed genes (Nachtigal *et al.* 1993). Therefore, it appears that both tissue-specific enhancers and silencers are required for hPL gene regulation. In contrast, the mouse (m) PL-I gene retains trophoblast-specific transactivation with 274 bp of 5'-flanking sequence (Shida *et al.* 1993). Analysis of this region indicates the involvement of three GATA sites and two activator protein-1 (AP-1) elements (Shida *et al.* 1993; Ng *et al.* 1994). Disruption of the GATA-2 or 3 genes of mice significantly reduced expression of the mPL-I gene, thus confirming the role of GATA factors in regulation of mPL-I gene expression *in vivo* (Ma *et al.* 1997). Basic helix-loop-helix transcription factors (bHLH), like Hand1, are thought to stimulate transactivation of the mPL-1 promoter by interacting with an E-box binding site (Cross *et al.* 1995; Scott *et al.* 2000).

The oPL gene has been structurally characterized and the single copy gene has 5 exons separated by 4 introns spanning about 11.2 kb of genomic DNA. An additional 4.5 kb of 5'-flanking sequence was isolated for the oPL gene to analyze transcription

regulation (Liang 1995; Liang *et al.* 1999). The proximal 1 kb of promoter region contains *cis*-acting elements that meditate transactivation in human (BeWo) and rat (Rcho-1) choriocarcinoma cell lines (Liang 1995; Liang *et al.* 1999). Subsequent transient transfection analysis of this region has distinguished the area between –383 bp and +16 bp, relative to the transcriptional start site, to possess trophoblast-specific elements (Limesand 1997; Liang *et al.* 1999). Six DNase I protected regions were identified in the proximal 383 bp with nuclear proteins purified from ovine binucleate cells (BNC). Furthermore, -124/+16 bp (minimal promoter) of oPL promoter maintained trophoblast-specific expression, but at reduced levels compared to the -383 bp region (Limesand 1997; Liang *et al.* 1999). These data indicate that the 3 footprints (FP1-3) observed in the minimal promoter region are capable of trophoblast specific expression, and two footprints (FP5 & 6) between -383 bp and -217 bp bind trophoblast-specific enhancers. Additionally, FP4 resides within the -217/-124 bp region, but the functional importance of this element is not apparent by deletion analysis. These data implicate important DNA binding sites for transactivation of the oPL gene. Previously defined *cis*-acting elements located within the protected regions include an AP-2 element (FP2), two GATA sites (FP2 & 3), and an E-box (FP4), as well as a previously undefined direct repeat (DR-1) in FP5 and FP6.

Considerable species diversity exists in PL structure, secretion, and transcriptional regulation. The pregnant sheep is an often-used model to examine placental-fetal interactions, and oPL appears to have somatogenic activities in the fetus, similar to hPL. An understanding of the transcriptional regulation of the oPL gene could provide a means to regulate PL expression *in vivo,* thereby generating a model by which to examine the *in*

vivo function of PL. Therefore, oPL transcriptional regulation mediated by the *cis*-acting elements identified with ovine binucleate nuclear extracts are defined in the present study.

Materials and Methods

Mutation Constructs

Dual polymerase chain reaction (PCR; Perkin Elmer, Branchburg, NJ) amplification was employed to generate block mutations at the GATA sites in the 124pGL2 vector. Over-lapping oligonucleotides containing a Not I restriction endonuclease recognition site at the 5' end were designed (Δ70F, 5'-CAG CGG CCG CTC CAC CCC AGG GCA TG-3'; Δ70R, 5'-GAG CGG CCG CTG CTT CTC TTT TAC CG-3'; Δ100F, 5'-ATG CGG CCG CAG AAT GCG GTA AAA GAG AAG-3'; Δ100R, 5'-TCT GCG GCC GCA TCA ATG CTG GTG CCT-3'), and these primers in conjunction with either the pGL2 forward or reverse primers (Promega) were used to amplify the 5' or 3' DNA fragment of the proximal 124 bp. The first PCR reaction contained 0.2 μM of forward and reverse oligonucleotides, 6.6 fmol 124pGL2, 0.1 μM of each dNTP, 1 U Taq DNA polymerase, in a 1X reaction buffer with 1.5 mM $MgCl_2$. PCR amplification parameters, following a 5 min incubation at 94°C, consisted of a denaturation step at 94°C for 1 min, a 2 min annealing step at 48°C, and an extension step at 72°C for 1 min. After 10 cycles the annealing temperature was increased to 60°C for an additional 30 cycles. The DNA fragments were agarose gel purified with DEAE membrane (Ausubel *et al.* 1995), and added in equal molar ratios to a second PCR reaction as the template DNA with the pGL2 forward and reverse oligonucleotides. The reconstructed DNA fragments were digested

with Kpn I and Hind III restriction endonucleases, agarose gel purified, and ligated into the pGL3-Basic vector (Promega). To mutate the GAGGAG and E-box sequences, the same approach was used, but the 380 pGL3 vector (Liang *et al.* 1999) was used as the template with the following primers: pGL3 forward; pGL3 reverse; Δ338F, 5'-CTG GCG GCC GCC ATG GCA ACC CAT-3'; Δ338R, 5'-ATG GCG GCC GCC AGG GGT CTT CCC T-3'; Δ283F, 5'-GAC GCG GCC GCC TGG TTG CTA CAG-3'; Δ283R, 5'-CAG GCG GCC GCG TCC AGG AGG ATT C-3'; ΔE-box 3'F, 5'-CTG CGG CCG CAT CAT CAC TGT GAT C-3'; ΔE-box 3'R, 5'-GAT GCG GCC GCA GTG ACC AGT GCG CT-3'; ΔE-box 5'F, 5'-GCA GCG GCC GCT TGA AGG CCA TCA T-3'; and ΔE-box 3'R, 5'-CAA GCG GCC GCT GCG CTG ACG AAT A-3'. The mutated fragments were ligated into the pGL-3 Basic vector, and the resulting plasmids were sequenced. Plasmid DNAs were prepared for each construct using an alkaline-lysis procedure (Ausubel *et al.* 1995) and purified by CsCl equilibrium gradient centrifugation for transient transfection experiments.

Cell Culture and Transfection

A rat choriocarcinoma cell line, Rcho-1, was a generous gift from Dr. M. J. Soares, (University of Kansas, Kansas City, KS). BeWo cells from a human choriocarcinoma were obtained from American Type Culture Collection (Rockville, MD). Rcho-1 cells were maintained in NCTC-135 medium (Gibco BRL, Gaitherburg, MD) supplemented with 1mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO) and 20% heat-inactivated fetal bovine serum (FBS; Gemini Bio-Products, Inc., Calabasas, CA). BeWo cells were

maintained in Waymouth medium MB (Gibco BRL) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 15% FBS. All cells were maintained in monolayer cultures at 37°C in 5% CO_2, 95% air with 100% humidity.

For transient transfections, the cells were plated at a density of 0.5 x 10^6 cells/well in 6-well plates. The polycationic lipid/DNA complex reaction was carried out at room temperature for 20 min, with 10 μl LipofectAmine reagent (Gibco BRL), 5 μg oPL promoter-pGL2 plasmid, 0.25 μg of pβgal control plasmid (RSV promoter and enhancer; ClonTech) and culture medium without serum and antibiotics in a total volume of 100 μl. The cells were washed twice in serum-free medium, the lipid/DNA mixture was diluted to 1 ml with serum-free medium, added drop wise to the cells and incubated at 37°C for 24 h. At this time the transfection mixture was replaced with 4 ml of serum-containing antibiotic-free medium and cultured for an additional 48 h.

After the incubation period, cells were lysed in 25 mM Gly-Gly ($C_4H_8N_2O_3$), 1 mM DTT and 0.1% Triton X100, and analyzed using a Luciferase Assay System (Promega, Madison, WI) or LumiGAL Chemiluminescent Assay System (ClonTech, Palo Alto, CA). The light emission generated by both activities was measured using a Turner TD-20e luminometer with an integration time of 10 seconds. The number of light units generated by luciferase activity was normalized to β-galactosidase activity for each transfection to control for intra-assay variation. Relative luciferase activity for each construct is expressed as a percent of wild type. Data are presented as percent mean±SEM for at least three separate replicate experiments, but a logarithm transformation was analyzed by least square analysis of variance using the Statistical Analysis System (Snedecor and Cochran 1976; SAS Institute 1993). Means were

separated using the Dunnett's T test, and statistical significance was accepted for $P<0.05$ (Steel and Torrie 1960).

Binucleate Cell Isolation

Mature cross-bred ewes were mated at behavioral estrus (day 0) and hysterectomized by midventral laparotomy after administration of pentobarbital and exsanguination at 100 days post coitus. Cotyledonary tissue was manually removed and rinsed three times in physiological saline and digested in the same manner as Reimers, *et al.* (Reimers *et al.* 1985). The cells were centrifuged at 500 X g for 10 minutes, resuspended in 4% glycerol-PBS for 30 min to lyse red blood cells, and subsequently washed twice in PBS at 200 X g. Prior to the final wash, placental cells were filtered though four layers of cheesecloth, a 150 μm nylon mesh and resuspended at a concentration of 1×10^7 cells/ml. To isolate binucleate cells, 4 ml of the cell solution were layered over a gradient of Ficoll-Histopaque with a density of 1.073 forming the lower layer and a 1:3 dilution with PBS generating the upper layer, and centrifuged for 30 min at 1600 X g (Morgan *et al.* 1990). The lower band of cells was removed, rinsed twice in PBS, counted and used in the Dignam *et al.* (1983) nuclear extraction procedure. This procedure resulted in 67.6±5.9% of the cells harvested being chorionic binucleate cells, which is comparable to earlier reports (Morgan *et al.* 1990). Consequently, 80.9% of the nuclei used for extraction were derived from chorionic binucleate cells.

Electrophoretic-Mobility Shift Assays

DNA binding studies were performed with antisense oligonucleotides radiolabeled with polynucleotide kinase radiolabeled and [γ-^{32}P] ATP (Ausubel *et al.* 1995). The oligonucleotide was heated to 100°C with an equal-molar ratio of sense oligonucleotide, and slowly cooled to 4°C. The double- stranded radiolabeled oligonucleotide was purified by G-25 size-exclusion chromatography, and quantified by scintillation spectrometry. Oligonucleotides generated for electrophoretic-mobility shift assays (EMSA) are oGATA, 5'-GCA GTG ATA GCT CC-3'; oAP2, 5'-GCT CCA CCC CAG GGC ATG-3'; E-box163, 5'-ACT GGT CAC TTG AAG GCC-3'; E-boxMut1, 5'-ACT GGT TTC TTG AAG GCC-3'; E-boxMut2 5'-ACT GGT CAC TAA AAG GCC-3'; o99, 5'-AAG AGA GAA GAA TGC GGT A-3'; and o580, 5'-GCC TGT CCT AGT TCT TTA ACC-3'. Binding reactions included 20 mM HEPES pH 7.9 (4°C; or 20mM Tris pH 8), 20 % glycerol (v/v), 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.25 mM DTT. Additionally, spermidine (1 mM), benzamidine (2mM), and BSA (25 μg/ml) were added, as well as Poly (dI-dC)-Poly (dI-dC) (10-1000 ng/reaction). The reaction buffer, unlabeled competitors and nuclear proteins (15-20 μg) were gently mixed and placed on ice for 10 min at which time 4.5 fmol of double stranded radiolabeled oligonucleotide (50,000 cpm) was added. To examine which GATA interacted with the oGATA sequence, mouse monoclonal antiserum (0.1 mg/ml) raised against GATA2 (CG2-96; Santa Cruz Biotechnology, Santa Cruz, CA) or GATA3 (HG3-31; Santa Cruz Biotechnology) was included in the reaction instead of unlabeled competitor. The addition of the AP-2α antiserum (C-18; Santa Cruz Biotechnology) was used in conjunction with the oAP-2 oligonucleotide in the same manner. Following a 30 min

incubation at 30°C, the reactions were placed on ice (5 min) and electrophoresed through a 5% polyacrylamide nondenaturing gel which had been pre-electrophoresed at 20 mAmps for 2 h. The gels were dried and exposed to x-ray film.

Results

Ovine PL Gene Minimal Promoter

Analysis of the oPL promoter in human and rat choriocarcinoma cell lines has identified two function regions (Liang *et al.* 1999). The minimal promoter region (-124/+16 bp) is capable of trophoblast-specific transactivation and three areas within this region are protected from DNase I digestion by protein-DNA interactions (Figure 1; Liang *et al.* 1999). The first protected region encompassed the oPL gene transcriptional start site, -12 to +7 (FP1), and suggests that the gene has an initiator element. Furthermore, a non-canonical TATA-box was not protected, which may confirm the necessity of an initiator element (InrE) to mediate transcription (Sharp 1992; Aso *et al.* 1994; Smale 1997).

Footprint 2 (FP2) encompassed a predicted activator protein-2 (AP-2) binding site (ACCCCAGGGCA) at –58 bp in the oPL gene (Imagawa *et al.* 1987; Meier *et al.* 1995; Liang *et al.* 1999). The recognition of an AP-2 element prompted analysis of protein-DNA interaction with this element. An oligonucleotide encompassing the oPL AP-2 element (-58) specifically interacted with a protein in ovine BNC and BeWo cell nuclear extracts (Figure 2). Furthermore, the addition of an antiserum against human AP-2α retarded the mobility of this complex in electrophoretic mobility shift assays (EMSA;

Ovine PL Gene Proximal Promoter Sequence

```
-410  GGTAAAGAAC CCACTGTCAG TGCAGGAGAG GTAAGAGACA CTGGTTTGAT CCCTGAGTAG
                    DR-1
-350  GGAAGACCCC TGGAGGAGGG CATGGCAACC CATTCCAGCA TTCTTGTCTG GCGAATCCTC
             DR-1
-290  CTGGACAGAG GAGCCTGGTT GCTACAGTCC ATAGGCTCTC AAAGAGCTGA ATACGCCTGA

-230 AACAATTTAG CATGCTCGCA TAGGGTGTAT AGAGATACTG AAATTTTCTA TTGCTCAGCG
           E-box
-170 CACTGGTCAC TTGAAGGCCA TCATCACTGT GATCTTTTCC CTATTGCTCT TATGCAGGCA
             GATA                                    GATA          AP-2
-110  CCAGCATTGA TAAGAGAGAA GAATGCGGTA AAAGAGAAGC AGTGATAGCT CCACCCCAGG

-50   GCATGAGTAA TCAGTAACAT CCTATTTCAG GTTGCCAGGT TTGCCTCCTT AGGCATTCCC
```

Figure 1. Ovine PL gene 5'-flanking sequence. The proximal 383 bp of oPL promoter was found to possess activity in human and Rcho-1 cell lines, and DNase I protected sites are underlined (derived from Liang *et al.* 1999). The transcriptional start site and subsequent nucleotides are in boldface type. Previously defined *cis*-acting elements that were protected are labeled above and include AP-2, GATA, and an E-box. The putative elements are marked by different underline thickness. A previously undefined element, DR-1 (GAGGAG), is designated by a bold underline.

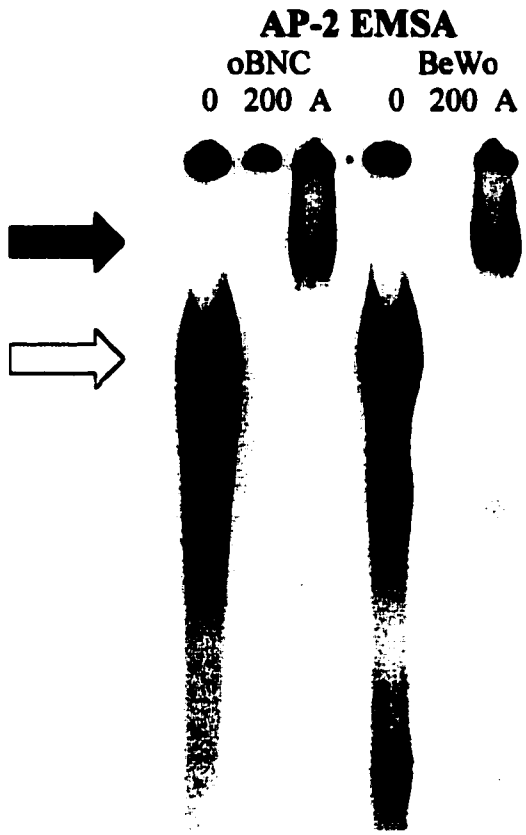


Figure 2. AP-2 electrophoretic-mobility shift assays. The oAP2 EMSA reactions were performed with 15 μg of oBNC or BeWo nuclear extract, indicated above the lanes. The specificity for the AP-2-DNA complex formed (labeled 0) was tested in the presence of 200 fold molar excess of unlabeled homologous competitor (labeled 200) or with the addition of 500 ng/reaction of AP-2α antiserum (labeled A). On the left an open arrow indicates the radiolabeled oAP-2 oligonucleotide bound to ovine AP-2, and the black arrow indicates AP-2:DNA complex bound to AP-2α antiserum.

Figure 2). These data confirm the presence of an AP-2 *cis*-acting element in the oPL promoter interacting with AP-2α from sheep and human trophoblast cells.

Two GATA sites (WGATAR; Ko and Engel 1993) were identified in FP2 and FP3 at –102 and –67 bp . Both of these GATA sequences are located within the proximal 124 bp, which was capable of stimulating trophoblast-specific transactivation. GATA elements have been described as functional elements regulating placental expression in both mouse and human genes (Ng *et al.* 1994; Steger *et al.* 1994). An oligonucleotide containing a GATA site (oGATA) within FP2 was generated for binding studies in EMSA. The oGATA specifically bound to an oBNC nuclear protein, and increasing concentration of unlabeled homologous oligonucleotide competed with the radiolabeled oligonucleotide, whereas heterologous oligonucleotides (o99 and o598) did not compete for complex formation (Figure 3A). Addition of a mouse monoclonal antibody raised against human GATA-2, to either oBNC or Rcho-1 nuclear extracts, inhibited protein-DNA complex formation in a dose dependent fashion (Figure 3B). The mouse monoclonal antibody raised against human GATA-3, at the highest concentration used, had no effect on complex formation (Figure 3B). Immunoblot analysis with GATA-2 and GATA-3 antiserum detected proteins in oBNC nuclear extracts with M_r of ≅43,000 and ≅48,000, respectively (data not shown). These data confirm that the proximal GATA site (-67 bp) is capable of specifically binding GATA-2 present in ovine BNC and Rcho-1 nuclear extracts.

The functionality of the GATA sites as transactivators of the oPL minimal promoter were analyzed by single block mutations. These mutations deleted the GATA elements residing at –67 bp (GATA→GGCC) and –102 bp (GATA→GATG), respectively. In

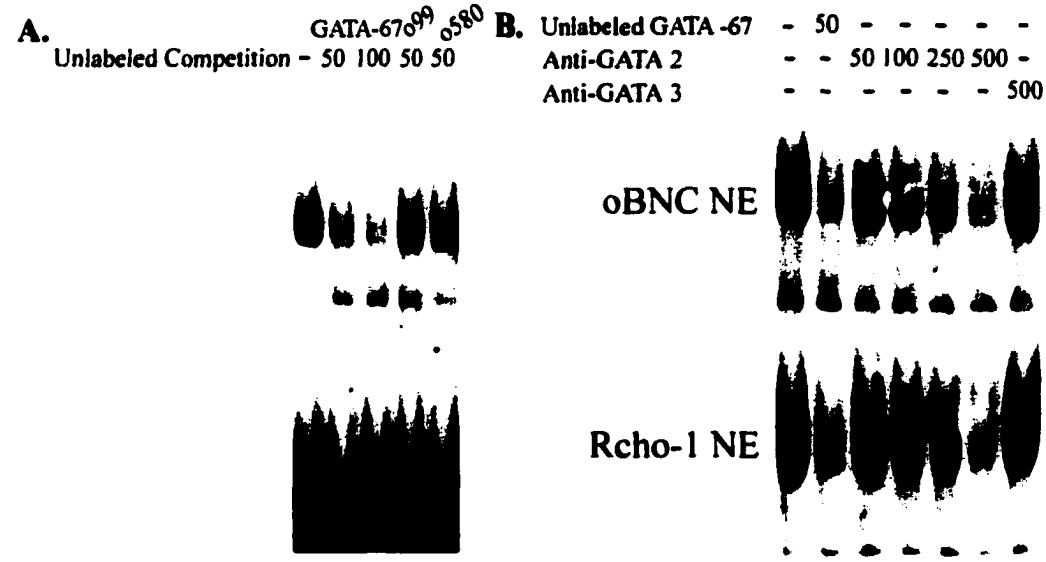


Figure 3. EMSA with the proximal ovine GATA element (-67 bp). Radiolabeled ovine GATA (50,000 cpm) was added to 20 μg of oBNC or Rcho-1 cell nuclear extract, and the interaction was separated from the unbound oligonucleotide by electrophoresis through a 5% polyacrylamide gel. GATA binding specificity was tested by adding either homologous (GATA-67) or heterologous (o99 and o580) competitors into the oBNC nuclear extracts binding reaction at 50 or 100-fold molar excess (Panel A). GATA factor binding to a radiolabeled GATA-67 was confirmed with the addition of antiserum raised against GATA-2 (50, 100, 250 or 500 ng/reaction) or GATA 3 (500 ng/reaction) in the presence of oBNC or Rcho-1 nuclear extracts (Panel B). Note the specific inhibition of complex formation by the GATA-2 antiserum, whereas GATA-3 antiserum at the highest concentration did not affect the complex.

both BeWo and Rcho-1 cells, the mutation of GATA site at -67 bp reduced transactivation, but only in BeWo cells was the reduction in activity significantly different than the wild type, although the decline in Rcho-1 cells approached significance (P=0.0534; Figure 4). The -102 bp GATA site appears less important in mediating transactivation of the oPL minimal promoter, but activity did decline upon mutation of this GATA element. Therefore, to fully examine GATA function within the minimal promoter, a double GATA mutation was generated within the minimal promoter. This construct depleted reporter activity to background levels in Rcho-1 (0.932±0.210) and BeWo (0.923±0.110) cell lines (Figure 4), suggesting that the GATA sites located within the minimal promoter of the oPL gene are capable of enhancing trophoblast transcription.

Ovine PL Gene E-box

Although important trophoblast *cis*-acting elements within the -217/-124 bp region were not identified by deletion analysis in the heterologous choriocarcinoma cell lines, a protein-DNA interaction was identified with oBNC nuclear extracts (FP4; -173/-137) (Limesand 1997; Liang *et al.* 1999;). A canonical E-box (CANNTG) at -163 bp was recognized within this protected region, and bHLH factors influence mPL-I transactivation, but more importantly play a role in trophoblast differentiation (Cross *et al.* 1995; Limesand 1997). The functionality of this binding site cannot be conclusively ruled out because it has not been tested in the oBNC. Binding studies with an oligonucleotide were performed to confirm a specific protein-DNA interaction to this *cis*-acting element. A double stranded oligonucleotide containing the E-box at –163 bp interacted specifically with proteins in oBNC nuclear extracts and formed two specific

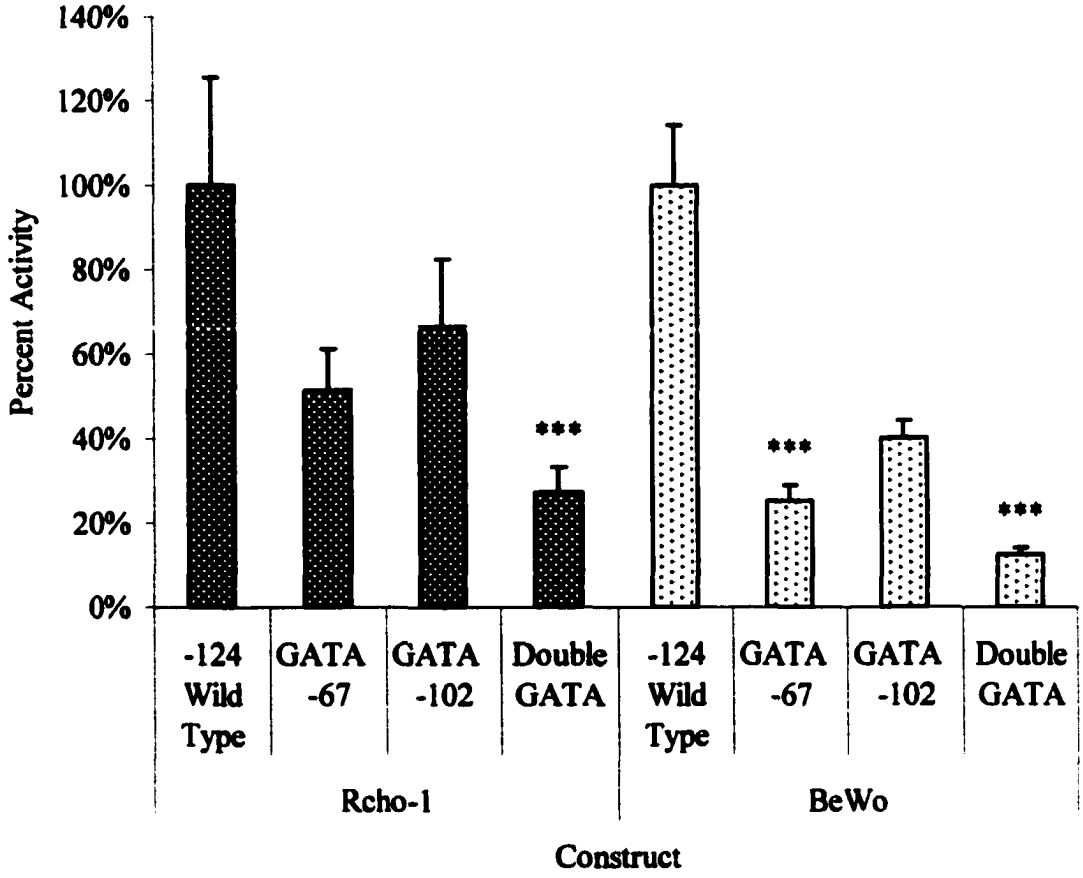


Figure 4. Mutational analysis of GATA. Transient transfection of the GATA mutation constructs in Rcho-1 (left) and BeWo (right) cells. The wild-type 124pGL3 is presented first in each series. Subsequent bars represent single block mutations of the GATA site at –67 bp or –102 bp, which is followed with the double GATA mutations at both –67 bp and –102 bp (see X-axis). The mean percent activity and the SEM is shown on the Y-axis, and significant ($P<0.05$) reductions in transactivation from wild-type were separated by a Dunnett's T test, are indicated by *** above the bar.

complexes when homologous and heterologous DNA competitors were added to the binding reactions (Figure 5). Furthermore, mutations in the –163 bp E-box (Mut1 CA→TT; Mut2 TG→AA) did not compete equivalently for the faster migrating protein-DNA complex formed in EMSA (Figure 5). The slower migrating complex was inhibited with the E-box Mut2 (TG) oligonucleotide, but complex formation was not drastically altered with the E-box Mut1 (CA; Figure 5). These data confirm that specific protein-DNA complexes are attributed to the conical E-box identified by DNase I protection assays, but multiple proteins or variation in heterodimer formation may result in different interactions with this *cis*-acting element. The E47 bHLH protein interacts with several trophoblast specific bHLH proteins, including Hand1, to facilitate DNA binding (Scott *et al.* 2000). Antiserum against E2A bHLH proteins (E47 and E12) did not alter the formation of the complexes with the –163 bp E-box and oBNC nuclear proteins in EMSA, which may suggest other bHLH factors or novel factors are binding to this element.

Deletion analysis in the choriocarcinoma cell lines indicated no adverse effect on promoter activity, but context-dependent transacting factors have been identified with other promoters (Fry and Farnham 2000). Therefore, the transacting factor interacting with the E-box may enhance transcription by altering the DNA architecture or removing histones to facilitate stimulation by other transacting factors (Fry and Farnham 2000; Brown *et al.* 2000). To analyze the function of this *cis*-acting element in a native context, block mutations were created to alter the downstream (D, TG→GC) and upstream (U, CA→GC) half sites of the E-box within the –380 bp pGL3 vector. Transient transfection of BeWo and Rcho-1 choriocarcinoma cell lines resulted in no decline in activity with the

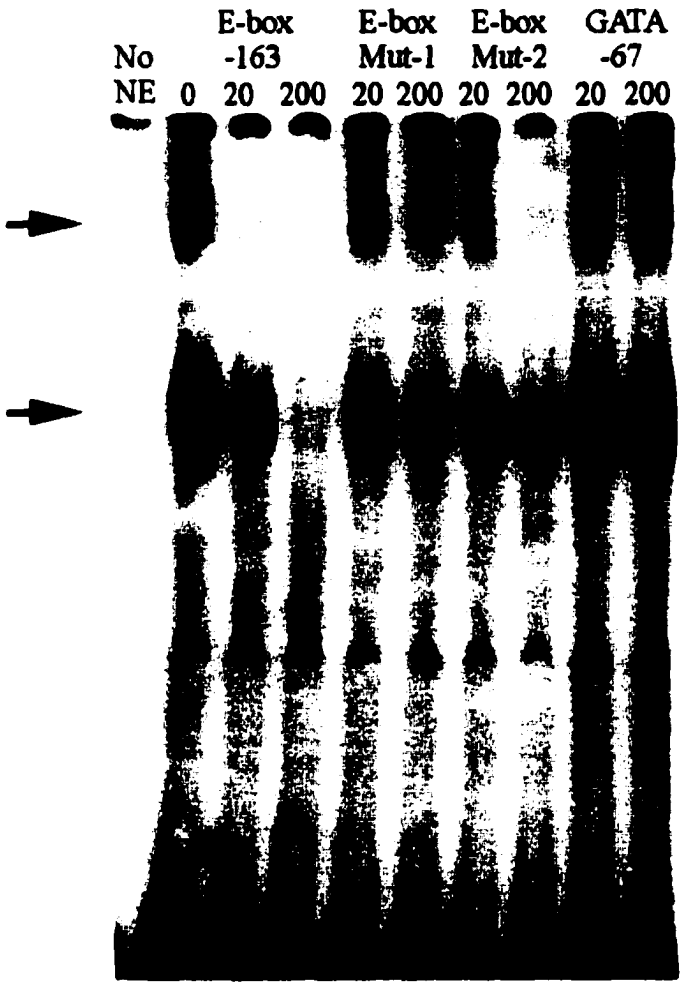


Figure 5. Electrophoretic-mobility shift assay of a putative E-box (CANNTG) at -163 bp. The left lane has 4.5 fmoles of radiolabeled E-box163 oligonucleotide only, and subsequent lanes contain 15 μg of oBNC nuclear extract that have 0, 20, or 200 fold molar excess of unlabeled competitors; E-box163, E-box Mut-1 (CA→TT), E-box Mut-2 (TG→AA) or GATA-67 (as labeled). The arrow indicates the protein-DNA complex.

mutation of the downstream portion of the E-box, whereas a significant decrease was observed with a mutation of the upstream region (Figure 6). The mutation of the upstream potion of the E-box reduced activity in BeWo cells 24.6 % and in Rcho-1 cells activity declined 43.7 %. These results indicate that the slower migrating complexidentified by EMSA may be functional in stimulating transactivation of the oPL gene in a context dependent fashion.

Ovine PL Gene Enhancer Region

The second region of the oPL gene (-383/-217 bp) contained enhancer elements that appeared to augment the transactivation of the minimal promoter (Liang *et al.* 1999). This 166 bp region had two DNase I protected areas that were identified with oBNC nuclear extracts. These areas reside between -286/-246 bp (FP-5) and -349/-319 bp (FP-6), and no previously identified mammalian *cis*-acting elements matched these protected regions. However, a direct repeat (DR-1) of GAGGAG resides within FP5 and FP6 (Figure 7A). The identification of these potential *cis*-acting elements in oBNC nuclear extracts and their importance in enhancing the oPL promoter in the choriocarcinoma cell lines prompted follow up studies. To demonstrate if the GAGGAG repeat was functional, block mutations were created to disrupt this sequence in the -380 bp pGL3 vector. Mutation of either DR-1 sequence significantly ($P<0.01$) reduced transactivation in BeWo cells, and a similar response was observed with Rcho-1 cells (Figure 7B). In BeWo cells, a mutation at DR-1 –338 resulted in a 86.8% reduction in activity and the mutation at DR-1 –283 decreased activity 78.5%. Similarly, a reduction of 77.9% and 78.9% for DR-1 –338 and DR-1 –283 was observed in Rcho-1 cells, respectively. These

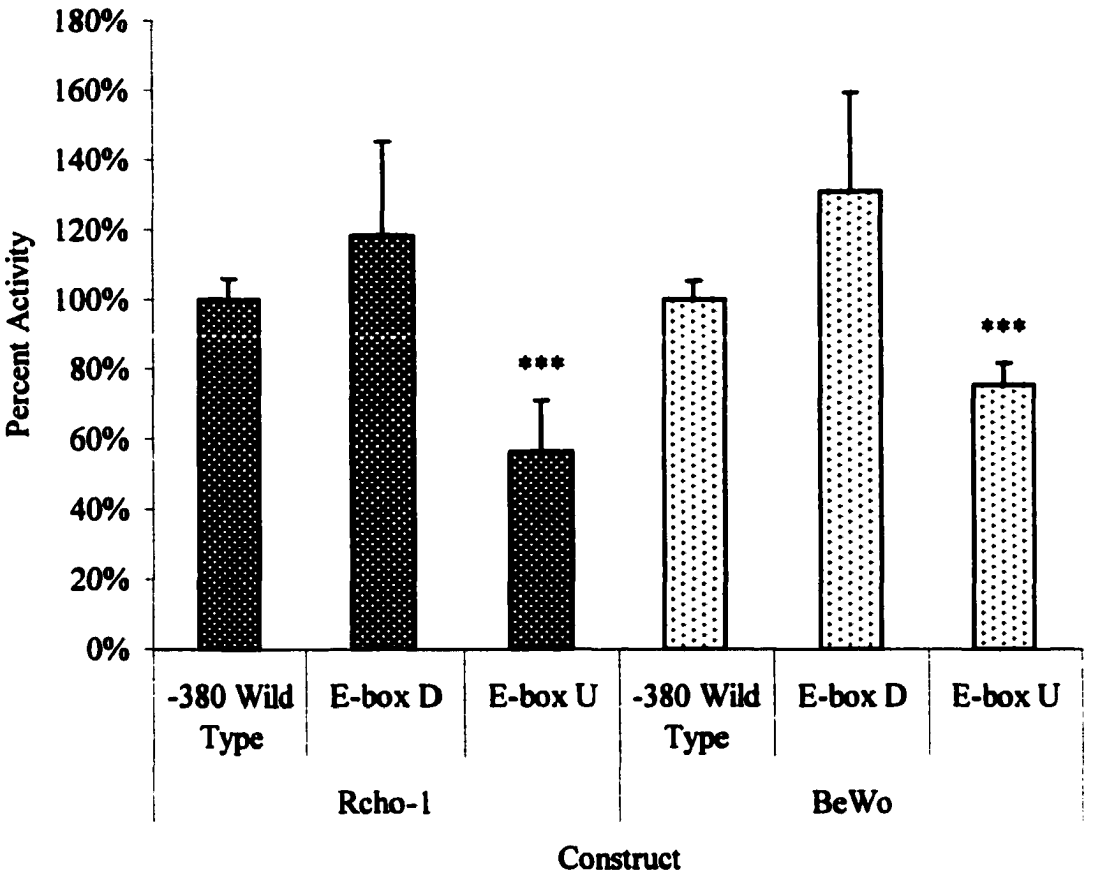


Figure 6. Mutational analysis of the E-box (-163 bp). The –163 bp E-box was analyzed by transient transfection analysis in Rcho-1 and BeWo choriocarcinoma cell lines. Wild-type (-380pGL3) vector is on the left and is compared to E-box mutations (E-box D, TG; E-box U, CA). The bars represent the mean percent activity (Y-axis) and the error bars indicate the SEM. Significant ($P<0.05$) reductions in transactivation were separated by a Dunnett's T test and are indicated by *** above the bar.

A.

```
-340   TGGAGGAGGGCAT   -328
       ||| ||  | |||
Δ338   TGGCGGCCGCCAT

-286   ACAGAGGAGCCTG   -275
       ||   |  |||||
Δ283   ACGCGGCCGCCTG
```

B.

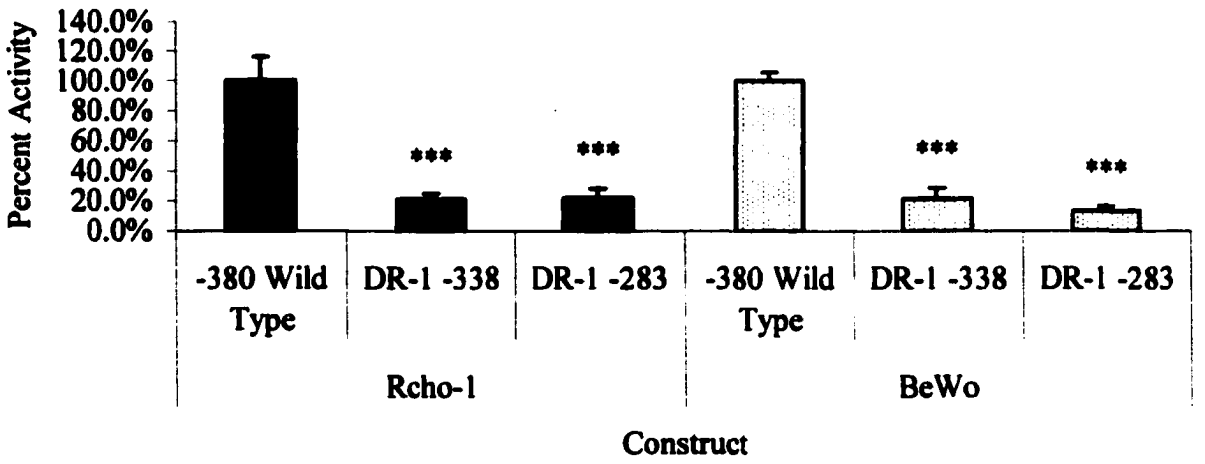


Figure 7. Mutational analyses of the DR-1 sequences (GAGGAG). The DR-1 sequences (-338 and -283) were mutated to Δ338 and Δ283 (Panel A) and compared to the wild-type (-380pGL3) construct in transient transfection assays in Rcho-1 and BeWo cells (Panel B). Mutation of either DR-1 element resulted in significant reduction in activity ($P<0.001$) indicated by ***.

data confirm that trophoblast enhancer elements reside within the protected regions identified by oBNC nuclear extracts and a novel *cis*-acting element, GAGGAG, may interact with the trophoblast-specific transacting factor.

Discussion

Considerable diversity exists between PLs in structure, secretion profiles, ancestral genes and transcriptional regulation, even between ruminants, while other GH/PRL gene family members are almost indistinguishable between species. To alter the production of the oPL gene *in vivo*, the transcriptional regulation of oPL must be defined. Functional data and DNase I protection assays reveal *cis*-acting elements which have been identified in other placentally expressed genes (Limesand 1997; Liang *et al.* 1999), but maximal expression from the oPL proximal promoter requires two additional sites which have not been previously described. The transacting factors that bind these elements remain to be determined, but they enhance oPL gene expression by interacting with trophoblast cell nuclear proteins.

Deletion analysis revealed the proximal 124 bp of the oPL gene 5'-flanking sequence retained trophoblast-specific transactivation in both choriocarcinoma cell lines (Limesand 1997; Liang *et al.* 1999). Three DNase I protected sites were observed in the minimal promoter, -124 bp to +16 bp. The first footprint, FP1 (Figure 1), protected a region encompassing the transcriptional start site, and may indicate that oPL transcription is mediated by an initiator element. The possibility of an initiator element in the oPL gene is pertinent since the non-canonical TATA-box for the oPL gene was not protected and is suspected not to be functional due to its lack of sequence homology. The atypical

nature or lack of a true TATA-box may have contributed to the variation observed in transcriptional start site mapping (Liang 1995; Liang *et al.* 1999), since faithful start sites for transcription require a TATA-box ≈30 bp upstream, and promoters that lack TATA-boxes usually lack clearly defined start points (Liang 1995; Smale 1997; Liang *et al.* 1999). For genes lacking putative TATA-boxes, an initiator element is required to nucleate the preinitiation complex at the appropriate site for transcription to occur (Aso *et al.* 1994). The sequence specificity for an initiator element has been mapped to RRA(+1)NWRR, and a pyrimidine is required at -1. This consensus sequence does not match the start site of the oPL gene since a G is located in the +3 position, which requires an A or T. An initiator element (InrE), as well as a consensus TATA-box, facilitates transcription initiation in the hPL promoter. The InrE was described by mutational analysis within -15 bp to +1bp of the hPL gene, which reduced basal and enhancer stimulated transcription in BeWo and JEG-3 cells (Jiang et al. 1995b). A protein with a M_r of 70,000 is expressed in human trophoblast cells which binds the InrE to aid in the control of trophoblast-specific transcription (Jiang *et al.* 1995b). The sequence of the transcriptional start site for hPL is CTA(+1)GGA, which also has a guanosine in the third position, but also has an adenosine rather than a pyrimidine in the fourth position. Further experiments will need to be carried out on the oPL gene to determine the sequence specificity and purpose of this non-canonical initiator element, and to determine whether it functions similarly to the hPL InrE.

An AP-2 binding site in the oPL gene minimal promoter starting at nucleotide -58 in FP2 was found to interact with AP-2α by EMSA (Figure 2). Human AP-2 was purified and determined to be a 50-52 kDa protein which recognizes a (T/C) C (C/G) C C (A/C) N

(G/C) (C/G) (G/C) sequence (Imagawa *et al.* 1987; Mitchell *et al.* 1987). Activator Protein-2 is thought to regulate several viral and mammalian genes in a tissue-specific manner, and is responsive to both protein kinase A and C signal transduction cascades, as well as being implicated in the regulation of cellular differentiation during embryogenesis (Imagawa *et al.* 1987; Mitchell *et al.* 1987; Meier *et al.* 1995). Additionally, the expression of AP-2 mRNA increases in primary cultures of human cytotrophoblast cells, which are differentiating into syncytiotrophoblast cells (Johnson *et al.* 1997). Human chorionic gonadotropin α- and β-subunit genes, which are transcribed in syncytiotrophoblast cells, are also regulated by AP-2 (Johnson *et al.* 1997). Therefore, AP-2 could be an important transacting factor mediating trophoblast-specific transcription of the oPL gene. However, its ubiquitous expression in a variety of cell types, including Hela cells, suggests that AP-2 is not acting alone (Mitchell *et al.* 1987; Meier *et al.* 1995). Therefore, GATA elements in FP2 and FP3 may indicate that GATA-2 and AP-2α are interacting through a protein-protein interaction to stimulate transcription of the oPL gene. GATA factors have been found to interact with AP-1 (Jun/Fos proteins) to stimulate transcription (Kawana *et al.* 1995). Additionally, GATA-1 has been shown to interact with a protein containing several zinc fingers, Fog (friend of GATA), which may facilitate protein-protein interactions between GATA and other transacting factors (Mackay and Crossley 1998), but there is no existing evidence that implicates an AP-2:GATA protein interaction.

GATA factors have been shown to convey transactivation of the mPL-I gene in a tissue-specific fashion by site mutation, as well as gene ablation experiments (Ng *et al.* 1994; Ma *et al.* 1997). Additionally, the hCG α-subunit, which is also produced in the

placenta, has been shown to use a GATA *cis*-acting element in the 5'-flanking sequence to activate transcription (Steger *et al.* 1994). Numerous putative GATA sites were found in the oPL 5'-flanking sequence, and the functionality of the two GATA elements found in the minimal promoter was examined. Monoclonal antibodies raised against GATA-2, but not ones raised against GATA-3, inhibited protein-DNA complex formation in EMSA with either oBNC or Rcho-1 nuclear extracts (Figure 3B). Mutation of the –67 bp GATA reduced activity in both choriocarcinoma cell lines, but mutation of the –102 bp site was less detrimental to transactivation (Figure 4). Therefore, it would appear that the –67 bp GATA serves a greater role in enhancing oPL gene transcription (Figure 4). However, mutation of both GATA sites within the oPL gene minimal promoter was required to reduce activity to background levels (Figure 4). These data, in conjunction with the GATA EMSA data suggest that GATA-2 is capable of binding to either the -67 or -102 sites to stimulate transcription in choriocarcinoma cells. Interestingly, the proximal (-67 bp) GATA site is absent from the proximal promoter of the bPL gene (Kessler and Schuler 1991).

Deletion from –217 bp to -124 bp in the oPL gene 5'-flanking region did not significantly alter the transactivation by the promoter in human and rat choriocarcinoma cell lines (Limesand 1997; Liang *et al.* 1999). However, DNase I protection assays and EMSA using oBNC nuclear proteins revealed a specific interaction with a putative E-box sequence within this 93 bp region at –163 bp. The functional significance of this element is questionable since deletion of this region (93 bp) did not reduce transactivation in heterologous trophoblast cell lines. However, mutational analysis of the E-box indicated that the upstream portion of this element, which interacts with a slower migrating band in

EMSA, is functional in both BeWo and Rcho-1 cell lines. The functionality of the nuclear protein interacting with the E-box may be context dependent (Fry and Farnham 2000), thereby facilitating transactivation of the oPL promoter in trophoblast cells by augmenting binding of the enhancer element located upstream in FP5 and FP6. While the reduction in activity for the upstream E-box mutation (CA) was significant, the effect was not as dramatic as that observed with the GAGGAG mutations. E-box domains have been described for bHLH proteins in myogenesis, and these transcription factors bind to DNA as dimers to stimulate cellular differentiation (Neville and Rosenthal 1996). Hand1, a bHLH protein found in sheep and mouse trophoblastic tissues, has been implicated in the enhancement of the mPL-I gene and differentiation of trophoblast cells to giant trophoblast cells in the rat and mouse (Cross *et al.* 1995). The binding site for Hand1 heterodimerized with E47 is NNTCTG (Th1 box; Scott *et al.* 2000). Scott and colleagues (2000) indicated that a Hand1 homodimer was able to interact with this element (CATCTG), but the affinity was much greater when it dimerized with E47. Therefore, additional binding sites for Hand1 may be distinguished for other bHLH proteins dimerizing with Hand1, such as ATF1, ITF2, Stra13, I-mfa and Hes-2, 3. These other bHLH factors have been identified in mouse trophoblastic giant cells and may interact with this E-box as homodimers or heterodimers to each other.

Trophoblast-specific regulatory elements reside within 383 bp of the oPL gene 5'-flanking sequence. A deletion from -383 bp to -217 bp resulted in a significant decline or loss of trophoblast-specific transactivation in both choriocarcinoma cell lines (Limesand 1997; Liang *et al.* 1999), suggesting that a species independent regulatory element(s) resides within this 166 bp. Two sites within this region were protected by ovine

binucleate cell nuclear extracts, both containing GAGGAG (DR-1; Figure 1). No previously defined *cis*-acting element matches the DR-1 sequence. Mutation of either DR-1 resulted in significant reduction of reporter activity in both BeWo and Rcho-1 cells (Figure 7). These data confirm the results from the deletion analysis, and provide functional evidence for the GAGGAG sequence as a trophoblast-specific element. Interestingly, our GAGGAG sequence resides between the two Hex sites identified in the human aromatase gene promoter, that have been implicated in placenta-specific expression (Kamat *et al.* 1998; Sun *et al.* 1998). However, the sequences flanking the oPL GAGGAG (FP-5, TGGACA**GAGGAG**CCTGGT; FP-6, CCCCTG**GAGGAG**GGCATG) do not resemble the Hex sites described for the human aromatase gene. Additionally, the GAGGAG sequence is part of the non-canonical Sp-1 binding site (GGGAGG) described in the proximal promoter region of the hPL genes (Fitzpatrick *et al.* 1990). All four of the guanosine residues within the GAGGAG sequence were determined to be involved in binding by methylation interferance assay (Fitzpatrick *et al.* 1990), which could implicate our DR-1 elements as binding sites for Sp-1. However, our DR-1 elements, and the GAGGAG sequence in the human aromatase gene do not possess the hallmarks of a GC box (Sp-1 binding site). Studies with the hPL promoter sequence (Fitzpatrick *et al.* 1990) incorporated the GAGGAG sequence, and the banding pattern observed in EMSA varied between JEG-3 choriocarcinoma cell and HeLa cell nuclear extracts. Antiserum raised against Sp-1 was not used (Fitzpatrick *et al.* 1990) to verify Sp-1 binding to the hPL promoter sequence. The combination of our data and the data previously reported (Kamat *et al.* 1998; Sun *et al.* 1998; Fitzpatrick *et al.* 1990) for the human aromatase and hPL genes may indicate

that the GAGGAG sequence is a crucial element for trophoblast cell activity, and that the transacting factor binding this site needs further investigation.

Our studies identify previously defined *cis*-acting elements, GATA, AP-2 and E-box, demonstrate protein-DNA interactions and functionally assess these elements in choriocarcinoma cell lines. Furthermore, a direct repeat (DR-1; Fig. 7) was identified in DNase I footprints of the oPL gene with oBNC nuclear extracts. The position of these two footprints suggests that they are required for maximal expression in transiently transfected choriocarcinoma cell lines. A mutation of either DR-1 element significantly reduced transcriptional activity, indicating that they may serve as trophoblast-specific enhancers. In summary, oPL gene transcription is modulated by *cis*-acting elements active in primate and rodent placenta, but also appears to require a unique element for enhancement, highlighting the importance of species-specific examination of transcriptional regulation of the placenta.

Summary

Ovine placental lactogen (oPL), a member of the growth hormone/prolactin gene family, is produced by chorionic binucleate cells at the maternal-fetal interface, and is thought to modulate metabolic processes and enhance fetal growth. The oPL gene was characterized and an additional 4.5 kb of 5'-flanking sequence was analyzed. Transient transfection of human (BeWo) and rat (Rcho-1) choriocarcinoma cell lines localized the trophoblast-specific *cis*-acting elements within the proximal 1.1 kb of oPL gene 5'-flanking sequence, and deletion constructs demonstrated that maximal activity resides

within the proximal 383 bp of oPL gene 5'-flanking sequence. DNase I protection analysis using ovine chorionic binucleate cell nuclear protein, identified six protected regions within the -383 bp to +16 bp region, relative to the transcriptional start site. Electrophoretic mobility shift assays and mutational analysis identified two functional GATA (-67 bp, -102 bp) sequences as transactivators of the oPL gene. The –67 bp GATA site was found to interact with GATA-2. An AP-2 element (-58 bp) was identified by DNase I protection assays and a specific interaction was confirmed with electrophoretic mobility shift assays and supershift assays, indicating that AP-2α may coordinate oPL transactivation. An E-box (-163 bp) was detected in the region that does not appear to enhance promoter activity (-217/-124 bp). Nuclear proteins were found to specifically interact with the E-box, and mutational analysis revealed that a protein interacting with the upstream region of the E-box facilitates transcription in trophoblast cells. However, a previously undefined direct repeat (DR-1, GAGGAG) residing at -338 bp and -283 bp was required for full transactivation, and mutation of either element significantly reduced promoter activity. Transcriptional regulation of human and rodent PL genes has been previously characterized, and our results indicate that tissue-specific regulation of oPL expression may result from *cis*-acting elements in common with human and rat genes expressed within the placenta. However, our data indicates that regulation of the oPL gene also results from novel *cis*-acting elements.

Chapter IV

NOVEL AP-2α SPLICE VARIANTS FUNCTION AS TRANSACTIVATORS FOR THE OVINE PLACENTAL LACTOGEN GENE

Introduction

One placental hormone, placental lactogen (PL), is a member of the growth hormone (GH)/prolactin (PRL) gene family and is thought to influence both maternal and fetal metabolism to promote fetal growth and development (Ogren and Talamantes 1994; Anthony *et al.* 1995a; Anthony *et al.* 1995b; Anthony *et al.* 1998). Chorionic binucleate cells synthesize ovine PL (oPL), a non-glycosylated 198 amino acid polypeptide with an apparent M_r of 22,000, and secrete it into both the maternal and fetal vasculature (Colosi *et al.* 1989; Warren *et al.* 1990; Kappes *et al.* 1992). The oPL gene has been structurally characterized, and 4.5 kb of 5'-flanking sequence was analyzed for promoter activity in choriocarcinoma cell lines (Liang *et al.* 1999). Transient transfection analysis revealed that the proximal 124 bp of oPL promoter was capable of trophoblast-specific transactivation (Liang *et al.* 1999). An activator protein-2 (AP-2) element was identified by DNase I protection assay, at –58 bp relative to the oPL gene transcriptional start site and confirmed by electrophoretic mobility shift (EMSA) and super shift assays (Liang *et*

al. 1999). These data combine to indicate that AP-2 is enhancing transactivation of the oPL gene.

Activator protein-2 has been shown to stimulate transcription of genes in human and mouse placentas. The α- and β-subunit genes of human chorionic gonadotropin (hCG) have functional AP-2 elements that are stimulated by AP-2α (Johnson *et al.* 1997). In addition, AP-2α mRNA increased during differentiation of cytotrophoblasts to syncytiotrophoblasts *in vitro* (Johnson *et al.* 1997). These data indicated an up-regulation of AP-2α during differentiation is correlated with the stimulation of hCG α- and β-subunit gene expression in syncytiotrophoblast cells. In the mouse, adenosine deaminase (ADA) is expressed in trophoblast cells of the chorioallantoic placenta, and an AP-2 element was identified by DNase I protection assays and EMSA / supershift assays in the region of the ADA gene responsible for placental expression (Shi and Kellems 1998). Mutation of the AP-2 element in the ADA gene resulted in a loss of transactivation in the placenta of transgenic mice. In addition to characterizing a tissue-specific AP-2 element, the expression of AP-2 was examined and found to be AP-2γ, which is expressed in all mouse trophoblast cells throughout development (Shi and Kellems 1998).

Tissue-specific regulation of placental genes via an AP-2 element and the species-specific expression of AP-2 genes in the trophectoderm prompted us to study AP-2 in the ovine placentome and it's ability to enhance oPL expression. Initially, functionality of the oPL gene AP-2 element was analyzed by mutational analysis in transiently transfected BeWo cells, indicating that the AP-2 element was functional. Therefore, subsequent experiments were performed to characterize AP-2 in ovine placenta. Four

full-length ovine AP-2α clones were purified from a cotyledonary cDNA library and nucleotide sequencing revealed they were potential splice variants of the ovine AP-2α gene. The ability of these four clones to stimulate transactivation of the oPL minimal promoter in transiently transfected HepG2 cells, which lack endogenous AP-2, was slice-variant dependent. Additionally, western analysis and reverse transcription-polymerase chain reaction of human and ovine placental tissues confirmed the presence of the AP-2α splice-variants. The present studies indicate that AP-2α activation of the oPL promoter is splice-variant dependent, and may suggest an alteration in AP-2α transcription during placental cell differentiation.

Materials And Methods

Construction and Preparation of Plasmid DNA

Activator protein-2 element mutation and pGL3 vector synthetic oligonucleotides used for DNA sequencing and polymerase chain reaction (PCR) are: pGL2 forward 5'-TGT ATC TTA TGG TAC TGT AAC TG-3', pGL2/3 reverse 5'-CTT TAT GTT TTT GGC GTC TTC C-3', oPL K5-6 5'-TCG CGG CCG CGG CAT GAG TAA TCA GTA ACA-3' and oPL K5-6 5'- CCG CGG CCG CGA GCT ATC ACT GCT TCT-3'. Oligonucleotide primers were synthesized by Macromolecular Resources at Colorado State University (Fort Collins, CO).

Dual PCR amplification was employed to generate single 8 bp block mutations within the 124 bp proximal promoter region of the oPL gene. The first reaction contained 0.2 μM of each primer, either the forward mutant primer (oPL K5-6) and reverse plasmid (pGL2) primer, or the forward plasmid (pGL2) primer and reverse mutant primer (oPL

K5-6R). In addition to the primers, 6.6 fmoles of 124pGL2, 0.1 μM of each dNTP, and 1 U *Taq*™ DNA polymerase (Gibco BRL) were added to a 1X reaction buffer with a final volume of 50 μL. A five min incubation (95°C) preceded the addition of *Taq* polymerase, and 10 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 2 min, extension at 72°C for 1 min were employed to generate DNA with mutated ends. An additional 30 cycles with an annealing temperature of 60°C were used to amplify mutated DNA fragments. PCR products were analyzed on a 1.6% agarose gel, and mutated DNA fragments were gel purified with DEAE membrane strips (Schleicher and Schuell, NH). The purified 5' and 3' mutated DNA fragments were used as template DNA (10 fmoles) in a second PCR reaction with pGL2 plasmid primers at 0.2 μM. The same PCR procedure, which created the first mutant fragment, was followed. The entire 124 bp mutant fragment was digested with restriction endonucleases (Kpn I and Hind III), gel purified, ligated into the pGL3 Basic vector, and transformed into competent DH5α *E. coli* cells. The constructs were screened by restriction endonuclease digestion (Not I) and confirmed by southern analysis. Positive constructs for each mutation were sequenced to verify the ligated ends and mutated region. Covalently closed circular DNA was prepared for each construct using an alkyline-lysis procedure and purified by CsCl equilibrium gradient centrifugation.

Non-radioactive Southern Hybridization of AP-2 clones

Digested DNA was separated on a 1.5% agarose gel, denatured and capillary blotted overnight in 1XSSC (0.150 M sodium chloride, 40 mM sodium citrate pH 7.0) to ZetaBind Transfer Membrane (Life Science Products, Inc., CO). Prehybridization

occurred in 5X SSC, 1 % nonfat dry milk, 0.1% N-lauroylsarcosine, 0.2% sodium dodecyl sulfate (SDS) for two hours at 55°C. The denatured, PCR generated, digoxigenin labeled DNA was added to the prehybrization solution at a concentration of 25 ng/ml and incubated overnight at 55°C. The membranes were washed twice for 5 min in 2X SSC with 0.1% SDS at room temperature, followed by two washes in 0.5X SSC containing 0.1% SDS at 65°C for 20 min. After the washes, membranes were equilibrated in 100 mM maleic acid, 150 mM sodium chloride; pH 7.5 and 3% Tween 20, and blocked in 1% nonfat dry milk in 100 mM maleic acid for 30 min at room temperature. Anti-digoxigenin conjugated with alkaline phosphatase (Boehringer Mannheim, GmbH, Germany) was add to the blocking buffer at a 1:5000 dilution and incubated at room temperature for 30 min. The membranes were washed twice in wash buffer at room temperature for 15 min. The membranes were equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5). The color substrate, 338 μg NBT and 175 μg 5-bromo-4-chloro-3-indolyl phosphate in detection buffer (10 ml), was added to the membrane and incubated at room temperature in the dark for 2-3 hr. After color development the membranes were washed several times in water for 30 min to stop development.

Mammalian Cell Lines and Transient Transfection

The BeWo and HepG2 cell lines were obtained from American Tissue Culture Collection (Rockville, MD). The Rcho-1 cell line was a gift from Dr. M. J. Soares (University of Kansas, Kansas City, KS). BeWo cells were cultured in F12K medium (Mediatech, Inc., Herndon, VA) supplemented with 100 U/ml penicillin, 100 μg/ml

streptomycin (Sigma Chemical Co.) and 10% fetal bovine serum (FBS; Gemini BioProducts, Inc., Calabasa, CA). The Rcho-1 cells were cultured in NCTC-135 supplemented with 20% FBS, 1mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin. The Hepatic carcinoma cell line (HepG2) was cultured in DMEM media (Mediatech, Inc.) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS. All cells were maintain in monolayer cultures at 37°C in 5% CO_2, 95% air with 100% humidity.

Transient transfections in choriocarcinoma cell lines, BeWo and Rcho-1, were performed with LipofectAmine reagent (Gibco BRL). Cells were plated at a density of 0.5 x 10^6 cells/well in 6-well 35 mm plates one day before transfection. The polycationic lipid/DNA complex reaction was a mixture of 10 μl lipid reagent, 1.5 pmoles of test plasmid, 50 fmoles of RSV-β-gal Control vector as a co-transfected DNA to normalize intra-assay variation, and the volume was adjusted to 100 μl with medium that did not contain serum and antibiotics. This mixture was incubated at room temperature for 20 min during which time the cells were washed twice in media that was serum and antibiotic-free. Serum and antibiotic-free media (890 μl) was added to the lipid/DNA mixture and placed on cells. The cells were incubated at 37°C for 24 hr in the presence of the lipid/DNA mixture after which time the medium was replaced with media supplemented with serum, and incubated for an additional 48 hr.

After the incubation period, cells were lysed in 25 mM Gly-Gly ($C_4H_8N_2O_3$), 1 mM DTT and 0.1% Triton X100, and analyzed using a Luciferase Assay System (Promega, Madison, WI) or LumiGAL Chemiluminescent Assay System (ClonTech, Palo Alto, CA). The light emission generated by both systems was measured using a Turner TD-

20e luminometer with a 5 second delay and an integration time of 10 seconds. Luciferase activity was normalized by β-galactosidase activity from the RSV-β-gal Control vector for each sample. Relative luciferase activity was expressed as fold stimulation over the relative background activity of a promoterless vector (pGL3-Basic) to adjust for inter-assay variation. Data are presented as the mean percent activity±SEM determined from multiple (n=8) transfection experiments. The transfection data were analyzed by least square analysis of variance using Statistical Analysis Systems (SAS Institute, 1993). In general linear model procedures (Snedecor and Cochran 1976), differences in the means between wild type vector and mutant vector were separated with a Student T test.

HepG2 cells were transiently transfected by calcium phosphate transfection procedures. Cells were plated at 2 X 10^6 cell/100mm plate and maintained in 10 ml of media for 16 hr at 37°C in 5% CO_2. Luciferase reporter constructs (-124pGL3 or -124mAP-2pGL3; 1.5 pmoles) and RSV-β-gal Control vector (50 fmoles) were transfected with either an AP-2 expression vector stimulated by the cytomegulovirus promoter or negative control DNA (pBlueScript; Stratagene) in 124mM $CaCl_2$ HEPES buffered saline (HBS; 25mM HEPES, 150mM NaCl) and incubated for 1 hr at room temperature. The precipitate was added to HepG2 cells and incubated at room temperature for 30 min. Ten ml of serum-free medium were added to the cells which were incubated at 37°C in 5% CO_2 for an additional 16 hr. HepG2 cells were washed 3 times with 10 ml of PBS, serum-containing media was added and cells were maintained for an additional 40 hr at 37°C. Cells were harvested and analyzed in the same manner as the choriocarcinoma cell lines.

Immunohistochemistry for AP2 Localization

Immunohistochemistry was completed using an immunoperoxidase procedure (Vectastain Elite ABC kit; Vector Laboratory CA). Briefly, paraffin embedded sections of ovine placentomes were deparaffinized in Histo D (Fisher) and rehydrated in a graded-ethanol series. Endogenous peroxidase activity was quenched with two 30 min incubations of 0.3% H_2O_2. The sections were microwaved twice in 10 mM citric acid (pH 6.0) for 5 min, before incubating in 1.5 % normal goat serum for 20 min to block non-specific binding. The sections were then incubated with 0.1 μg/ml AP-2α antiserum (AP-2α; C-18; Santa Cruz Biotechnology, Santa Cruz, CA) for 45 min. Sections were then rinsed in PBS (pH 7.4), and incubated with biotinylated goat anti-rabbit immunoglobulin G antiserum and avidin-peroxidase conjugate for 1 hr, and diaminobenzidine tetrahydrochloride (DAB) substrate (Vector Laboratory) for 5 min. The procedure was repeated with oPL antiserum (1:25,000) and identified with VIP substrate (Vector Laboratory). The sections were dehydrated, mounted and photographed.

RNA Isolation and Northern Hybridization Analysis

Total cellular RNA was isolated from 100 day post coitus cotyledonary tissue (Ausubel et al. 1995). Tissue was quickly frozen in liquid nitrogen and stored at less than –70°C until RNA isolation. Isolation of the RNA began with grinding tissue in liquid nitrogen with a pestle and mortar. Ground tissue was placed in 4 M guanidine thiocynate, 25mM sodium citrate, 0.5% N-lauroyl sarcosine and 0.1 M β-mercaptoethanol at 0.1 g of tissue per ml and aspirated and expelled though an 18-guage

needle three times. The solution was transferred to a polypropylene tube and centrifuged at 12000 X g for 15 min. Seven ml of supernatant were placed on a 3 ml 5.7 M cesium chloride cushion, and total cellular (tc) RNA was pelleted by centrifugation at 147,000 X g for 16 hr (22°C). The tcRNA was resuspended in RNase-free 50 mM potassium citrate, 100mM potassium chloride and 1 mM EDTA. Phenol:chloroform:isoamyl solution (25:24:1) was added in equal volume, vortexed and centrifuge for 5 min, and the upper aqueous phase with the tcRNA was saved. The tcRNA was precipitated with 0.1 volume of 8 M lithium chloride and 2 volumes of 100% EtOH that was incubated overnight at -20°C, microcentrifuged for 20 min at 4°C (13,000 rpm) and washed once with ice cold 70% ethanol. After air-drying, tcRNA was resuspended in 0.5 ml diethyl pyrocarbonate-treated (DEPC; 0.1%) H_2O and concentration was determined by absorbance at 260 nm.

Cotyledon tcRNA was analyzed for degradation by electrophoresis though a 1.5% agarose, 66 mM formaldehyde, MOPS-buffered (20 mM 3 N-morpholine propane-sulfonic acid, 5 mM sodium acetate and 1 mM EDTA) gel. The gel was equilibrated with 1X TAE (40 mM Tris-acetate pH 8.5 and 2 mM EDTA) buffer solution by washing the gel 4 times for 20 min each, and electro-blotted on to Zetabind nylon membrane. Transferred tcRNA was crosslinked (CL-1000 Ultraviolet Crosslinker UVP), dried and soaked in 5% acetic acid for 15 min. To visualize 18S and 28S bands a 0.5 M sodium acetate (pH 5.2), 0.04 % methylblue solution was incubated with the membrane for 5 min and subsequently washed in DEPC H_2O to remove excess stain and photographed.

Northern hybridization was performed on membranes to determine size of AP-2 mRNA in placental tcRNA. An AP-2α cDNA fragment was generated by reverse transcription-polymerase chain reactions (RT-PCR) using synthetic oligonucleotides

homologous to human and mouse AP-2α sequences (AP-2F; 5'-TCC ACC TCG AAG TAC AAG GT-3' (exon 4) and AP-2R; 5'-GCT GAG GTA CAT TTT GTC CAT G-3'(exon 7)). Synthesized products of ~500 bp were inserted into pCR 2.1, confirmed by sequencing, and the insert was purified and radiolabeled by random prime labeling reactions (Multi-prime kit; Amersham). The membrane was incubated in pre-hybridization solution containing 6X SSPE, 1% SDS, 10% dextran sulfate, 50% formamide, 200 μg/ml herring sperm DNA and 50 μg/ml yeast tRNA for 5 hr at 42°C. After blocking, 2 X 10^6 cpm/ml of radiolabeled AP-2 insert were added to the membrane and incubated at 42°C overnight. The membrane was washed for 5 min in 2X SSC with 0.1% SDS once at room temperature and once at 42°C. Two stringent washes in 0.1X SSC with 0.1% SDS at 60°C for 20 min were performed to remove nonspecific binding. Following washes the membrane was exposed to Kodak x-ray film for 48-72 hr.

Construction and Screening of an Ovine Placental cDNA Library

Poly-adenylated (poly-A^+) RNA was isolated for construction of the cotyledonary cDNA library. Briefly, 2 mg of tcRNA in 0.5 M LiCl, 10mM Tris-HCl, 0.5mM EDTA and 0.1% SDS were heated to 70°C for 10 min. The RNA was passed though an oligo-dT cellulose (BioLab) column (0.4 ml), which was prewashed three times with 2 ml 0.1 NaOH, 10 ml H_2O and 10 ml buffer to maximize binding. The oligo dT column with mRNA bound was washed with 2 ml of 0.15 M LiCl, 10mM Tris-HCl, 0.5mM EDTA and 0.1% SDS, and eluted with 2ml of 2mM EDTA with 0.1% SDS. The eluted mRNA was precipitated, suspended in DEPC H_2O and checked for purity on a formaldehyde-agarose gel.

Complementary DNA was generated using the Stratagene cDNA synthesis kit. Prior to first stand synthesis, 5 μg of poly-A^+ RNA in 20 μl were heated to 65°C for 5 min, and cooled to room temperature. The mRNA was treated with 2 μl of 100 mM methylmercury hydroxide for 1 min, followed by a 5 min incubation with 4 μl of 0.7 M β-mercaptoethanol to relax the secondary structure and reduce truncated cDNA fragments. Annealing of the linker-primer (2.8 μg; GAGAGAGAGA GAGAGAGAGA ACTAGTCTCG AGTTTTTTTT TTTTTTTTTT) in the presence of 1X first-strand buffer (0.6 mM dGTP, 0.6 mM dTTP, 0.6 mM dATP, 0.3 mM 5-methyl dCTP, 125 μM [α-^{32}P] dATP, 800 Ci/mmol; ICN, Inc.) and 40 U RNase Block Ribonuclease inhibitor was accomplished by incubation for 10 min at room temperature. The first stand synthesis of the cDNA was performed with Moloney murine leukemia virus reverse transcriptase (75 U) at 37°C for 1 hr. Second strand synthesis was performed in 1X second-strand buffer (0.3 mM dGTP, 0.3 mM dTTP, 0.3 mM dATP, 0.78 mM dCTP, 125 μM [α-^{32}P] dATP, 3 U Rnase H, 100 U DNA polymerase I) that was incubated for 2.5 hr at 16°C. The ends of the cDNA were blunted with 5 U of *Pfu* DNA polymerase for 30 min at 72°C, phenol-chloroform (1:1) extracted and precipitated. The pellet was resuspended in 9 μl of 0.4 μg/μl Eco RI adapters (AATTCGGCACGAG) in 1X ligase buffer, 1 mM rATP, and 4 U T4 DNA ligase and incubated for 2 days at 8°C. The ligation reaction was inactivated by incubation at 70°C for 30 min and subjected to Xho I restriction endonuclease digestion. The cDNA (60 μl) was added to a 1 ml Sephacryl S-500 spin column in 0.1 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10 mM EDTA buffer and centrifuged at >400 x g. This was repeated 4 times with the addition of 60 μl of buffer

each time. The fractionated cDNA (1/10 of reaction) was electrophoresed over a 5% nondenaturing polyacrylamide gel to confirm integrity. The first fraction was extracted with phenol-chloroform (1:1), chloroform, precipitated and resuspended in 3 μl of water and quantified with ethidium bromide intercalation. The Eco RI/Xho I cDNA (100 ng) was ligated into ZAP Express Eco RI / Xho I vector (1 μg), with 1 mM rATP, and 2 U T4 DNA ligase for 2 days at 4°C. The ligated vector DNA was packaged using the Gigapack III Gold Packaging Extract (Stratagene) and the titered was 6.9 x 10^7 pfu/μg. The library was amplified and stored at -80°C.

The cotyledonary cDNA library was screened to identify phage with an AP-2 cDNA. Briefly, 50,000 pfu per 150 mm NZY agar plate were incubated overnight at 37°C, chilled for 2 hr at 4°C, and transferred on to Magna Graph nylon filters (Micron Separations, Inc., Westborough, MA). The DNA on the filters were denatured in 1.5 M NaCl and 0.5 M NaOH solution for 2 min and neutralized in 1.5 M NaCl/0.5 M Tris-HCl (pH 8.0) for 5 min. The filters were then rinsed in 2X SSC, blotted dry on Whatman paper and crosslinked. Duplicate filters were prehybridized in 6X SSPE, 1% SDS, 0.1% (w/v) Ficoll 400, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) bovine serum albumin (BSA), 50% formamide, 200 μg/ml herring sperm DNA and 50 μg/ml Yeast tRNA for 5 hr at 42°C and hybridized overnight with 1X10^6 cpm/ml of AP-2 cDNA insert. Hybridized filters were washed at the same stringency as the Northern blots and exposed to x-ray film. Positive plaques were picked and purified to homogeneity in subsequent rounds of DNA hybridization screening.

Positive phage clones had the pBK-CMV plasmid excised using the helper phage, ExAssist (Stratagene). The pBK-CMV phagemid produced was used to infect the

XLOLR *E. coli* strain (Stratagene), which were plated on LB/Kanamycin (50 μg/ml) plates. Plasmid DNA for 3 colonies of each positive plaque were purified, digested with EcoRI and Xho I restriction endonucleases, separated on an agarose gel and subjected to Southern hybridization to confirm AP-2 inserts. The largest clones were sequenced with T3, T7, AP-2F, and AP-2R synthetic oligonucleotide primers. Additional primers generated for sequencing entire AP-2 cDNA inserts include: AP-2F4, 5'-TTC CCT GGA ATC AAC TCC-3'; AP-2R4, 5'-GAC TTC GCC CTC CTC AGC AC-3'; AP-2F5, 5'-TCC CGC ACT TAA AAA CCT TCC-3'; AP-2R9, 5'-GAG AGA AGA CGG CAT CAG-3'; AP-2R10, ACG TGG GAG TAA GGA TC-3'; AP-2F6 5'-CAT GGA CAA AAT GTA CCT CAG-3'; and AP-2F11, 5'-ATC GCG GCA GGA TAG AGA C-3'. Additionally, the AP-2 cDNAs were digested with BamHI and/or EcoRI and fragments were subcloned into pBlueScript (Stratagene) for sequencing (Davis Sequencing, Davis, CA).

Electrophoretic Mobility Shift Assays (EMSA)

Cellular proteins were isolated from HepG2 cells that were transiently transfected with the ovine AP-2 expression vectors. Cells were removed from the tissue culture dish with 0.25% trypsin/EDTA (Sigma) and washed 3 times with 1X PBS. The cells were resuspended in 20 mM Hepes (pH 7.9), 450 mM KCl, 20% glycerol (v/v), 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT and subjected to 3 freeze-thaw cycles. The cellular debris was removed with a 10 min centrifugation at 16,000 x g.

DNA binding assays were performed with antisense oligonucleotide radiolabeled with polynucleotide kinase and [γ-^{32}P] ATP (Ausubel *et al.* 1995). The oligonucleotide

was heated to 100°C with an equal-molar ratio of sense oligonucleotide, and slowly cooled to 4°C. The double- stranded radiolabeled oligonucleotide was purified by G-25 size-exclusion chromatography, and quantified by scintillation spectrometry. Oligonucleotides generated for AP-2 EMSA are oAP-2 sense, 5'-GCT CCA CCC CAG GGC ATG-3' and oAP-2 antisense 5'-CAT GCC CAG GGG TGG AGC-3'. Binding reactions included 20 mM HEPES (pH 7.9), 20 % glycerol (v/v), 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.25 mM DTT. Additionally, spermidine (1 mM), BSA (25 μg/ml) and Poly (dI-dC)-Poly (dI-dC) (1 μg/reaction) were added. The reaction buffer, unlabeled competitors and cellular extracts (10 μg) were gently mixed and placed on ice for 10 min at which time 5 fmol of double stranded radiolabeled oligonucleotide (50,000 cpm) were added. After a 30 min incubation at 37°C, the reactions were placed on ice (5 min) and electrophoresed through a 5% polyacrylamide nondenaturing gel which had been pre-electrophoresed at 20 mA for 2 h. The gels were dried and exposed to x-ray film.

Western Immunoblot Analysis

Nuclear proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Ausubel *et al.* 1995). Nuclear proteins were transferred to nitrocellulose membrane (Micron Separations, Inc.) in 2 mM Tris pH 8.3, 150mM glycine, 20% methanol, and 0.1% SDS buffer. The membrane was blocked in 0.5% bovine serum albumin (BSA), 1X TBST (10 mM Tris pH 8, 150 mM NaCl, 0.05% Tween 20) for 1 hr. Immunoblot detection of AP-2α was accomplished with an AP-2α antiserum (Santa Cruz Biotechnology). The membrane was incubated with the antibody

(0.1 μg/ml) in 1X TBST and 0.5% BSA overnight at 4°C. The membrane was washed 3 times with 1X TBST for 10 min, and incubated with anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology) at 1:2000 for 1 hr at room temperature. The membrane was washed 2 times in 1X TBST and one time in 1X TBS, incubated for 1 min in Western Blotting Luminol Reagent (Santa Cruz Biotech) and exposed to Kodak x-ray film.

RT-PCR Analysis

Ovine cotyledonary tcRNA, mouse placental tcRNA, BeWo cell tcRNA, and Hela cell tcRNA was purified over a cesium chloride cushion (see above), and the human placental RNA was a gift from Dr. Tim Regnault, University of Colorado Health Science Center, Denver CO. The mRNA was reverse transcribed with Superscript II reverse transcriptase (Gibco BRL) using an oligo-dT primer. Variant AP-2α cDNAs were amplified with specific forward primers and the AP-2R oligonucleotide as the reverse primer. The specific forward oligonucleotide primers synthesized by Gibco BRL are: AP-2v1 (human exon 1a), 5'-CAC GCC GAT CCA TGA AAA TGC T-3'; AP-2v3 (ovine AP-2 clone 6501), 5'-CTT CGC AGA TGT TAG TTC ACA G-3'; AP-2v6 (ovine AP-2 clone 6602), 5'-AGC CGC GAT GTC CAT ACT TG-3'; and AP-2v7 (ovine AP-2 clone 1812 or 242), 5'-GCC TGA TGC CGT CTT CTC TC-3'. PCR reactions were setup in the same manner as previously described for generating the mutation construction, except the new oligonucleotide primers were used and 1 μl of the RT reaction. These PCR reactions were subjected to 25 cycles of 95°C for one min, 59°C for 2 min and 72°C for 2 min with Recombinant Taq Polymerase (Gibco BRL). Amplified

products were separated on a 1% agarose gel and subjected to Southern hybridization to confirm sequence identity.

Results

Functional Analysis of the AP-2 Cis-acting Element in Choriocarcinoma Cells

Previous studies identified an AP-2 element at –58 bp relative to the oPL gene transcriptional start site. The AP-2α-DNA interaction was confirmed by electrophoretic-mobility shift (EMSA) and supershift assays and resides within the minimal promoter region (-124 to +16 bp) of the oPL gene (Liang *et al.* 1999). Mutation of the AP-2 element (CCCCAGGGC→GGCCGCGGC) in the oPL minimal promoter sequence was used to test the functionality of the *cis*-acting element. The -124 wild-type pGL3 (-124pGL3) and the -124 mutant AP-2 (-124mAP2) vectors were transiently transfected into BeWo and Rcho-1 cells. In BeWo cells a 74% reduction in activity was observed with the mutation, indicating that the AP-2 element in the oPL minimal promoter was functional (Figure 8). The transfections performed in Rcho-1 cells exhibited no reduction in transactivation (data not shown). Therefore, differences in choriocarcinoma cell lines suggest a species variation in the transactivation potential at the AP-2 *cis*-acting element in the oPL minimal promoter.

AP-2α in Ovine Placenta

Functionality of the AP-2 element in BeWo cells suggested that AP-2 may be an important transactivator for oPL, but these data were obtained from a heterologous system and were not confirmed in Rcho-1 cells. Currently there is no ovine placental cell

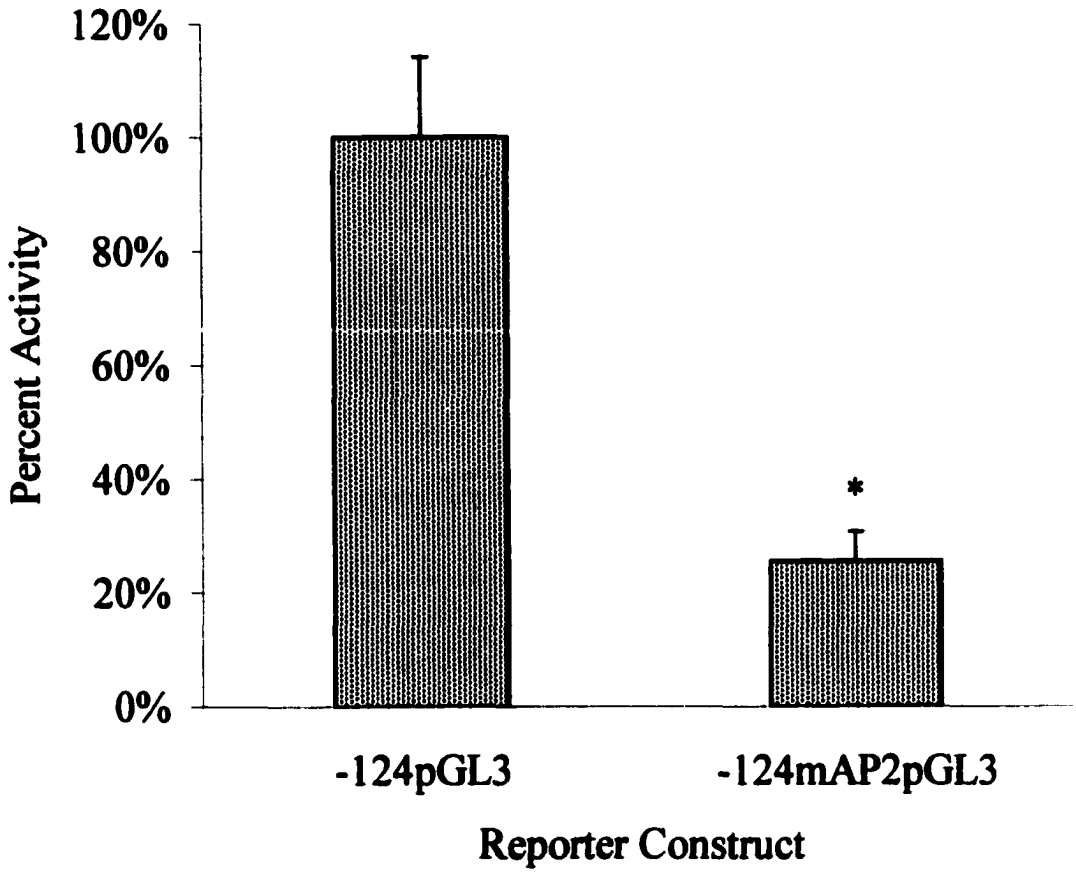


Figure 8. Functional analysis of the AP-2 *cis*-acting element in the oPL gene. An AP-2 element at –58 bp, relative to the oPL gene transcriptional start site was assessed in transient transfection assays. Human choriocarcinoma (BeWo) cells were transiently transfected with the oPL gene minimal promoter (-124pGL3) or a vector with a mutation at the AP-2 element (–124mAP2pGL3). The mean percent activity of the wild-type vector was designated 100%. The decline in transactivation (percent activity) between the wild type and AP-2 mutation was significant ($P<0.05$) and is distinguished with an asterisk.

line, and various attempts to transiently transfect ovine binucleate cells have failed. Therefore, AP-2α was identified in ovine cotyledonary tissue by Northern hybridization and immunolocalized in the sheep placentome at day 40 and 120 of gestation. Northern hybridization of ovine cotyledonary tcRNA identified a single transcript that was ~3 kb in size (Figure 9). Within the placentome, AP-2 was co-localized to ovine binucleate cells within the fetal chorionic epithelium at both 40 and 120 days post coitus (Figure 10). AP-2 was not exclusively expressed by ovine binucleate cells, and was found in other epithelial cells within the cotyledon, but not in uterine epithelium.

To confirm the involvement of AP-2 as a transactivator for the oPL gene, a cotyledonary cDNA library was screened and 103 positive plaques were purified to homogeneity from 4.9×10^7 plaques. Four independent clones encoded full-length ovine AP-2 as determined by nucleotide sequencing. The comparison of the deduced amino acid sequences of these clones revealed that they varied at the amino terminus, but were homologous to the C-terminal region of human AP-2α. Clone 242 and 1812 were 100% identical throughout their coding region, and encode a protein with a predicted M_r of 53,927. Clone 242 contained an additional 516 bp of 5' untranslated region (UTR) and clone 1812 had an additional 596 bp of 3' UTR, but both were approximately 3 kb in length. The 5' segment encoding the N-terminal amino acid residues for these 2 clones did not match previously described AP-2α isoforms, and indicates alternative splicing of ovine AP-2α mRNA (Figure 11). Significant nucleotide identity exists between sheep AP-2α and human and mouse AP-2α sequences from exon 2 to the stop codon, 97% and 94% respectively. The first 1220 bp of clone 242 shares 67% identity to human AP-2α gene intron A, at 5381 bp to 6562 bp (Bauer *et al.* 1994). This sequence is located

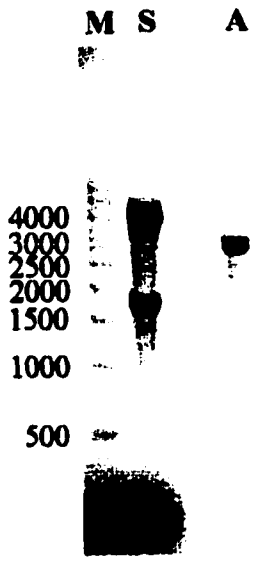
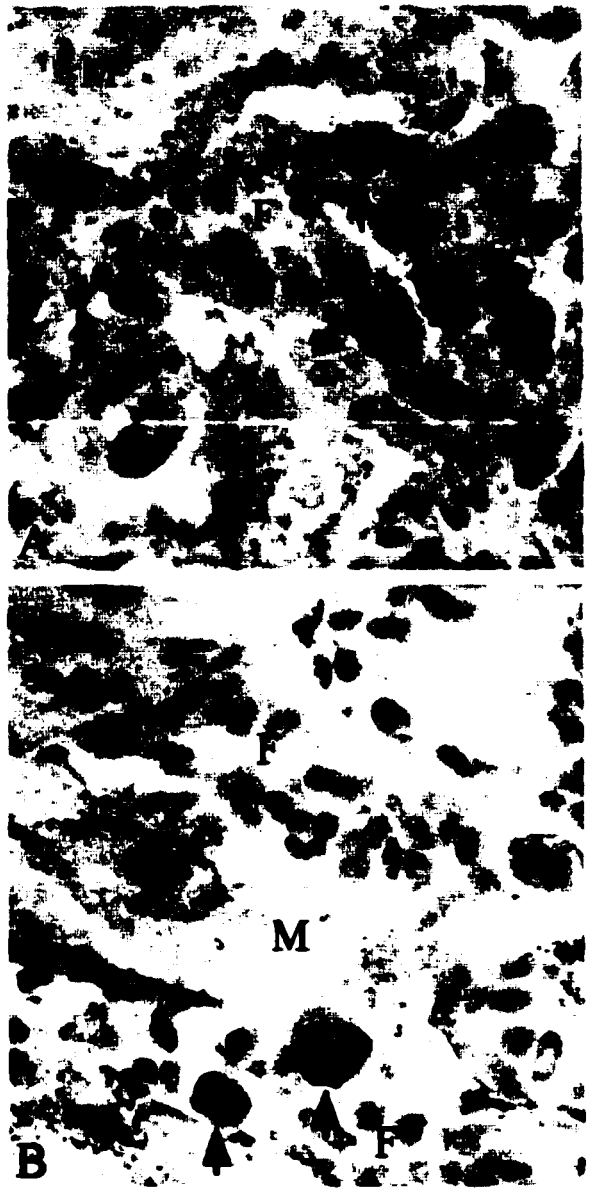


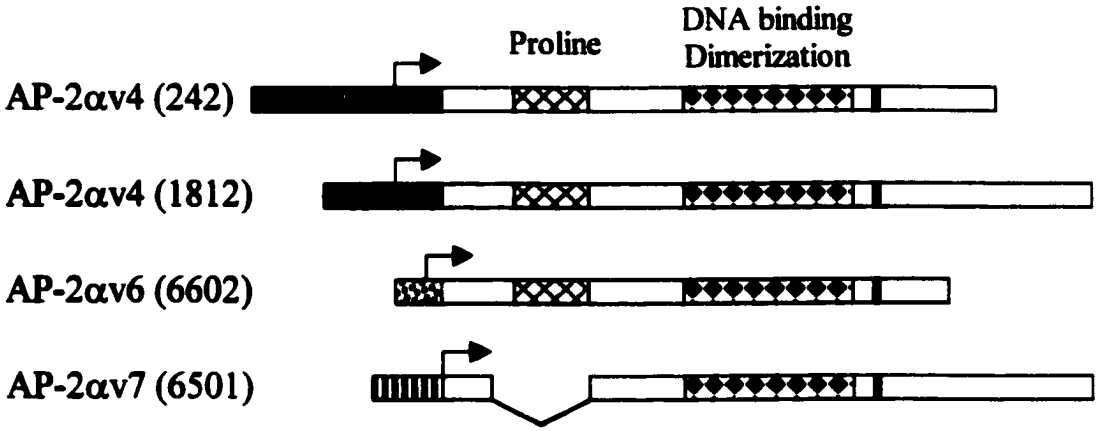
Figure 9. Northern hybridization analysis AP-2α. Ovine cotyledonary tcRNA was separated though a 1.5% agarose gel and transferred to a nylon membrane. On the left, methylblue staining was used to visualize the nucleotide markers (M), and the 28S and 18S ribosomal RNA in cotyledonary tcRNA (S). A predominant AP-2α transcript at ~3000 nucleotides was distinguished by an autoradiograph (A) of the AP-2 Northern blot hybridization, and confirms the presences of AP-2α mRNA in ovine cotyledonary tcRNA.

Figure 10. Immunohistochemical localization of AP-2α in the ovine placentome. Activator protein-2α was identified in the nuclei of the chorionic epithelium by immunoperoxidase staining with DAB substrate (brown), and localized to the binucleate cells by dual staining with an oPL antiserum detected with VIP substrate (purple). Micrograph A. represents immunostaining of AP-2 and oPL in the placentome collected at day 120 of gestation. The second micrograph (B) is a gestational day 40 placentome, and the third micrograph (C) is a negative control for the primary antiserum. The black arrows identify the chorionic binucleate cells and maternal (M) and fetal (F) tissues are indicated in the micrographs.



C

A.



B.

AP-2αv4	1 MPPRLASVKIPYDWGRKGPFRFWRIFCQSRAVGW 35 FLAAACGRAGRFRTQPAEWPTPDAVFSPLGLALFQ
AP-2αv6	1 MSILAKMGDWQ
AP-2αv7	1 MLVHSFSAM

Figure 11. Schematic representation of ovine AP-2α variant cDNA. Alternative splice-variants of the ovine AP-2α is depicted in A. The N-terminal regions, proline-rich region and DNA binding and dimerization domain are indicated by various box patterns for each variant labeled on the left. The translational start site (arrow) and stop site (black bar) are shown. The variation in length of the 5'-UTR and 3'UTR are indicated by the length of the boxes and deletions within the coding region is described with a line. The variant N-terminal predicted amino acid residues encoded by the first exon for each AP-2α variant cDNAs are shown (B). The predicted initiating methionine residue for each AP-2α variant is underlined.

immediately upstream of exon 2 in the human AP-2α gene (Bauer *et al.* 1994), and was also described for mouse AP-2α variant 4 (v4) and designated exon 1c (Meier *et al.* 1995). Only 60% identity between mouse exon 1c and the 5' nucleotide sequence of clone 242 was found, and the predicted amino acid sequence has no similarity to mouse AP-2αv4. In addition to the diversity between the species, the translational initiation site for AP-2 clone 242 (or 1812) does not conform to a preferred initiation site (Kozak 1984). Therefore, AP-2 clone 242 and 1812 may be the ovine homolog of mouse AP-2αv4, which may indicate evolutionary pressures on the region of the AP-2α gene immediately upstream of exon 2 (Figure 11).

A third AP-2 clone (6602) encodes an AP-2α protein with a predicted M_r of 46,708, which also differs at the N-terminus from previously described mammalian AP-2α variants, but is conserved from exon 2 to the stop codon (Figure 11). Sequence comparisons with the 5' UTR revealed 61% identity to nucleotides 3977 to 4045 of intron A in the human gene (Bauer *et al.* 1994), and no resemblance to any of the mouse variant exons (Meier *et al.* 1995). Five amino acid residues separate two methionine residues at the N-terminus of clone 6602's predicted amino acid sequence. The first methionine residue in the open reading frame of 6602 does not conform to a preferred initiation sequence (G/ANNATGG; Kozak 1984). The nucleotide sequence encompassing the second methionine residue does conform, with an adenosine at position –3 and a guanosine at position +4, suggesting that this is the initiating methionine for clone 6602. Therefore, 5 amino acid residues are encoded by a new variant exon 1 region in this ovine clone (Figure 11). Significant identity (80%) was found between clone 6602 exon 1 region and the 5 amino acid residues encoded by exon 1 of the *Xenopus laevis* AP-2α

(Winning *et al.* 1991), with a conserved substitution (Glu to Asp) at the third residue in the ovine amino acid sequence. The nucleotide sequence identity for these two AP-2α cDNAs within the coding region of the first exon was 93%, but when the combined 5' UTR and coding nucleotides were analyzed the similarity dropped to 75%. Comparison of AP-2α clone 6602 coding region and the *Xenopus laevis* AP-2α coding sequence yielded 80 % identity. Identification of this clone (6602) in an ovine cDNA library was the first recognition of its existence in a mammalian system and it was designated AP-2α variant 6 (v6).

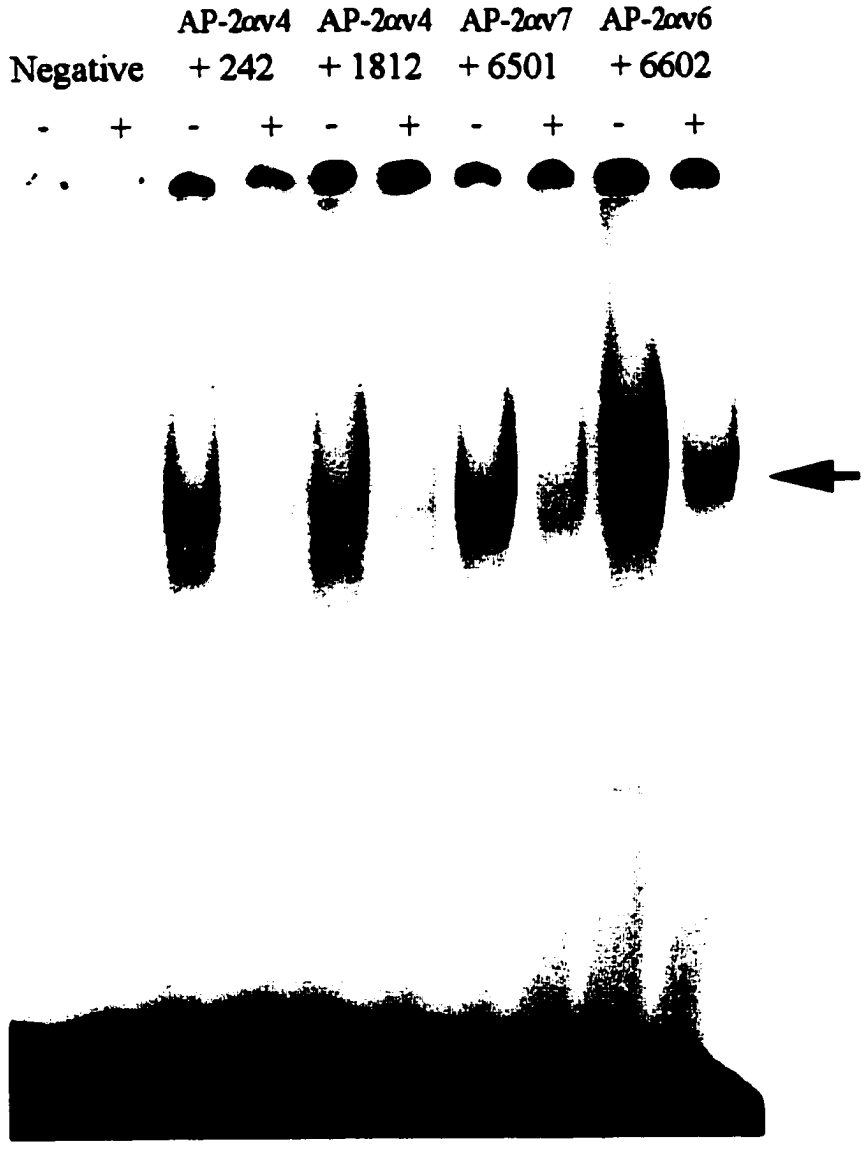
The fourth AP-2α clone (6501) distinguished a new mRNA variant in the ovine cotyledon. The 5'untranslated sequence and amino terminal nucleotide sequence of this clone shared 72 % identity with murine exon 1b and 81% identity to the human AP-2α gene between nucleotides 4774 and 5381 within intron A (Meier *et al.* 1995; Bauer *et al.* 1994). This region of the human and mouse genes has been described as exon 1b (Meier *et al.* 1995), and the nucleotides of clone 6501 encoding the amino terminus shares 100% identity to the mouse exon 1b translated region identified as AP-2α variant 3 (v3). The initiating methionine for clone 6501 was also predicted to be the second methionine residue in the open reading frame (Figure 11). This methionine residue was predicted because a preferred translational start site was observed in the nucleotide sequence surrounding this methionine residue in clone 6501 (Kozak 1984). An altered splice site within exon 2 of clone 6501 was created by a 144 bp deletion, in the region with sequence identity to human exon 2 (Figure 11). This deletion removes 48 amino acids within AP-2α and appears to be the result of an unconserved GT to GC splice site when compared to the human AP-2α gene and other ovine AP-2α clones. Clone 6501 encodes

a protein with a predicted M_r of 40,848 and represents a new AP-2α, designated variant 7 (v7).

Functionality of the Ovine AP-2α Variant Clones

Ovine AP-2α clones were co-transfected with the oPL minimal promoter (-124pGL3) in HepG2 cells, a heptoma cell line that lacks endogenous AP-2, to analyze AP-2α variant activity. Initially, ovine AP-2α v4 (242 or 1812), AP-2α v6 (6602), and AP-2α v7 (6501) were transfected alone, and whole cell extracts from the transfected HepG2 cells were purified and analyzed for protein expression by EMSA with the radiolabeled ovine AP-2 element (Figure 12). Ovine AP-2α expression vectors were co-transfected with -124pGL3 reporter vector to ascertain the functionality of these ovine AP-2α variants with the ovine AP-2 *cis*-acting element (Figure 13). Two AP-2α variants, v6 (6602) and v7 (6501), stimulated transactivation of the oPL minimal promoter when compared to HepG2 cells co-transfected with equivalent quantities of pBluescript (Figure 13). To confirm that the function was mediated through the AP-2 element in the oPL promoter, the expression vector was co-transfected with the -124mAP2 pGL3 vector. Enhancement of reporter activity was not observed with a mutation in the AP-2 site (Figure 13). Therefore, ovine AP-2αv6 and AP-2αv7 act through the putative AP-2 *cis*-acting element within the oPL gene minimal promoter to stimulate transcription. Interestingly, both AP-2αv4 clones (242 or 1812) were unable to stimulate activity with the oPL minimal promoter and a slight decline in activity was noticed for this AP-2α variant (Figure 13).

Figure 12. AP-2α variant expression in transiently transfected HepG2 cells assessed by EMSA. An AP-2 radiolabeled oligonucleotide was added to the binding reaction with cellular proteins from non-transfected HepG2 cells, HepG2 cells transiently transfected with AP-2α clone 242 (AP-2αv4), AP-2α clone 1812 (AP-2αv4), AP-2α clone 6501 (AP-2αv7), or AP-2α clone 6602 (AP-2αv6) and labeled above (-). A second binding reaction for each of the cellular extracts was performed in the presence of 100 fold molar excess homologous unlabeled competitor (+). The reactions were electrophoresis through a 5% polyacrylamide non-denaturing gel and the resultant autoradiograph is presented with the specific protein-DNA interaction identified on the right with an arrow.



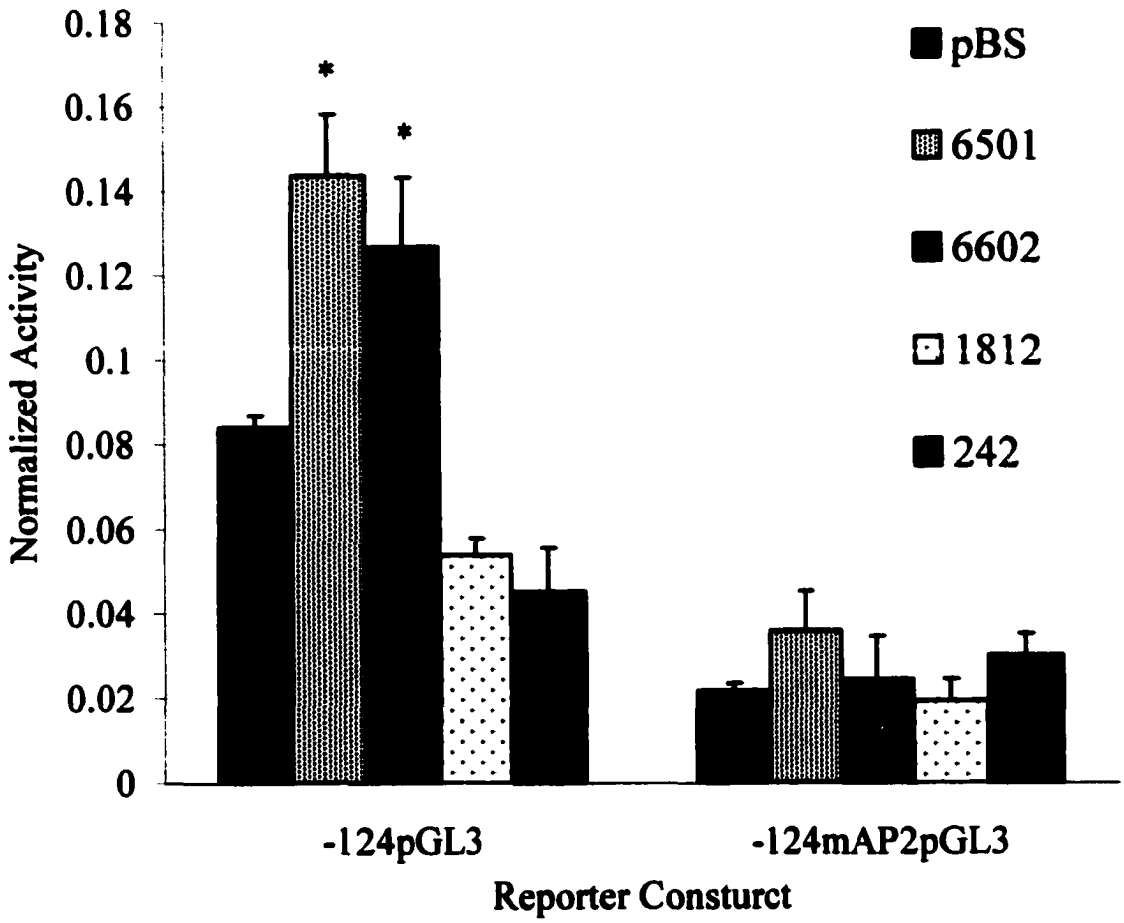


Figure 13. Functional analysis of ovine AP-2α variants. HepG2 cells were co-transfected with a reporter vector and an expression vector containing one of the ovine AP-2α clones. The two reporter vectors used were the wild-type (–124pGL3) vector containing the oPL gene minimal promoter or the –124mAP2pGL3 vector which has a mutation in the AP-2 element (labeled on the x-axis). The bars represent the mean activity of the reporter constructs co-transfected with 10 μg of pBluescript (pBS), AP-2αv7 (6501), AP-2αv6 (6602), AP-2α4 (1812 or 242), which are indicated in the legend at the upper right hand corner. The luciferase expression was normalized with a β-galactosidase control vector and the normalized activity is represented on the y-axis. The mean activities were compared to the pBS control by Dunnett's T test, and statistical ($P<0.05$) differences are indicated with an asterisk.

AP-2α Splice Variants in Placental Tissue

Western immunoblot analysis with an AP-2α antiserum identified different AP-2α nuclear proteins by their apparent molecular weight as the principal transacting factors in Hela, BeWo, and ovine binucleate cell nuclear extracts (Figure 14). The predominant AP-2α factor in Hela cells has a M_r ≅52,000, whereas the predominant protein in nuclear extracts from BeWo and ovine binucleate cells had an apparent M_r of ≅43,000. A second protein was identified in ovine binucleate cell nuclear extracts, which exhibited a M_r of ≅47,000. The identification of these two variant AP-2α nuclear proteins in ovine binucleate cells suggests that AP-2αv6 (6602) and AP-2αv7 (6501) are responsible for oPL gene enhancement. Additionally, the confirmation of an AP-2α splice variant in BeWo nuclear extracts by western analysis suggests that stimulation of the oPL minimal promoter in BeWo cell transient transfections is transactivated by an AP-2α splice variant.

The presence of AP-2α variants was examined in human, mouse and ovine placenta, as well as in BeWo cells by RT-PCR. Primers designed to amplify an AP-2α region between exon 4 and exon 7 of the human AP-2α sequence demonstrated that AP-2α mRNA was present in all the tissues and cell lines examined (Figure 15). Specific forward oligonucleotide primers for ovine AP-2α variant clones (v4, 242 or 1812; v6 6602; and v7, 6501) as well as human AP-2α (variant 1) were generated. The mRNA was reverse transcribed with an oligo-dT primer and a cDNA was amplified with a specific forward primer for each AP-2α variant and the reverse primer (AP-2R). The products were confirmed to be AP-2α by Southern blot hybridization (Figure 16). The

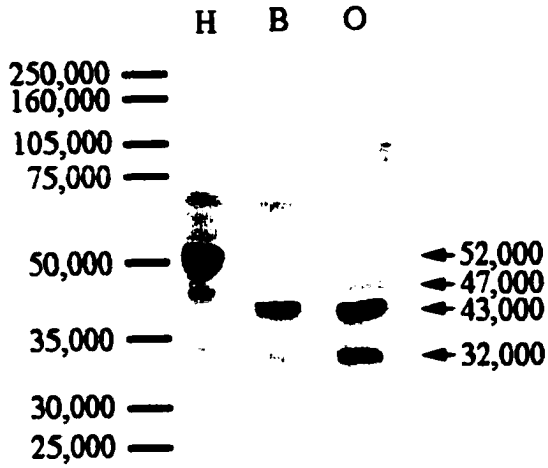
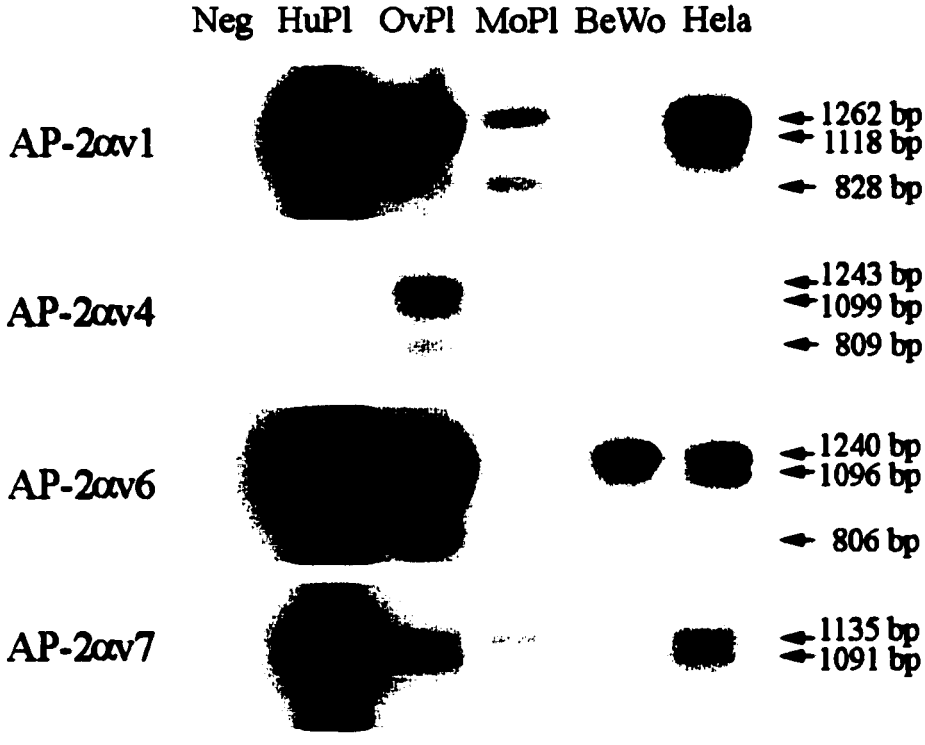


Figure 14. Western immunoblot analysis of AP-2α. Hela (H), BeWo (B), and ovine binucleate cell (O) nuclear extracts (15 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. AP-2α was identified with an antiserum raised against AP-2α. Molecular markers are indicated on the left and AP-2α proteins and their predicted M_r are labeled on the right.

Figure 15. RT-PCR confirmation of AP-2α transcripts in the placenta. AP-2α transcripts were identified in placental tcRNA from humans, sheep, and mice, as well as two cell lines. Total cellular RNA was reverse transcribed with and oligo-dT primer and AP-2α transcripts were synthesized in a PCR reaction with specific primers to the human AP-2α gene derived from exon 4 and exon 7. The products from human (HuPl), ovine (OvPl) and mouse (MoPl) placental RNA and those from BeWo and Hela cell RNA were separated through a 1% agarose gel and transferred to a nylon membrane. Southern hybridization of this membrane revealed that AP-2α is present in all the tissues and cell lines tested, but not in a negative control reaction (Neg).

Figure 16. AP-2α splice-variants in human, mouse and sheep placenta. RT-PCR reactions were performed with no RNA (Neg), human placental tcRNA (HuPl), ovine placental tcRNA (OvPl), mouse placental tcRNA (MoPl), BeWo cell tcRNA, and Hela cell tcRNA. PCR reactions were performed using specific primers for AP-2α variant 1 (AP-2αv1), AP-2α variant 7 (6501), AP-2α variant 6 (6602), and ovine AP-2α variant 4 (242 or 1812). Each panel represents the products of the different RT-PCR reactions (labeled on the left) that were confirmed by Southern hybridization with a radiolabeled AP-2α cDNA. The arrows to the right of the autoradiographs identify the transcripts, and their respective sizes, that hybridized AP-2α cDNA.



AP-2α variant 1 mRNA was confirmed in the placenta for all the species, but was not found in BeWo cells (Figure 16). Additional hybridization signals at ~1100 and ~800 bp were found in the transcripts amplified from human and ovine placental RNA, and may indicate alternative splicing of exon 2. Alternative AP-2α splicing of exon 2 has been reported (Ohtaka-Maruyama *et al.* 1998; Meier *et al.* 1995). In the human and mouse AP-2α variant 2 (v2), does not contain exon 2 and exon 1 and exon 3 are joined in frame (Meier *et al.* 1995). In other instances, portions of the exon 2 have been removed by alternative splice sites, such as sheep AP-2αv7 (6501) which has 144 bp removed or mouse AP-2α variant 5 which has 165 bp removed (Meier *et al.* 1995; Ohtaka-Maruyama *et al.* 1998). Only two hybridization signals were identified in the mouse placenta and their sizes coincide with the predicted sizes for AP-2αv1 and AP-2αv2. Ovine AP-2αv4 (242 or 1812) shares nucleotide sequence identity to human intron A region immediately upstream of exon 2. Products amplified with specific primers to this cDNA indicate that it was only present in ovine placental tcRNA, and smaller products may correlate to deletions due to alternative splicing in exon 2 (Figure 16).· AP-2αv6 (6602) was found in human placenta, ovine placenta, BeWo cells and Hela cells, but was not present in mouse placental tcRNA by RT-PCR analysis. Once again 3 products hybridized in the ovine and human placental tcRNA and are thought to result from alternative slicing of exon 2. No signal was identified in mouse placental tcRNA for this variant (Figure 16). These data suggest that clone 6602 is the predominant AP-2α splice variant in BeWo cells and may be the functional AP-2α variant stimulating transactivation of the oPL minimal promoter in these cells. The AP-2αv7 (6501) was identified in human, ovine and mouse placentas. In the ovine placenta, human placenta and Hela cells two products hybridized,

and may represent the 144 bp splice variant within exon 2. Only a single hybridization signal was identified from mouse placental mRNA and no signal was found in BeWo cells, and these results were confirmed by a longer exposure (Figure 16).

These data confirm that all AP-2α variants purified from the cotyledonary cDNA library are present in the ovine placenta, and indicate that additional variation of these proteins may exist in placental tissue. Two new AP-2α splice variants, equivalent to AP-2αv6 (6602) and AP-2αv7 (6501), are also present in human placental tcRNA. However, only a transcript analogous to AP-2αv7 (6501), with an N-terminus similar to mouse AP-2αv3, was identified in mouse placental tcRNA. Since only an AP-2αv6 (6602) homolog was identified in BeWo cells, this suggests that this variant is responsible for oPL minimal promoter activity in the cells, supporting the co-transfection data and indicating that AP-2αv6, which shares identity to *Xenopus laevis* AP-2α, functions as a transactivator in placental tissue.

Discussion

The oPL gene is constitutively transcribed in chorionic binucleate cells that reside at the maternal-fetal interface in the ovine placenta (Kappes *et al.* 1992). An AP-2 *cis*-acting element was identified in the oPL gene minimal promoter at –58 bp relative to the transcriptional start site, and the physical interaction with AP-2α at this site was demonstrated by DNase I footprinting, EMSA, and supershift assays (Liang *et al.* 1999). In the present study the functionality of this *cis*-acting element was verified by mutational analysis in BeWo cells, a human choriocarcinoma cell line. Functional AP-2 *cis*-acting elements have been described for other genes expressed in the placenta, and include

mouse adenosine deaminase (ADA) gene (Shi *et al.* 1997; Shi and Kellems 1998) and human chorionic gonadotropin (hCG) α- and β-subunit genes (Johnson *et al.* 1997; Johnson and Jameson 1999). Other genes, which possess AP-2 *cis*-acting elements within their promoter regions, and are expressed during placental development include transforming growth factor α (Berkowitz *et al.* 1997; Wang *et al.* 1997), vascular endothelial growth factor (Garrido *et al.* 1993; Gille *et al.* 1997), matrix metalloproteinases (Fini *et al.* 1994; Aho *et al.* 1997), tissue inhibitors of metalloproteinases (De *et al.* 1994; Hammani *et al.* 1996), and the estrogen receptor (McPherson *et al.* 1997; McPherson and Weigel 1999).

Placental regulation of the ADA gene was analyzed in transgenic mice, and localized to a 770 bp region 5.4 kb upstream of the transcriptional start site (Shi *et al.* 1997). Protein-DNA interactions in this region were analyzed, an AP-2 *cis*-acting element was identified and demonstrated to enhance trophoblast-specific expression of the ADA gene (Shi and Kellems 1998). Activator protein-2γ was identified as the enhancing factor for the mouse ADA gene, since it is abundantly expressed in mouse trophoblast cells (Shi and Kellems 1998). Although AP-2α and AP-2β transcripts are present in mouse extra-embryonic trophoblast cells, disruption of these genes did not have adverse affects on fetal development, which has been shown to occur with a disruption in the ADA gene (Williams *et al.* 1988; Blackburn *et al.* 1996; Schorle *et al.* 1996; Zhang *et al.* 1996; Moser *et al.* 1997). Therefore, AP-2γ was suggested to mediate the transactivation of the ADA gene in mouse trophoblast cells. Activator protein-2γ expression was identified in all trophoblast lineages throughout gestation, whereas ADA expression was restricted primarily to the extra-embryonic ectoderm (Chazaud *et al.* 1996; Shi and Kellems 1998).

These data indicate that AP-2γ is essential for ADA transactivation, but other transacting factors interacting with additional *cis*-acting elements in the 770 bp region may be required for ADA gene expression (Shi and Kellems 1998).

Activator protein-2α has been shown to transactivate hCG α- and β-subunit genes in JEG-3 choriocarcinoma cells (Johnson *et al.* 1997). The functional AP-2 element in the α-subunit was identified between –186/-156 bp, and two AP-2 elements at –311/-279 bp and –221/-200 bp were found in the hCG β-subunit gene (Johnson *et al.* 1997). Johnson *et al.* (1997) demonstrated functionality of these AP-2 elements in the hCG α- and β-subunit gene promoters by mutational analysis in JEG-3 cells, and by co-transfection experiments with an AP-2αv1 expression vector in HepG2 cells. Western immunoblot analysis of human trophoblast or JEG-3 cells identified a predominant protein with an apparent M_r of 52,000. However, proteins in the human trophoblast nuclear extracts with different molecular weights were also recognized with this AP-2 antiserum, but were not identified in JEG-3 nuclear extracts (Johnson *et al.* 1997). The authors do not indicate which epitope the AP-2 antiserum was raised against or test nuclear extracts of trophoblast cells at different gestational time points. Expression of the hCG β-subunit gene during the first trimester switches from cytotrophoblast cells to syncytiotrophoblast cells at the sixth week of gestation, whereas the expression of the α-subunit gene is found in both trophoblast lineages throughout gestation (Hoshina *et al.* 1982a; Hoshina *et al.* 1982b; Maruo *et al.* 1992). In addition to spatial control, hCG is temporally regulated with high expression during the first trimester that diminishes at term (Maruo *et al.* 1982a), suggesting that changes within trophoblast cells during gestation are occurring.

This may reflect alteration in gene expression, regulated by transcription factors like AP-2α or splice variants of AP-2α.

AP-2α expression in the human placenta has been studied in primary cultures of cytotrophoblast cells purified from term placentas and mRNA transcripts of AP-2α were identified in purified cytotrophoblast cells by RT-PCR (Johnson *et al.* 1997). Cytotrophoblast cells undergo differentiation and fuse to form syncytiotrophoblast cells in culture (Ringler and Strauss 1990). Analysis of tcRNA from these cultures indicates that AP-2α RNA increases gradually over a 12 day period. Additionally, mRNA for α-subunit in the purified cytotrophoblast cells increased during trophoblast differentiation, until day 4 and then declined. The hCG β-subunit RNA was not identified until day 2 of culture, when it increased dramatically until day 4, and appeared to level off for the remainder of the culture period (Johnson *et al.* 1997). These data suggest AP-2α expression is not directly correlated to the expression of the hCG α– or β-subunit genes. The data indicate additional requirements such as other functional elements or alteration in AP-2α transactivation potential between these two genes. The RT-PCR analysis of the AP-2α gene in these cultures was performed with primers designed to amplify a region between exon 5 to exon 7 of the human gene, which amplifies a region encoding the C-terminus. Therefore, subtle changes in AP-2α at the N-terminus are not accounted for, and transactivation potential of AP-2α splice-variants may affect transcription of these genes in primary cultures. Furthermore, changes in AP-2α expression and/or splicing during gestation may also alter the transactivation potential of this transcription factor, which in turn could alter the expression of these genes.

The presence of AP-2α in ovine placenta was confirmed by Northern hybridization and immunocytochemistry (Figure 9 and 10). A predominant hybridization signal of ~3000 nucleotides was identified in cotyledonary tcRNA, which is smaller than that identified in Hela cells that exhibit a broad range of transcripts from 3300 to 3600 nucleotides (Williams *et al.* 1988). Subsequently, the cellular location of AP-2α within the placentome was examined to determine: 1) if it was expressed in the chorionic binucleate cells; and 2) if the expression was cell-specific. AP-2α was immunolocalized to the chorionic binucleate cells in day 40 and 120 ovine placentomes by dual immunostaining with oPL antiserum (Figure 10). However, AP-2α was not restricted to the chorionic binucleate cells and was present in all fetal epithelial cells, but was not found in the maternal epithelial layer in the uterus (Figure 10). These data coincide with AP-2α expression in the human (Johnson *et al.* 1997) and mouse placenta (Mitchell *et al.* 1991) indicating that expression is not restricted to differentiated trophoblast cells. The data for AP-2α expression in cotyledonary tissue of the ovine placentome implicates AP-2α as a potential transactivator for the oPL gene, but suggests that its activity is not exclusive for oPL gene expression. Therefore, similar conclusions provided for other placental genes enhanced by an AP-2 element may be true to oPL expression. First, an AP-2 interaction is required for trophoblast transactivation, but other interactions within the promoter are required to restrict expression to the chorionic binucleate cells. The ambiguity between the mutational analysis observed between human and rat choriocarcinoma cell lines implicates factors other than AP-2 mediating trophoblast-specific expression. The transcripts identified in the cotyledonary tcRNA indicated a size variation, suggesting possible differences between ovine AP-2α and human AP-2α.

Therefore, to confirm a functional interaction between ovine AP-2α and its element in the oPL minimal promoter, ovine AP-2α had to be examined in co-transfection experiments in a cell line lacking endogenous AP-2α.

A cotyledonary cDNA library was screened and four independent AP-2α clones, which appeared to contain full-length cDNAs, were purified. Nucleotide sequencing of the clones revealed significant homology between them and the human or mouse AP-2α nucleotide sequences. However, sequence variablility at the 5' end of the ovine AP-2α cDNA was recognized. The differences between the cDNAs result from alternative RNA splicing in the first and second exons of the AP-2α gene (Figure 11). AP-2α variants due to differential mRNA splicing have been demonstrated previously, and six independent mRNA transcripts for AP-2α have been identified. AP-2α variant 1 (AP-2αv1) was first characterized in Hela cells (Imagawa *et al.* 1987; Mitchell *et al.* 1987; Williams *et al.* 1988), and a dominant negative AP-2α factor, AP-2B, is generated by differential splicing at exon 5/6, producing a truncated AP-2α that lacks the dimerization domain (Buettner *et al.* 1993). Meier *et al.* (1995) was unable to find a homolog of AP-2B in the mouse embryo, but did identify three new AP-2α variants that were temporally and spatially regulated during development. Alternative splicing that eliminates exon 2 creates AP-2α variant 2 (AP-2αv2), whereas variant 3 (AP-2αv3) and 4 (AP-2αv4) are generated by exon 1b and exon 1c located within intron A, between exon 1a (AP-2αv1) and exon 2 of the human gene (Bauer *et al.* 1994; Meier *et al.* 1995). The fifth AP-2α variant (AP-2αv5) was identified in mouse ocular lens tissue and is generated by an alternative splicing event in AP-2αv3, which deletes 165 bp of exon 2 (Ohtaka-

Maruyama *et al.* 1998). Clone 6501 (AP-2αv7) was found to encode a region similar to exon 1b of the mouse (AP-2αv3), which shares 81% identity with the human AP-2α gene between 4774 and 5381 bp. However, this clone lacked a 144 bp region in exon 2, which is identical to AP-2αv5 at the 5' slice site identified in exon 2, but differs at the 3' splice site (Ohtaka-Maruyama *et al.* 1998). The cDNA for clone 6602 (AP-2αv6) does not resemble any of the previously described mammalian AP-2α variants at the N-terminus, and shares 61% identity with a 68 bp region at 3977 bp of intron A in the human gene. However, similarity between the N-terminus of AP-2αv6 and *Xenopus laevis* AP-2α was found. They only differed by a single amino acid substitution at the third residue (Glu to Asp), which was a result of a signal nucleotide substitution (Winning *et al.* 1991). The cDNA encoding the open reading frames of clone 1812 and 242 were identical, and the nucleotide sequence coding N-termimal amino acids and the 5' UTR shared 67% identity to the human gene located immediately upstream of exon 2, or 60% identity to the mouse exon 1c. In the mouse this splice-variant was designated AP-2αv4, but transcripts were not confirmed in human tcRNA. Meier *et al.* (1995) suggested that homologous sequence to the predicted human exon 1c is required to confirm the presence of this AP-2α splice-variant in human tissues. Therefore, ovine AP-2αv4 is suggested to be an analog of the mouse AP-2αv4 even though there was very little similarity between these species. The functionality of these AP-2α variants identified in the mouse and frog remains to be examined, but speculation on their functionality has resulted from characterization of motifs within AP-2αv1 (Williams and Tjian 1991a; Meier *et al.* 1995).

The AP-2αv1 protein was initially purified from Hela nuclear extracts by its affinity for the SV40 early promoter and human metallothionein IIA promoter (Mitchell *et al.* 1987). AP-2αv1 has a M_r of 52,000 and stimulates transcription *in vitro* through the *cis*-acting element GCCNNNGGC (Mitchell *et al.* 1987; Williams and Tjian 1991a). Characterization of AP-2αv1 revealed a distinct DNA binding and dimerization motif at the C-terminus, which was classified as a basic helix-span-helix motif required for protein-DNA interaction (Williams *et al.* 1988; Williams and Tjian 1991b). The transcriptional activation domain was localized to a proline-rich region (Pro^{38} to Pro^{97}) at the N-terminus of the AP-2αv1 (Williams *et al.* 1988; Williams and Tjian 1991b). Additionally, a glutamine-rich domain (Gln^{63} to Gln^{103}) overlaps the proline-rich region and an acidic region (Pro^{123} to Asp^{171}) was recognized in AP-2α (Williams *et al.* 1988). All of these domains have been shown to mediate AP-2αv1 transactivation to some extent (Williams and Tjian 1991a). The DNA binding and dimerization domains were present in all of the ovine AP-2α clones, but a portion of the proline-rich activation domain was absent from AP-2αv7 (6501), due to the variant splicing of exon 2. Activator protein-2αv2 and AP-2αv5 were speculated to be repressors of transcription because they lack the proline-rich activation domain, which was confirmed by an internal deletion in the AP-2αv1 removing amino acid residues between Leu^{31} and Asn^{77} (Williams and Tjian 1991a). However, AP-2αv7 (6501) stimulated transactivation of the oPL minimal promoter in co-transfection experiments in HepG2 cells (Figure 13). This may be a result of a proline-rich region (29%) between residues Pro^{23} and Pro^{43}, or a glutamine-rich domain (27%) between amino acid residues Gln^{24} and Gln^{49}. In summary, AP-2αv7 has a glutamine-rich region greater than that of AP-2αv1 (22%), and the

proline-rich region (30%) is similar to that identified in AP-2αv1 (Williams and Tjian 1991a). Therefore, AP-2αv7's ability to transactivate the oPL minimal promoter may not be significantly different from AP-2αv1's transactivational potential. Both of the other ovine clones (AP-2αv4 and AP-2αv6) possessed the activation domains identical to the human AP-2αv1 with the exception of a histidine to glutamine substitution at residue 92, which also occurs in the mouse AP-2αv1 (Williams *et al.* 1988; Moser *et al.* 1993).

Activator protein-2αv6 (6602) stimulated transactivation through its *cis*-acting element at –58 bp in the oPL gene, thus confirming that this splice-variant, previously identified in *Xenopus laevis* and presently described in the ovine and human placenta, is functional (Figure 13). Western analysis of BeWo cell and chorionic binucleate cell nuclear extracts indicate that the predominant AP-2α transacting factor has a M_r of ≅43,000 (Figure 14). Since the only AP-2α variant identified in BeWo cell RNA was homologous to AP-2αv6 (6602), this AP-2α splice-variant is implicated as the transacting factor in the human choriocarcinoma cell line (Figure 16). Additionally, transcripts with exon 2 splice-variations were identified within ovine placental tcRNA, but these variant forms were not confirmed by western immunoblots and are not found in BeWo cell RNA. These data suggest that the AP-2αv6 (6602) is the AP-2α transacting factor for the oPL gene. Additionally, an AP-2α protein with the apparent Mr of ≅47,000 was identified in ovine binucleate cell nuclear extracts (Figure 14). Two AP-2α isoforms generated by alternative RNA splicing may translate a protein with this apparent M_r. The first being AP-2αv1 with the 48 amino acid residues excised from exon 2, which was identified by AP-2αv7 (6501) and confirmed in the RT-PCR products of ovine placental

tcRNA. The second mRNA transcript may be AP-2αv3 with the 48 amino acid residue deleted from exon 2, which was also demonstrated in ovine placental tcRNA by RT-PCR. Neither of these cDNA clones were purified from the ovine placental library, but further screening may be required to identify all the cDNAs encoding AP-2α splice variants.

This study was designed to confirm the functionality of an AP-2 element in the minimal promoter of the oPL gene. In the process, AP-2α expression within the ovine placenta was identified as a potential regulatory step that might change during trophoblast cell differentiation. Regulation of AP-2α is hypothesized because: 1) the lack of functionality in Rcho-1 cells which possess an isoform of AP-2α; 2) the identification of AP-2α splice-variants in ovine placental RNA; and 3) the fact that not all AP-2α splice-variants are capable of stimulating activity with the oPL gene minimal promoter. Two of the three splice-variants of the AP-2α gene isolated from ovine cotyledonary cDNA library stimulated transactivation (Figure 14) and were present in human placenta mRNA (Figure 16). An AP-2α variant (ovine AP-2αv4), specific for sheep, was not able to transactivate the oPL gene minimal promoter and the protein was not found in ovine chorionic binucleate cell nuclear extracts. This may reflect the lack of a preferred translational start site effecting protein production in binucleate cells. However, the protein was translated following transfection into HepG2 cells, indicating that the lack of function may be due to an extended N-terminus or to a lack of abundance. Combined these data suggest regulation of AP-2α expression exists at a post-transcriptional level, within the RNA splicing process, and at the translational level of AP-2α during trophoblast differentiation. Post-transcriptional influences may be revealed by AP-2α variants with different N-termini. Alternatively, the variations observed may represent

different transcriptional regulatory events during chorionic binucleate cell development (Meier *et al.* 1995; Ohtaka-Maruyama *et al.* 1998). Variation in use of a gene's first exon to regulate tissue-specific transactivation has been described for the CYP19 (P450 aromatase) gene, which has tissue-specific first exons (Means *et al.* 1989; Toda *et al.* 1990; Means *et al.* 1996). The RNA splicing mechanism for AP-2α appears to be species dependent as well. In human and ovine placenta, different products thought to be alternative splicing of exon 2, were observed by RT-PCR analysis for all the AP-2α N-terminal splice-variants, but similar splicing of AP-2α in the mouse placenta and BeWo cell RNA was not identified for all the transcripts. Therefore, it could be hypothesized that splicing machinery may be regulated in a cell-dependent manner. To confirm this hypothesis, AP-2α variants must be localized to the trophoblast cells in the ovine chorionic epithelium. Temporal and spatial restrictions of AP-2α variant transcripts in the ovine placenta, which have been identified in mouse embryonic tissues and the mouse ocular lens (Williams *et al.* 1988; Ohtaka-Maruyama *et al.* 1998), will confirm AP-2α regulation during trophoblast differentiation. The temporal regulation of AP-2α during binucleate cell differentiation may indicate that variants described in the present study stimulate oPL expression by up regulating AP-2αv6 or AP-2αv7 expression.

Summary

Activator protein-2 (AP-2), a sequence specific transacting factor, has been implicated as a potential transactivator of the ovine placental lactogen (oPL) gene. Transcriptional enhancement though an AP-2 *cis*-acting element has been described for other placentally expressed genes, but the regulation by AP-2 isoforms generated by

different genes appears to be species dependent. The competence of AP-2 to transactivate the oPL minimal promoter (-124 bp to +16 bp) by interacting at its *cis*-acting element was confirmed by mutational analysis in transiently transfected human choriocarcinoma (BeWo) cells, but not rat choriocarcinoma cells (Rcho-1). AP-2α was immunolocalized within the ovine placentome to the ovine binucleate cells, but was also found in the nuclei of other trophoblast cells within the chorionic epithelium. Northern hybridization of ovine cotyledonary total cellular RNA identified a single AP-2 transcript approximately 3 kb in length. Four nearly full-length AP-2 cDNAs were isolated from an ovine cotyledonary cDNA library. Nucleotide sequencing of these cDNAs revealed that the AP-2 mRNA expressed in the ovine placenta is homologous to the human AP-2α gene, but differs at the N-terminus. Three new AP-2α splice-variants were identified in the ovine placenta. Expression of ovine AP-2α variants in a human hepatoma (HepG2) cell line, devoid of endogenous AP-2, indicates that enhancement through the AP-2 element in oPL minimal promoter is variant dependent. RNA transcripts for all of the ovine AP-2α splice-variants were confirmed in the ovine placenta by RT-PCR, and a homolog for the two functional clones were identified in human placental RNA. However, only one AP-2α variant, which has also been identified in Xenopus, was expressed in BeWo cells. An identical protein was identified in the ovine binucleate cell nuclear extracts by western immunoblot analysis. These data indicate the presence of a new mammalian AP-2α splice-variant augments transactivation of the oPL gene in the ovine chorionic binucleate cells.

Chapter V

TRANSACTIVATION OF THE OVINE PLACENTAL LACTOGEN GENE IS MEDIATED BY A NOVEL *CIS*-ACTING ELEMENT INTERACTING WITH THE TRANSCRIPTION FACTOR PURα

Introduction

A developing fetus requires a substantial nutrient flux from the mother to the fetus for normal growth and development. Placental lactogen (PL), a member of the growth hormone (GH)/prolactin (PRL) gene family, is thought to alter maternal metabolism to increase the pool of nutrients available for the fetus. Additionally, PL has been suggested to act on fetal tissues to stimulate nutrient uptake (Talamantes and Ogren 1988; Handwerger 1991; Anthony *et al.* 1998). Ovine chorionic binucleate cells synthesize ovine (o) PL, and secrete it into both the maternal and fetal vasculature as a nonglycosylated 198 amino acid polypeptide with an apparent molecular weight (M_r) of ~22,000 (Anthony *et al.* 1995a; Anthony *et al.* 1995b; Anthony *et al.* 1998). The oPL gene has been structurally characterized, and 4.5 kb of 5'-flanking sequence was studied for trophoblast-specific transactivation in heterologous choriocarcinoma cell lines (Liang *et al.* 1999). Transient transfection analysis of oPL gene 5'-flanking sequence identified

the proximal 383 bp as the promoter region capable of stimulating maximal activity in human (BeWo) and rat (Rcho-1) choriocarcinoma cell lines. In addition a region between –124/+16 bp (minimal promoter), relative to the transcriptional start site, was able to retain trophoblast-specific activity, albeit reduced from the activity obtained with the -383/+16 bp region (Liang *et al.* 1999).

DNase I protection and electrophoretic mobility shift assays were performed with ovine binucleate cell (BNC) nuclear extracts to identify specific protein-DNA interactions within the oPL minimal promoter. Three footprints (FP) at -12/+7 bp (FP1), -74/-48 bp (FP2) and -123/-95 bp (FP3) were identified and previously recognized *cis*-acting elements that augment trophoblast expression for other placental genes were located within FP2 and FP3 (Liang *et al.* 1999). Two GATA elements at -67 bp (FP2) and -102 bp (FP3) and an AP-2 element at -58 bp (FP2) were confirmed to interact with oBNC nuclear proteins (Liang *et al.* 1999). Furthermore, GATA-2 and AP-2α were confirmed as the transacting factors interacting with their putative elements in the oPL minimal promoter (Liang *et al.* 1999). Functional GATA elements have been described in the human chorionic gonadotropin (hCG) α-subunit gene (Steger *et al.* 1994), mouse adenosine deaminase gene (ADA) (Shi *et al.* 1997) and mouse PL-I gene (Ng *et al.* 1994). Activator protein-2 sites have been demonstrated to enhance placental activity for the mouse ADA gene (Shi and Kellems 1998), hCG α- and β-subunit genes (Johnson *et al.* 1997).

Functionality of these previously identified elements within the oPL minimal promoter was studied by mutational analysis (Chapter III; Liang *et al.* 1999). The mutation disrupting the GATA element at –67 bp exhibited a greater reduction in activity

for both choriocarcinoma cell lines, whereas the mutation of the –102 bp GATA element was less detrimental to transactivation (Liang *et al.* 1999). However, mutation of both GATA elements reduced activity to basal levels in Rcho-1 and BeWo cells and confirmed that both GATA elements are functional (Liang *et al.* 1999). Since the GATA element in FP3 has less influence on oPL minimal promoter activity, another mutation was created in FP3 to identify additional *cis*-acting elements functioning in this region. A mutation at –102/–109 bp identified a functional *cis*-acting element, distinct from the GATA element, in BeWo cells, but not Rcho-1 cells. This region of FP3, upstream of the GATA element (-102 bp), was able to interact specifically with a protein in both oBNC and BeWo cell nuclear extracts, but no interaction was observed with Rcho-1 nuclear proteins. The specific nucleotides making up the *cis*-acting element were defined by mutational binding studies and the size of the transacting factor was determined by Southwestern analysis. The transacting factor interacting with this *cis*-acting element was identified in a cotyledonary cDNA expression library. The single-stranded DNA binding protein, Pura, was identified, and binding studies and functional assessment of Pura supported its role in augmenting expression of the oPL minimal promoter.

Materials And Methods

Plasmid Construction and Purification

Dual PCR amplification was employed to generate site directed mutations in the oPL minimal promoter. Over-lapping sense and antisense oligonucleotides with a Not I restriction endonuclease site at the 5' end were generated: Δ90F, 5'-AGG CGG CCG CAA AAG AGA AGC AGT GAT AGC-3'; Δ90R, 5'-TTT GCG GCC GCC TTC TCT

CTT ATC AAT GCT-3'; Δ110F, 5'-ACG CGG CCG CAT AAG AGA GAA GAA TGC GGT-3'; and Δ110R, 5'-TAT GCG GCC GCG TGC CTG CAT AAG AGC-3' (Macromolecular Resources, Colorado State University, Fort Collins, CO). These primers in conjunction with the pGL2 (Promega, Madison WI) forward and reverse primers were used to create a mutation in the oPL minimal promoter as described by Liang *et al.* (1999). The mutated minimal promoter products were digested with Kpn I and Hind III restriction endonucleases and ligated into the pGL3 Basic vector (Promega). Mutations in the minimal promoter region were confirmed by dideoxy-nucleotide sequencing (Sanger *et al.* 1977). Positive plasmid DNAs were prepared for each construct using an alkaline-lysis procedure (Ausubel *et al.* 1995), and purified by CsCl equilibrium gradient centrifugation.

Cell Culture and Transient Transfection

The Rcho-1 cell line, a rat choriocarcinoma cell line, was a generous gift from Dr. M. J. Soares, (Kansas City, Kansas) and the Bewo cell line, a human choriocarcinoma cell line, was obtained from American Type Culture Collection (Rockville, MD). Rcho-1 cells were maintained in NCTC-135 medium (Gibco BRL, Gaitherburg, MD) supplemented with 1mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO) and 20% heat-inactivated fetal bovine serum (FBS; Gemini Bio-Products, Inc., Calabasas, CA). BeWo cells were maintained in Waymouth medium MB (Gibco BRL) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 15% FBS. All cells were maintained in monolayer cultures at 37°C in 5% CO_2, 95% air with 100% humidity.

For transient transfections, cells were plated (5 x 10^5 cells/well) in 6-well plates one day before transfection. The polycationic lipid-DNA complex reactions were carried out at room temperature for 20 min, with 6 μl (Rcho-1) or 10 μl (BeWo) of LipofectAmine (Gibco BRL, Gaithersburg, MD), 5 μg of pGL3 Basic, -124pGL3 (wild-type), -124Δ90pGL3, -124Δ100pGL3 or -124Δ110pGL3, and 0.25 μg RSVβgal-Control plasmid, in serum and antibiotic free medium (100 μL). Cells were washed twice with serum-antibiotic free media prior to addition of the lipid-DNA complex, and volumed to 1 ml with medium. After 24 hr the transfection medium was replaced with 4 ml of serum-containing medium, and cultures were maintained for an additional 48 hr.

After the second incubation, cells were lysed in 25 mM Gly-Gly ($C_4H_8N_2O_3$), 1 mM DTT and 0.1% Triton X100, and analyzed using a Luciferase Assay System (Promega, Madison, WI) or LumiGAL Chemiluminescent Assay System (ClonTech, Palo Alto, CA). The light emission generated by both systems was measured using a Turner TD-20e luminometer with a 5 second delay and an integration of time of 10 seconds. Luciferase activity was normalized for intra-assay variation by the RSVβgal vector β-galactosidase activity for each sample. Data are presented as the mean percent activity±SEM determined from replicated transient transfection experiments (n≥4). The transfection data were analyzed by least square analysis of variance using Statistical Analysis Systems (SAS Institute, 1993). In general linear model procedures (Snedecor and Cochran 1976), differences in the means between wild-type vector and mutant vector were separated with a Dunnett's T test ($P<0.05$).

Electrophoretic-Mobility Shift Assay (EMSA)

Cotyledonary tissue was collected from ewes at 100 days of gestation. Cells were disassociated and binucleate cells were isolated in the same manner as described by Liang *et al.* (1999). BeWo cells were expanded in culture and cells from fifteen 150 mm plates were removed for nuclear extract isolation. The Rcho-1 cells were cultured to confluency and maintained for an addition 6 days. The stem cells were removed with a trypsin-EDTA solution (Sigma) leaving the differentiated Rcho-1 cells, which were scraped off the plates, and used to isolate differentiated Rcho-1 cell nuclear proteins. The nuclear proteins were obtained by the procedures of Dignam *et al.* (1983), precipitated with ammonium sulfate (1/3 to 2/3 fraction), and dialyzed (10,000 MWCO) against 500 X the volume of buffer (20 mM Hepes pH 7.9 @ 4°C, 20 % glycerol (v/v), 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.25 mM DTT). Nuclear protein concentrations were assessed with a Bradford assay, aliquots were snap frozen and stored at less than -70°C.

Electrophoretic-mobility shift assays were performed with a radiolabeled synthetic oligonucleotide using T4 polynucleotide kinase (United States Biochemicals, Cleveland, OH) to phosphorylate the 5' end with [γ-^{32}P] ATP (ICN). The binding reactions were performed in 20 mM Hepes (pH 7.9 at 4°C), 20 % glycerol (v/v), 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.25 mM DTT (Dignam D buffer). In addition, spermidine (1 mM) and poly (dI-dC)-poly (dI-dC) (50 ng/μl) were added to the reactions. The reaction buffer, unlabeled competitors and nuclear proteins (25 μg) were gently mixed, placed on ice for 5 min, incubated 5 min at 50°C and placed on ice for an additional 5 min. Five fmoles of labeled oligonucleotide (50,000 cpm) were added to the reaction and subsequently incubated at 30°C for 20 min. The reaction mixture was cooled on ice for 5

min and electrophoresed through pre-run 5% nondenaturing polyacrylamide gels (4°C) for 2-3 hr. The gel was then dried and exposed to x-ray film. Oligonucleotides (Macromolecular Resources) for the EMSA were: o110, 5'-CAG GCA CCA GCA TTG-3'; h110, ATC CCA GCA TGT GTG-3'; oAP2, GCT CCA CCC CAG GGC ATG-3'; o99, 5'-AAG AGA GAA GAA TGC GGT A-3'; and oGATA, 5'-CTT CCT GAT AAA ACC ACT GG-3' (sense strands represented). In addition synthetic oligonucleotide with two bp transversion across o110 (–116 to –102) were generated and used as competitor in binding reactions.

Southwestern Analysis

A 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate 100 μg of BeWo nuclear extract. The size-fractionated proteins were transferred to a 0.22 μm nitrocellulose membrane (Micron Separation, Inc., Westborough, MA) and renatured in 10mM HEPES pH 7.9, 50 mM KCl, 10% glycerol, 0.1% Nonidet P-40, 0.5 mM DTT, and 0.5 mM spermidine for 16 hr at 4°C with a buffer change after the first hr. The membrane was blocked for 3 hr at 4°C with 20 mM HEPES pH 7.9, 100 mM KCl, 10% glycerol, 0.1% Nonidet P-40, 0.5 mM DTT, 1 mM spermidine, 5 μg/ml sonicated Herring Sperm DNA, 25 μg/ml Yeast tRNA, 100 μg/ml BSA and 5% non-fat dry milk. The binding reaction contained 20 mM HEPES pH 7.9, 100 mM KCl, 10% glycerol, 0.1% Nonidet P-40, 0.5 mM DTT, 1 mM spermidine, 5 μg/ml sonicated Herring Sperm DNA, 2.5 μg/ml Yeast tRNA, 10 μg/ml BSA, 0.5% non-fat dry milk and $1X10^6$ cpm/ml radiolabeled double stranded oligonucleotide (Concat o110, 5'-GATCT TAATG CTGGT GAATG CTGGT GAATG CTGGT GGTAC-3'),

which was end labeled with T4 polynucleotide kinase and [γ-^{32}P] ATP. Binding reactions were carried out at 16°C for 2 hr. Following the binding reaction, the membranes were washed in ice cold 10 mM HEPES pH 7.9, 50 mM KCl, 10% glycerol, 0.5 mM DTT and 1 mM spermidine three times for 2 min and exposed to x-ray film.

Expression Library and Screening

An ovine cotyledonary cDNA library (6.9 x 10^7 pfu/μg) was constructed in ZAP Express EcoR I/Xho I vector (Stratagene, La Jolla, CA; Chapter IV). Bacteriophage infection and plating of the infected XL1-Blue MRF' *E. coli* cells on NZY agar plates was according to Young and Davis (1983) and modified by Vinson *et al.* (1988). Plates were cultured for 3 hr at 42°C, IPTG impregnated nitrocellulose filters (Micron Separation, Inc.) were overlayed and plates were incubated at 37°C for 6-8 hr. Overlaying a second IPTG impregnated filter for an additional 2 hr generated a duplicate filter. After the filters were removed, they were allowed to dry slightly on Whatman paper, submerged into ice-cold Dignam D buffer containing 6 M guanidine hydrochloride and gently agitated at 4°C for 10 min to denature the proteins. The buffer was changed once and the process repeated. After the second incubation, the filters were washed twice for 5 min at 4°C with Dignam D buffer containing 3 M guanidine hydrochloride. This process was repeated with Dignam D buffer containing 1.5 M, 0.75 M, 0.375 M and 0 M guanidine hydrochloride. After denaturation, the proteins on the filters were renatured, blocked and probed in the same manner described for Southwestern analysis. Multiple exposures were used to confirm positive plaques on the duplicate filter. The plaques were picked and subjected to secondary and tertiary rounds of screening to purify

positive plaques. To confirm specific binding interaction, a heterologous radiolabeled oligonucleotide (oAP2) was used on a duplicate set of filters at the tertiary screening, and plaques with no affinity for the heterologous DNA were confirmed. Single plaques were isolated, and using the helper phage ExAssist to *in vivo* excise, pBK-CMV phagmid were generated, which were used to infect XLOLR *E. coli* cells to isolate pBK-CMV plasmids. The pBK-CMV plasmid DNA was isolation from 5 colonies for each plaque purified cDNA. Restriction endonuclease digestion and Southern hybridization, using multiprime radiolabled cDNA inserts from the isolated plaques, were used to confirm sequence identity between the cDNA inserts. Six independent clones cross-hybridized to one another. The 5' and 3' ends of each of these cDNAs were sequenced (Davis Sequencing, Davis, CA) with T3 and T7 oligonucleotide primers and found to contain identical cDNA inserts. Additionally, internal synthetic oligonucleotides were designed (925 F1, 5'-CTA CAT GGA TCT CAA GGA G-3'; and 925 R1, 5'-CTA GTC GTC GAT GAG CTT G-3') to sequence the entire sense and antisense strands of the cDNA inserts.

Purα Binding Studies

Ovine Purα cDNA (clone 925) was used as a template in an *in vitro* transcription- and translation-coupled wheat germ lysate system (Promega, Madison, WI). Briefly, 1 μg of pBK-CMV clone 925 was added to a reaction containing 50% wheat germ lysate, amino acid mixture (20 μM), T3 RNA polymerase, 0.8 U/μl RNasin Ribonuclease inhibitor (Promega) in 1X reaction buffer, with a final volume of 50 μl, and incubated at 30°C for 1.5 hr. EMSA were performed with 2 μl of the *in vitro* translated Purα or the positive (luciferase) control reaction. Binding reactions were performed as previously

described, but the radiolabeled MF0677 single stranded oligonucleotide (5'-GGA GGT GGT GGA GGG AGA GAA AAG-3') or o110 oligonucleotide was used. Additionally, only 5 μg of nuclear extracts were used in the presence of radiolabeled MF0677, and a heterologous competitor o99 (5'-AAG AGA GAA GAA TGC GGT A-3') was included to detect non-specific interactions. The MF0677 oligonucleotide was the preferred binding site for human Purα (Bergemann *et al.* 1993). Purα antiserum was obtained from Dr. Naomasa Miki, Osaka University, Osaka, Japan. Supershift assays with the MF0677 and o110 oligonucleotides were performed with the addition of 1 μl of the antiserum raised against Purα, or 1 μl of normal rabbit serum (negative), to the binding reaction containing *in vitro* translated Purα, BeWo cell nuclear extracts or oBNC nuclear extracts.

The *in vitro* translated ovine Purα (5 μl of reaction) was separated through a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Micron Separations, Inc.) in 2 mM Tris pH 8.3, 150mM glycine, 20% methanol, and 0.1% SDS buffer. The membrane was blocked in 5% non-fat dry milk 1X TBST (10 mM Tris pH 8, 150 mM NaCl, 0.05% Tween 20) for 1 hr. Immunoblot detection of Purα was accomplished by incubating Purα antiserum at a 1:500 dilution in 1X TBST and 5% non-fat dry milk overnight at 4°C. The membrane was washed 3 times with 1X TBST for 10 min, and anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology) at 1:2000 was incubated for 1 hr at room temperature. The membrane was washed 2 times in 1X TBST and once in 1X TBS, incubated for 1 min in Western Blotting Luminol Reagent (Santa Cruz Biotech) and exposed to Kodak x-ray film.

Co-transfection of Pur α and Reporter Constructs

Functionality of the expression vector was tested in BeWo cells by co-transfecting 5 μg reporter vector (124pGL3 or Δ110pGL3) with 0.1 μg, 0.5 μg and 2.5 μg of Purα 925 pBK-CMV vector or a control vector (pBlueScript; Stratagene). Transfections were carried out in the same manner as the mutation constructs, but only the intra-assay control was used to normalize the luciferase activity. The data was analyzed by general linear model procedures (SAS Institute, 1993) and a Tukey's test was used for multiple comparisons of the means ($P<0.05$).

Results

DNase I protection assays identified a protein-DNA interaction encompassing -123/-95 bp (FP3), and a GATA element (-102 bp) was located in the 3' end of this protected region. However, mutational analysis of this GATA element (-124Δ100pGL3) did not significantly reduce oPL minimal promoter transactivation in transiently transfected BeWo cells (Liang *et al.* 1999). Since the GATA element resides at the 3' end of the footprint a mutation in the middle of the FP3 at –109/-102 bp (-124Δ110pGL3) was created. These mutations (Δ100 & Δ110), the wild-type (124pGL3) and an additional mutation at -89/-82 bp (-124Δ90pGL3) were transiently transfected into BeWo and Rcho-1 cell lines (Figure 17). The -124Δ90pGL3 mutation was included to show inserting a Not I site within the oPL minimal promoter does not have deleterious affects on promoter activity. The response of this mutation, not located in a protected region defined by oBNC nuclear extracts, did not significantly alter transactivation and a

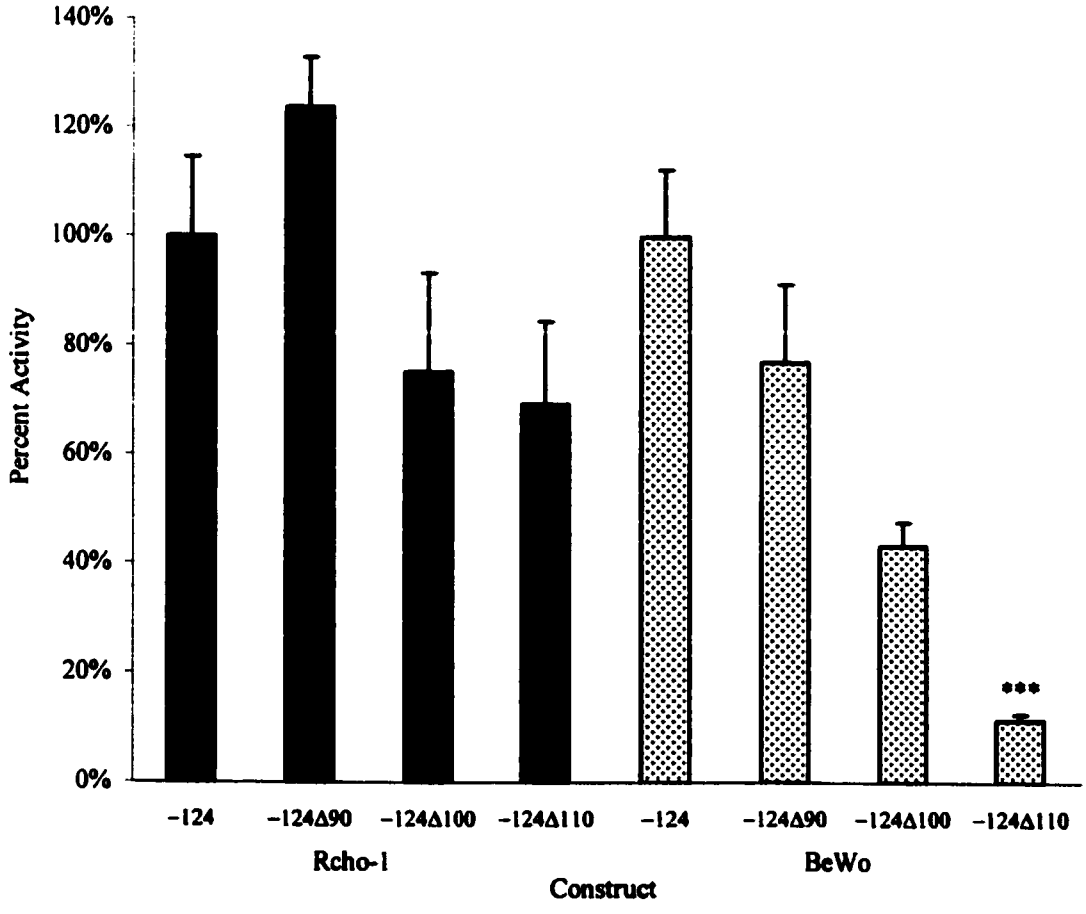


Figure 17. Footprint 3 mutational analysis in choriocarcinoma cell lines. Mutational analysis of the oPL gene minimal promoter in rat (Rcho-1) and human (BeWo) choriocarcinoma cell lines is shown. The cell lines and the pGL3 vectors, -124pGL3 (wild-type), -124Δ90 (-89/-82 bp), -124Δ100 (-99/-92 bp), -124Δ110 (-109/-102 bp) are indicated on the ordinate axis. The bars represent the mean percent activity (n≥4) with the wild-type designated 100%, and the error bars are the SEM (labeled on the abscissa). The differences in the means were separated by a Dunnett's T test and "***" represents a $P<0.05$.

decrease of 23% was observed in BeWo cells, whereas an increase of 24% was found in Rcho-1 cells. Transient transfections in Rcho-1 cells with -124Δ100 and -124Δ110 reduced reporter activity to 25% and 31%, respectively, but neither reduction varied significantly from the wild-type. The reduction in Rcho-1 cells may account for mutation of the GATA site (-102 bp), which is disrupted with both mutations. BeWo cell transfections portrayed a different situation between the two mutations. No significant effect was found between the wild-type and the -124Δ100 mutation, which was reduced 67%, but -124Δ110 decreased activity 89% ($P<0.01$). Therefore, a *cis*-acting element located in the center of FP3, distinct from the GATA element (-102 bp), was believed to mediate transactivation in BeWo cells.

The mutational analysis suggests protein-DNA interaction within –109/-102 bp region in human choriocarcinoma cells. The protein-DNA interaction was studied by electrophoretic-mobility shift assays (EMSA) with ovine binucleate cell (oBNC) nuclear extracts to identify if a nuclear protein specifically interacted with this sequence. An oligonucleotide encompassing -116/-102 bp (o110) was radiolabeled and binding of a single protein-DNA complex identified (Figure 18). However, several nonspecific complexes were observed in the binding reactions. Therefore, EMSA binding reactions were heated at various temperatures prior to addition of the radiolabeled o110 to assess the heat stability of the oBNC nuclear protein (Figure 18). The protein interacting with o110 was found to be heat stable to 55°C, but not at 65°C or higher. The heat stability of the protein (55°C) was used in subsequent EMSA binding reactions to remove nonspecific complexes.

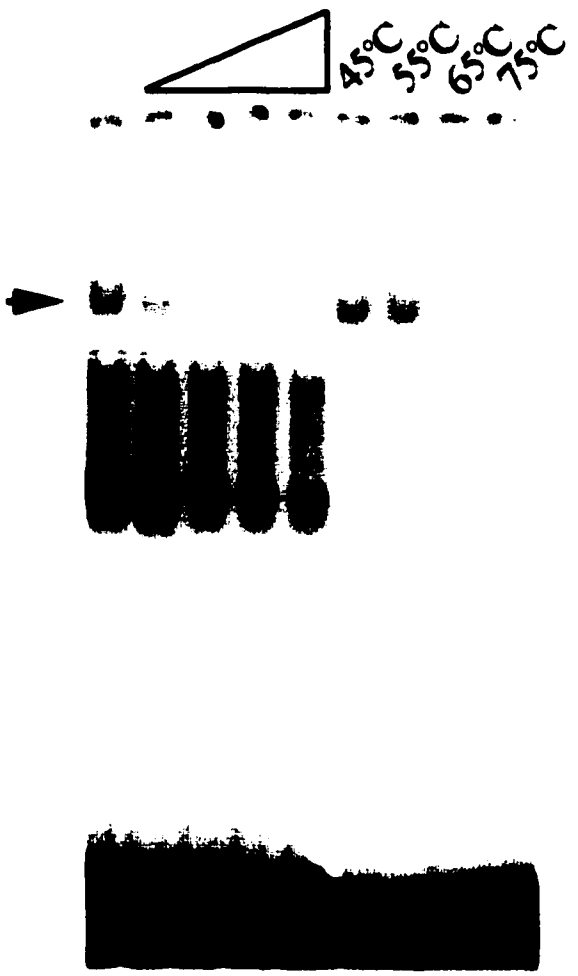


Figure 18. Ovine 110 oligonucleotide EMSA and temperature sensitivity. An o110 oligonucleotide (-116/-102 bp) derived from the oPL gene minimal promoter bound a nuclear protein in ovine binucleate cells (oBNC). The first lane contains 25 μg oBNC nuclear extract with no competitor, and the subsequent lanes with the triangle above them have 10, 50, 100 and 250 fold molar excess of non-radiolabeled homologous competitor. To further examine this protein-DNA interaction, the reactions were heated (5 min) prior to the addition of the radiolabeled oligonucleotide at 45°C, 55°C, 65°C and 75°C.

The specificity of this protein-DNA interaction was examined in oBNC, BeWo and Rcho-1 nuclear extracts in the presence of unlabeled heterologous oligonucleotides (oGATA and oAP2). The protein-DNA complex formed with oBNC nuclear extracts was unaffected by unlabeled heterologous competitors, whereas addition of unlabeled homologous oligonucleotides reduced the complex formation with radiolabeled o110 (Figure 19). BeWo nuclear extracts also reduce the mobility of the o110 oligonucleotide in a similar fashion seen with oBNC nuclear extracts by EMSA, and complex formation was inhibited with the addition of unlabeled homologous competitor (Figure 19). In contrast, differentiated Rcho-1 cell nuclear extracts were unable to form a protein-DNA interaction with twice the quantity (50 μg) of nuclear proteins. Binding studies indicate a specific protein-DNA interaction within FP3 for a BeWo and oBNC nuclear protein, which is distinct from the previously defined GATA element. This interaction located upstream of the GATA element was not identified in Rcho-1 cells, but was present in the oBNC and BeWo cells. Combined with the mutational analysis, these data suggest that the protein-DNA interaction functions as a transacting factor to augment expression from the oPL minimal promoter in BeWo cells.

Experiments were designed to identify the nucleotides interacting with the nuclear protein in the oBNC and BeWo cells. Two base pair transversions across the o110 oligonucleotide were generated and are described in Figure 20A. These mutant oligonucleotides were used as competitors in EMSA binding reactions. The ability of the protein-DNA complex to form in the presence of 100 fold molar excess unlabeled mutant competitor was analyzed with a phosphorimager. The volumetric intensity of each mutation was expressed as percent binding, relative to the complex formation containing

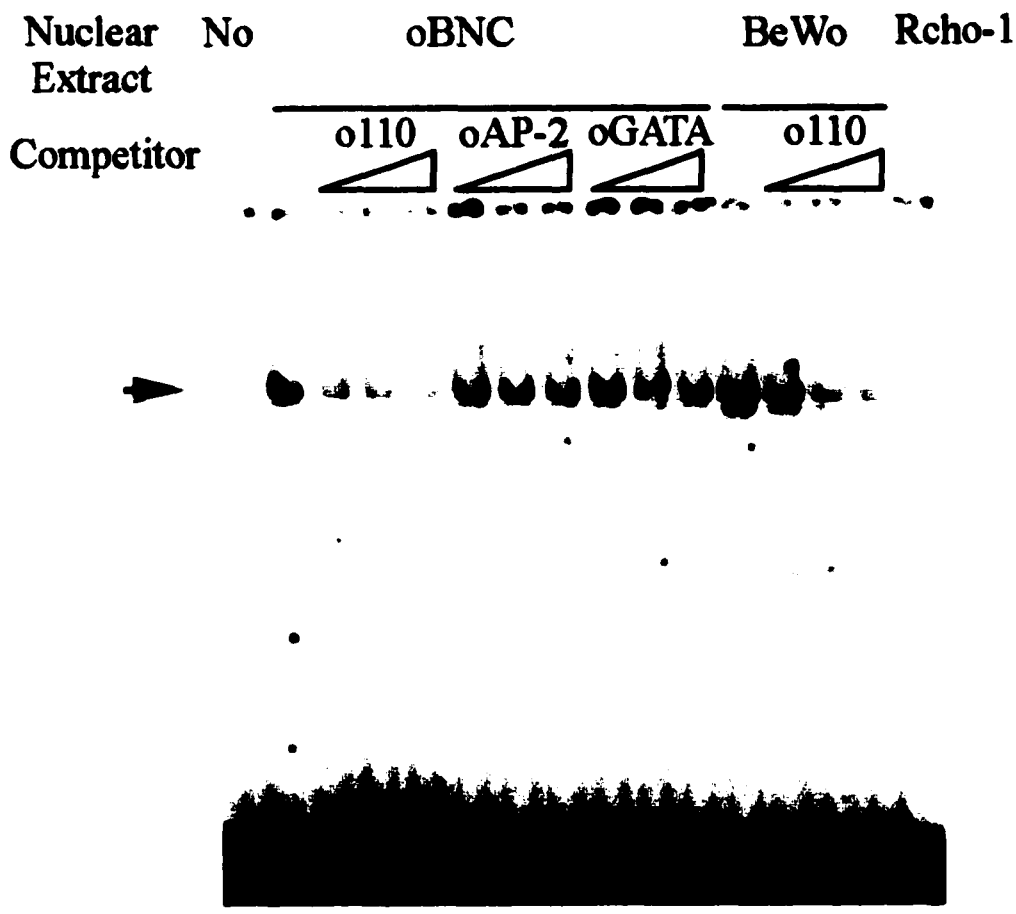


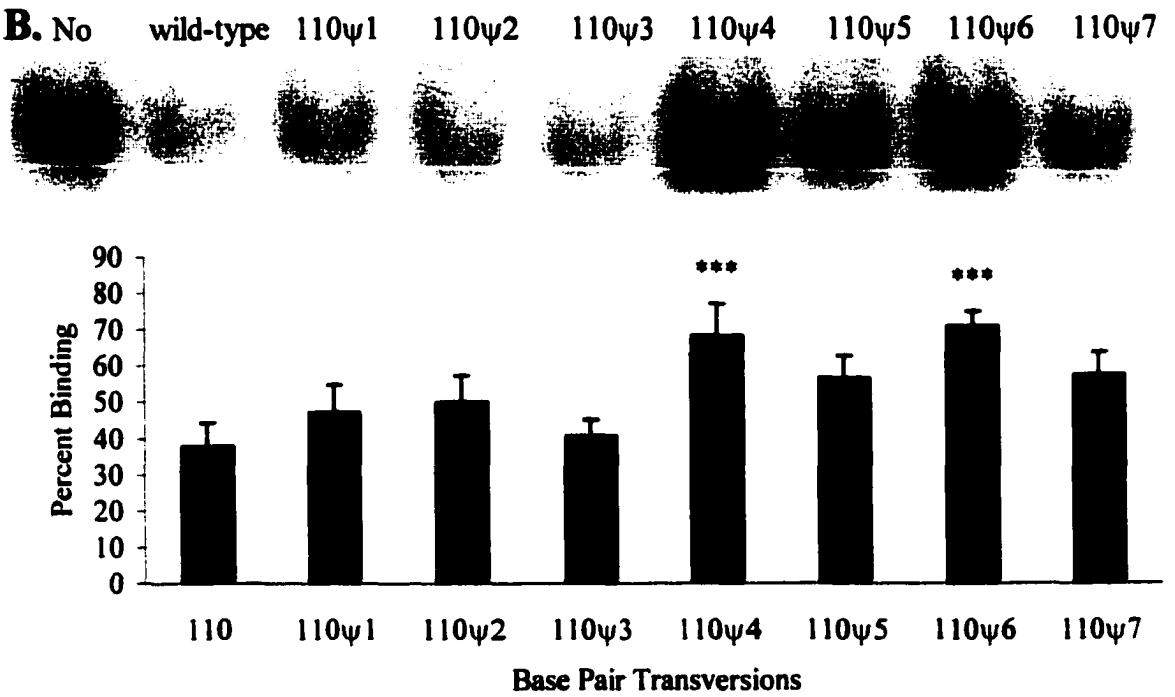
Figure 19. Specificity of the o110 oligonucleotide binding by EMSA. Binding studies using a radiolabeled o110 oligonucleotide in the presence of no, oBNC (25 μg), BeWo (25 μg) and Rcho-1 (50 μg) nuclear extracts are indicated above the lanes. Both oBNC and BeWo nuclear extracts reduced the mobility of the radiolabeled o110 oligonucleotide (arrow), when no competitor was added. Subsequent lanes for oBNC nuclear extracts included the addition of homologous (o110) or heterologous (oAP2 or oGATA) unlabeled competitors (labeled above) in increasing amounts (50, 100 or 250-fold molar excess), indicated with a triangle. Furthermore, binding reactions with BeWo cell nuclear extracts were competed with 50, 100, or 250-fold molar excess of homologous promoter. The Rcho-1 nuclear extracts were not able to form a complex with the o110 oligonucleotide, even when the amount of nuclear extract was doubled.

Figure 20. EMSA competition analysis with mutant o110 oligonucleotides. EMSA binding reactions with the o110 radiolabeled oligonucleotide was incubated in the presence of o110 mutant oligonucleotides, which had two base pair transversions. **Panel A** represents the wild type sequence of the o110 oligonucleotide and the two bp transversions created in the mutant oligonucleotides as labeled. These mutated o110 oligonucleotides were used as unlabeled competitors (100-fold molar excess) in the EMSA binding reactions with the radiolabeled wild-type o110 oligonucleotide. Additionally, no competitor and unlabeled wild-type o110 oligonucleotide at 100-fold molar excess was included in separate binding reactions. In **Panel B,** a representative EMSA with oBNC nuclear extracts is shown above the composite bar graph, which contains 4 independent repetitions. The binding percentage was calculated by dividing the volumetric intensities of the complex formed in lanes containing a competitor oligonucleotide by the intensity of the complex formed with no competitor. The bar graph summarizes mean percent binding±SEM for each competitor labeled on the ordinate axis. A Dunnett's T test was use the separate the mean percent binding of the mutant competitors from the wild type competitor and significance was accepted at $P<0.05$ (***). **Panel C** indicates the representative EMSA for BeWo cell nuclear extracts and the composite bar graph generated by 4 independent EMSA experiments.

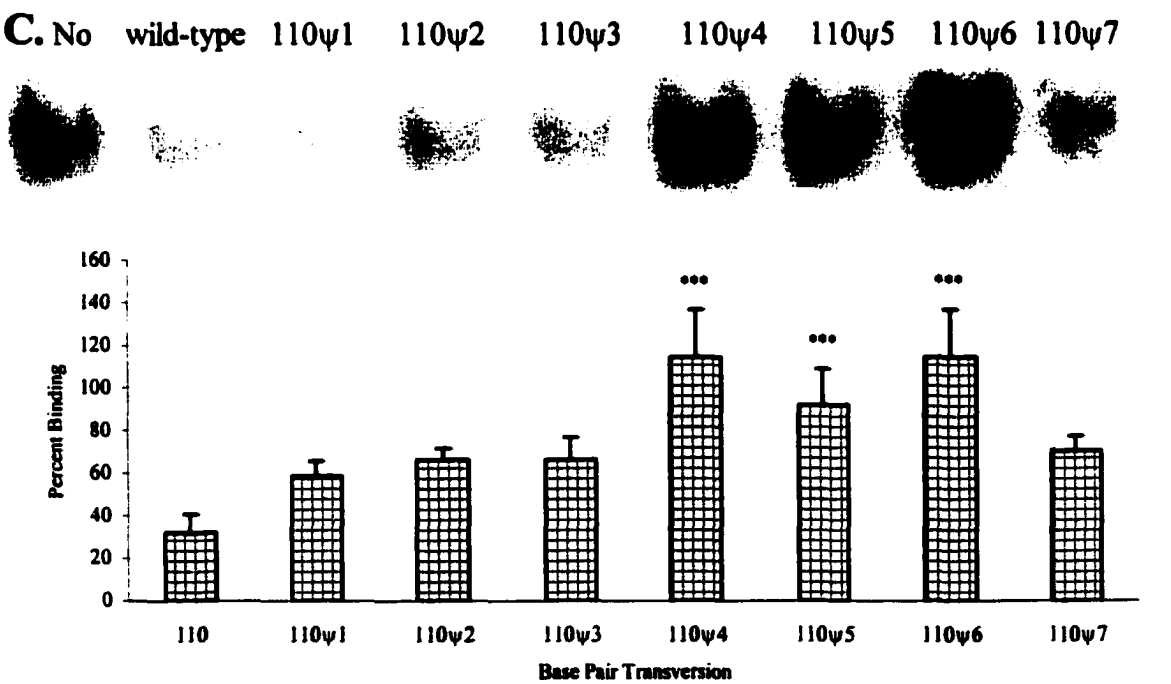
A.

Wild-type	-116	CA	GG	CA	CC	AG	CA	TT	G -102
Label		110ψ1	110ψ2	110ψ3	110ψ4	110ψ5	110ψ6	110ψ7	
Transversion		GT	CC	GT	GG	TC	GT	AA	

B.



C.



no competitor, and the mean percent binding (n≥4) was statistically compared to the wild-type o110 competitor (Figure 20B). The data identified a transversion at 110ψ4 (CC→GG) and 110ψ6 (CA→GT) with oBNC nuclear extracts that were unable to inhibit the protein-DNA complex formation with the wild-type competitor (Figure 20B). The competition EMSA studies with BeWo cell nuclear extracts demonstrated that 110ψ4 and 110ψ6 were unable to inhibit complex formation, as well as 110ψ5 (AG→TC; Figure 20C). Therefore, the nucleotides, 5'-CCAGCA-3', located at -110/-105 bp were identified as the *cis*-acting element, which interacted with a nuclear protein in oBNC and BeWo cells.

The nucleotides identified to interact with the nuclear protein appear to be a novel *cis*-acting element, because no previously defined elements were identified by database searches of transcription factor elements. However, an identical 6 bp region was found in the human PL genes at -147 bp relative to the transcriptional start site. An oligonucleotide encompassing -150/-136 bp (h110) of the hPL-4 gene containing this *cis*-acting element was synthesized. No similarity between o110 and h110 oligonucleotides was observed outside of these 6 identical nucleotides. Radiolabeled h110 in an EMSA with both BeWo and oBNC nuclear extracts identified a specific protein-DNA interaction, when homologous and heterologous oligonucleotides were added to the binding reactions (Figure 21). Furthermore, the formation of the complex was inhibited with the addition of o110, confirming the interaction is specific for CCAGCA (Figure 21).

The identification and confirmation of this novel *cis*-acting element in both the ovine and human PL genes prompted studies to identify the transacting factor. Southwestern

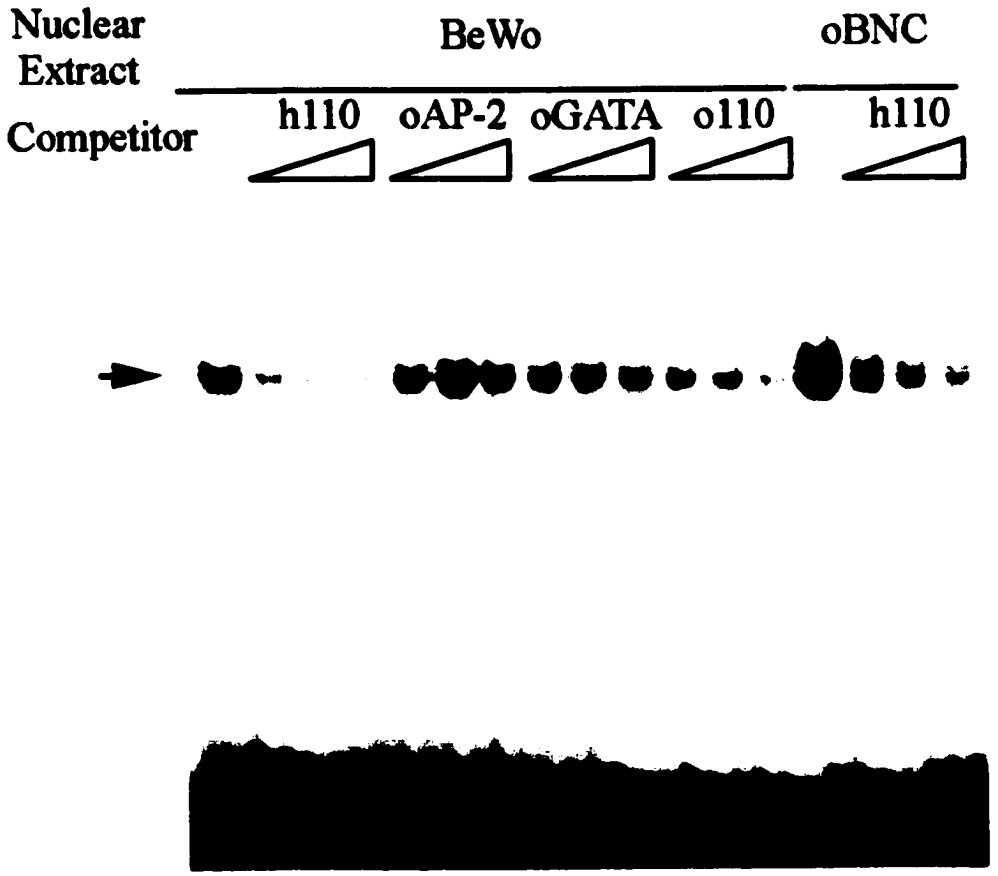


Figure 21. EMSA of h110 in BeWo and oBNC nuclear extracts. An oligonucleotide encompassing –150/-136 bp (h110) of the human PL gene with a CCAGCA site at -147 bp relative to the transcriptional start site, was tested by EMSA for protein-DNA complex formation. The protein-DNA complex (arrow) for radiolabeled h110 was tested with homologous (h110 and o110) as well as heterologous competitors (oAP2 and oGATA) in BeWo nuclear extracts (labeled above the lanes) at 50, 100 and 250-fold molar excess unlabeled competitor (indicated by the triangle). Radiolabeled h110 formed a specific complex with oBNC nuclear extracts, which had a similar mobility in comparison to the BeWo complex.

analysis was used to determine the molecular mass of the transacting factor. A concatamer of a triplicate o110 sequence was used to identify the transacting factor in BeWo cell nuclear extracts, and specificity of the protein-DNA interaction was confirmed with the addition of 50 fold molar excess unlabeled homologous competitor. Southwestern analysis (Figure 22) identified a nuclear protein with the o110 concatamer that migrates with an apparent $M_r \cong 41{,}000$.

The ability to detect the protein by Southwestern analysis allowed the screening of an ovine placental expression library with the o110 concatamer to purify a cDNA encoding the transacting factor. After screening 1×10^6 plaques of the ovine placental ZAP Express library with both homologous and heterologous probes, 11 positive clones were purified to homogeneity. The cDNA inserts within these clones were radiolabeled and used in southern hybridizations to verify homology between the isolated clones. Six of the eleven positive clones crossed hybridized with one another indicating they possess sequence identity. These cDNA clones were sequenced and found to share identity with one another as well as with human and mouse Purα, a single-stranded DNA binding protein. Both the sense and antisense strands of clone 925 and 1114 were entirely sequenced and found to contain identical cDNAs. The cDNA for clone 925 encoding the ovine Purα cDNA was 1114 bp with 15 bp of 5' untranslated sequence (UTR) and 130 bp of 3' UTR (Figure 23). The open-reading frame encoded 322 amino acids (966 bp) and had a preferred translation start site and a polyadenylation signal at 1078 bp. The protein encoded by this clone had a predicted M_r of 34,881. The ovine Purα cDNA nucleotide sequence was 97% and 95% identical to human (Bergmann and Johnson 1992) and mouse Purα (Ma *et al.* 1994) cDNAs, respectively. At the amino acid level, ovine

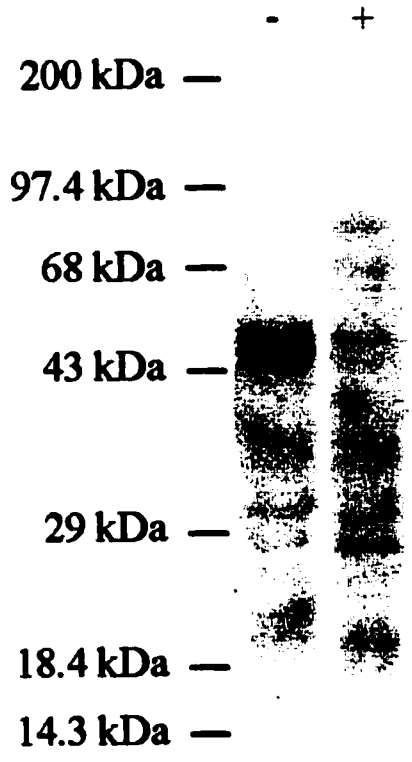


Figure 22. Southwestern analysis of the nuclear protein interacting with o110. The molecular weight standards are indicated on the left of the figure adjacent to membranes with SDS-PAGE gel separated nuclear proteins transferred to them. A concatamer with triplicate o110 binding sites was radiolabeled (-) and used to identify the nuclear protein. In the second lane a 50 fold molar excess (+) of unlabeled concatamer was added to the binding reaction. A specific protein interaction (arrow) with an apparent M_r of ≅41,000 was found.

Figure 23. Ovine Purα nucleotide and amino acid sequence. Both the sense and antisense strands of the cDNA insert in clone 925 were sequenced. The predicted amino acids are identified above the nucleotide sequence. A polyadenylation signal is identified in bold. Three class I repeats are indicated with a single underline and two class II repeats are identified with a double underline. Additionally, a glycine-rich region is identified at the amino terminal region, and a glutamine/glutamate-rich region (282-322) is located at the carboxy-terminus.

```
   1                  M  A  D  R  D  S  G  S  E  Q  G  G  A  A  L
   1  GCGGAGCGCAGCATCATGGCGGACCGAGACAGCGGCAGCGAGCAGGGTGGTGCGGCGCTG
  16   G  S  G  G  S  L  G  H  P  G  S  G  S  G  G  G  G  G  G  G
  61  GGCTCGGGCGGCTCCCTGGGGCACCCAGGCTCGGGCTCCGGCGGGGGCGGTGGTGGCGGC
  36   G  G  G  G  G  G  S  G  G  G  G  G  G  G  A  P  G  G  L  Q
 121  GGGGGCGGCGGCGGCGGCAGTGGCGGCGGCGGCGGCGGCGGGGCCCCGGGGGGGCTGCAG
  56   H  E  T  Q  E  L  A  S  K  R  V  D  I  Q  N  K  R  F  Y  L
 181  CACGAGACGCAGGAGCTGGCCTCCAAGCGGGTGGACATCCAGAATAAGCGCTTCTACCTG
  76   D  V  K  Q  N  A  K  G  R  F  L  K  I  A  E  V  G  A  G  G
 241  GACGTGAAGCAGAACGCCAAGGGCCGCTTTCTAAAGATCGCTGAGGTGGGCGCGGGCGGC
  96   N  K  S  R  L  T  L  S  M  S  V  A  V  E  F  R  D  Y  L  G
 301  AACAAGAGCCGCCTTACTCTTTCCATGTCAGTGGCCGTGGAGTTCCGCGACTACCTGGGC
 116   D  F  I  E  H  Y  A  Q  L  G  P  S  Q  P  P  D  L  A  Q  A
 361  GACTTCATCGAGCACTACGCGCAGCTGGGCCCCAGCCAGCCGCCCGACCTGGCCCAGGCG
 136   Q  D  E  P  R  R  A  L  K  S  E  F  L  V  R  E  N  R  K  Y
 421  CAGGACGAGCCGCGCCGGGCGCTCAAGAGCGAGTTCCTGGTGCGCGAGAACCGCAAGTAC
1561   Y  M  D  L  K  E  N  Q  R  G  R  F  L  R  I  R  Q  T  V  N
 481  TACATGGATCTCAAGGAGAACCAGCGCGGCCGCTTCCTGCGCATCCGCCAGACGGTCAAC
 176   R  G  P  G  L  G  S  T  Q  G  Q  T  I  A  L  P  A  Q  G  L
 541  CGGGGGCCCGGCCTGGGCTCCACGCAGGGCCAGACCATTGCACTGCCCGCACAGGGGCTC
 196   I  E  F  R  D  A  L  A  K  L  I  D  D  Y  G  V  E  E  E  P
 601  ATCGAGTTCCGCGACGCTCTGGCCAAGCTCATCGACGACTACGGAGTGGAGGAGGAGCCG
 216   A  E  L  P  E  G  T  S  L  T  V  D  N  K  R  F  F  F  D  V
 661  GCTGAGCTGCCCGAGGGCACCTCCTTGACTGTGGACAACAAGCGCTTCTTCTTCGATGTG
 236   G  S  N  K  Y  G  V  F  M  R  V  S  E  V  K  P  T  Y  R  N
 721  GGCTCCAACAAGTACGGCGTGTTTATGCGAGTGAGCGAGGTGAAGCCCACCTACCGCAAC
 256   S  I  T  V  P  Y  K  V  W  A  K  F  G  H  T  F  C  K  Y  S
 781  TCCATCACCGTGCCCTACAAGGTGTGGGCCAAGTTTGGACACACCTTCTGCAAGTACTCG
 276   E  E  M  K  K  I  Q  E  K  Q  R  E  K  R  A  A  C  E  Q  L
 841  GAGGAGATGAAGAAGATTCAAGAGAAGCAGAGGGAGAAGCGAGCTGCCTGTGAGCAGCTT
 296   H  Q  Q  Q  Q  Q  Q  Q  E  E  T  A  A  A  T  L  L  L  Q  G
 901  CACCAGCAGCAGCAGCAGCAGGAGGAGACAGCCGCTGCCACCCTGCTGCTGCAGGGT
 316   E  E  E  G  E  E  D  -
 961  GAGGAAGAAGGGGAAGAAGATTGATCAAACTGAATGGAAAAAACCCACACACACATGCAC
1023  ACACACACACACACACACAGCCACACACAGAGAAACTATACTGTAAAGAAAGAGAGAAAA
1081  TAAAAAGTTAAAAAAGTTAAAAAAAAAAAAAAA
```

and human Purα share an identity of 99 %, and only three amino acid variations were found between human and ovine Purα: Ser to Gly[30], Ser to Gly[43] and Gly to Ser[42].

EMSA with oBNC and BeWo nuclear extracts were analyzed in the presence of both double stranded and single stranded unlabeled competitors to identify if ovine Purα is binding to a single-stranded or double-stranded template in the oPL minimal promoter. Both double-stranded and single-stranded competitors influenced the protein-DNA complex generated with the radiolabeled double-stranded probe. The o110 sense strand exhibited an affinity equal to the double-stranded o110 unlabeled competitor, whereas the antisense strand had little to no effect on complex formation (Figure 24). In these reactions only the antisense strand was labeled indicating that the protein-DNA complex is associated with double-stranded DNA, but the interaction appears to interact specifically with the sense strand. This confirms that the single-strand DNA binding protein, Purα, will interact with double-stranded DNA. Antiserum raised against mouse Purα (Osugi *et al.* 1996) was added to the EMSA binding reaction after the 55°C heat treatment, and a supershift was identified for both oBNC and BeWo cell nuclear extracts, but was not identified with the addition of normal rabbit serum (Figure 24).

The *cis*-acting element identified in the oPL minimal promoter is not similar to the putative Purα element, $(GGN)_n$, which was previously identified to specifically interact with the single-strand DNA binding protein. Therefore, an oligonucleotide containing the putative Purα element (MF0677; Bergemann and Johnson 1992) was synthesized and tested in EMSA assays. EMSA binding reactions with BeWo cell and oBNC nuclear extracts were able to retard the mobility of the MF0677 single-strand oligonucleotide specifically, when unlabeled homologous and heterologous competitors were tested.

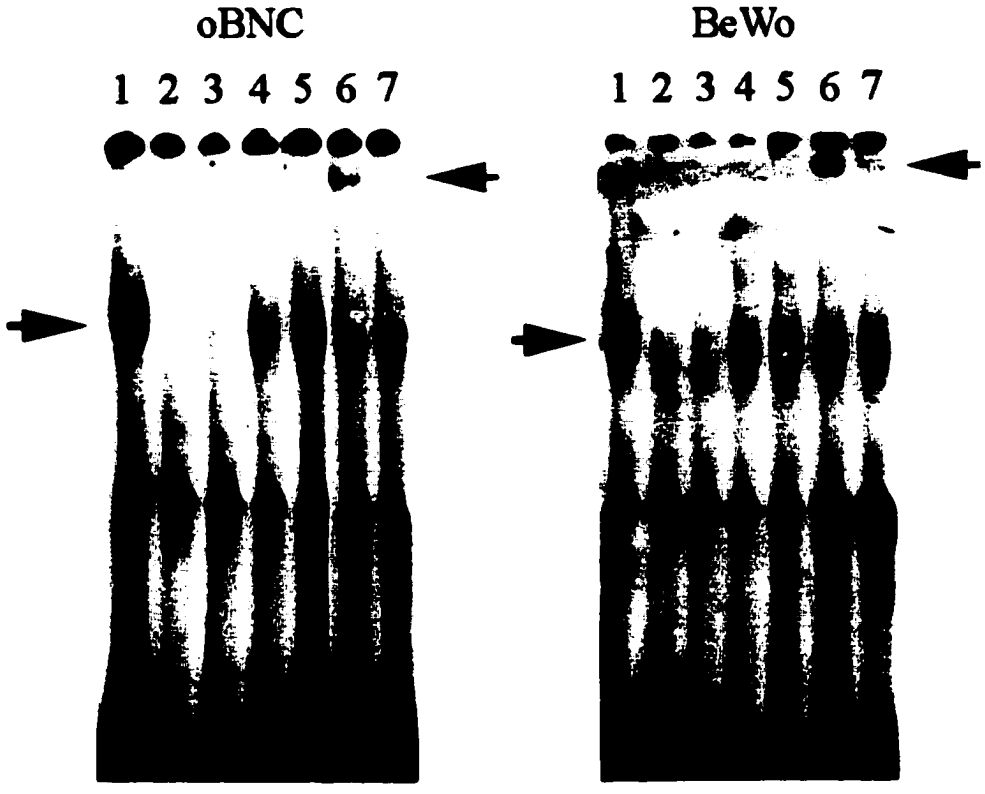


Figure 24. EMSA and supershift with o110. EMSA with either oBNC or BeWo cell nuclear extracts are shown. The lanes are number along the top. Lane 1 contains no competitor, where lanes 2-5 have 100-fold molar excess of double-strand o110, single-strand o110 sense, single-strand o110 antisense and single-strand o99 sense competitors, respectively. Lanes 6 contains rabbit antiserum raised against mouse Puroα and lane 7 contains normal rabbit serum. The arrow on the left indicates the protein-DNA complex and the arrow on the right identifies the supershift complex.

Furthermore, the addition of double-stranded o110 also inhibited the formation of the MF0677complex (Figure 25). However, less influence with single-strand o110 competitors was observed, but complex formation was not equivalent to that observed in binding reactions containing no competitor or heterologous competitor (Figure 25). Addition of Purα antiserum to the binding reaction further reduced the mobility of the complex, and this reduced mobility was specific because the addition of normal rabbit serum did not change the mobility of the MF0677 complex (Figure 25). Moreover, *in vitro* transcribed and translated ovine Purα was demonstrated to form a complex with MF0677, and the mobility was reduced with the addition of Purα antiserum. Western immunoblots with the *in vitro* translated Purα confirmed that the protein migrates with the apparent M_r of ≅41,000, which is similar to the M_r observed in Southwestern analysis (data not shown).

An expression vector containing the ovine Purα cDNA was co-transfected in BeWo cells with the –124pGL3 vector to assess its ability to stimulate transactivation. Co-transfections with 2.5 μg of Purα clone 925 significantly enhanced the activity of the oPL minimal promoter in BeWo cells, whereas a control vector (pBlueScript) did not augment transactivation (Figure 26). Additionally, a significant enhancement in promoter activity was not identified when the *cis*-acting element within the minimal promoter was mutated (-124Δ110pGL3). These data indicate that ovine Purα was able to stimulate transactivation of the oPL minimal promoter in BeWo cells by interacting with a novel *cis*-acting element, CCAGCA.

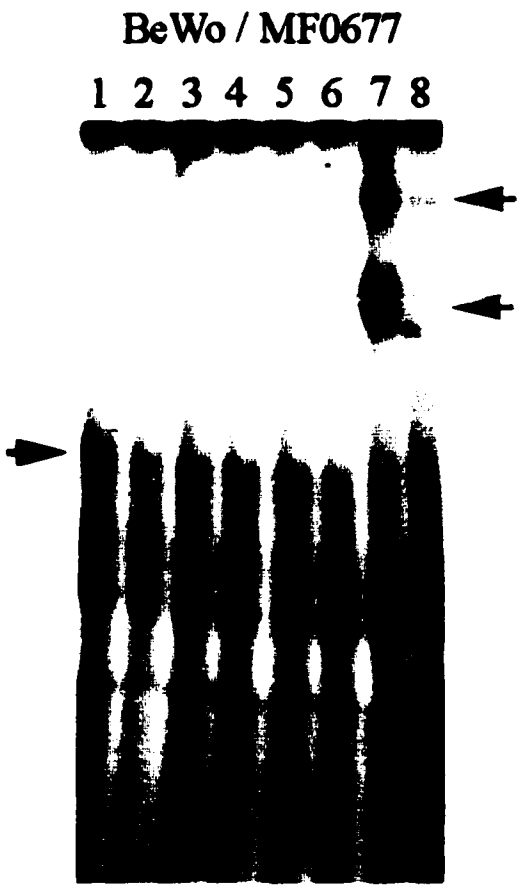


Figure 25. EMSA and supershift with putative Puroα element. The MF0677 single-stranded oligonucleotide was radiolabeled and complex formation with BeWo cell nuclear extracts (5 μg) was tested by EMSA. The first lane (1) contained no unlabeled competitor, whereas lanes 2-6 had 100-fold molar excess of homologous (MF0677), o110 sense strand, o110 antisense strand, o99 sense strand and double stranded o110, respectively. Lanes 7 and 8 had rabbit anti-mouse Puroα and normal rabbit serum added into the binding reaction, respectively. On the left an arrow indicates the specific protein-DNA complex, and arrows on the right recognize two supershift complexes formed with the addition of Puroα antiserum.

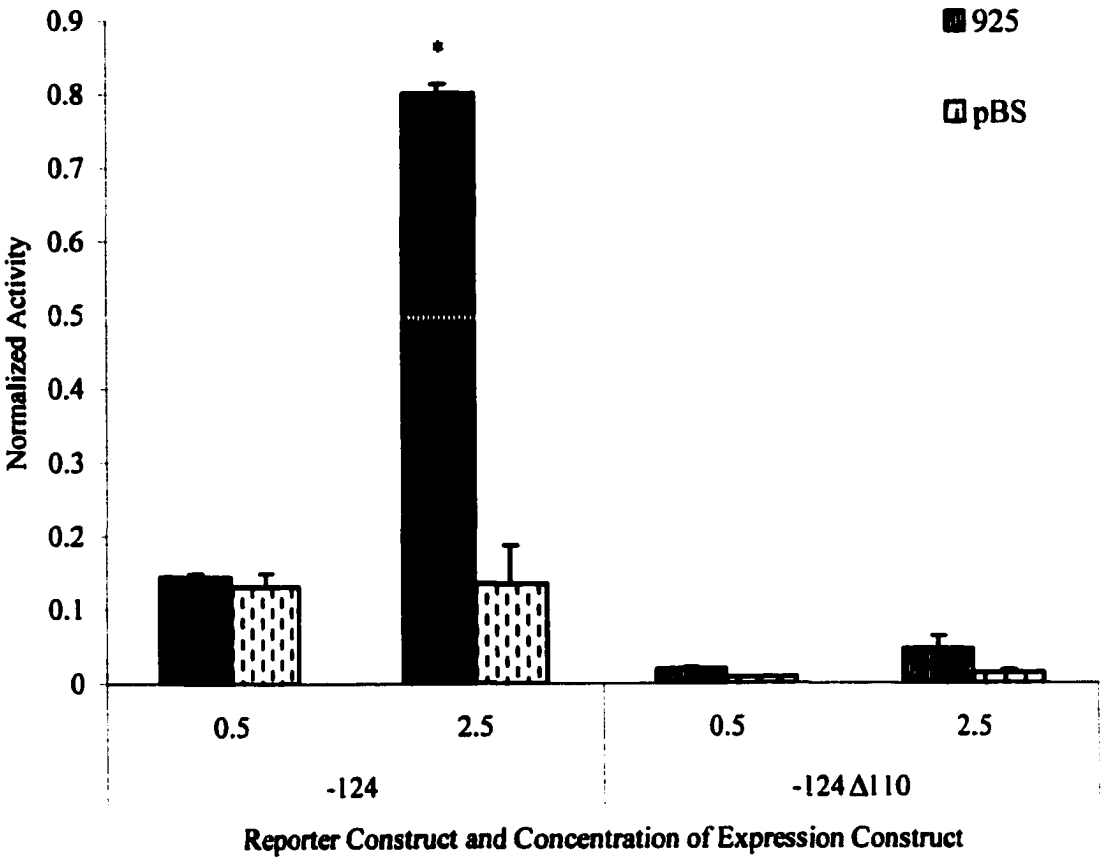


Figure 26. Functionality of a Purα expression vector in BeWo cells. BeWo cells were co-transfected with the Purα expression vector (925) or a control plasmid (pBluescript; pBS), and either a reporter vector -124pGL3 with the *cis*-acting element or a mutated site (-124Δ110pGL3) to analyze the affect of Purα. Two concentrations of the expression vector were tested (0.5 μg and 2.5 μg) and are indicated on the ordinate. The bars represent the mean activity and the error bars indicate the SEM. A Tukey's multiple comparison test was used to separated the means and significance accepted at $P < 0.05$.

Discussion

Mutational analysis of footprint 3 identified a novel *cis*-acting element (-109/-102 bp) distinct from the previously identified GATA element (-102 bp) within the oPL minimal promoter. A specific protein-DNA interaction was identified by EMSA, and competition EMSA with mutant oligonucleotides identified a protein-DNA interaction at CCAGCA (-110/-105 bp). Screening of an ovine placental expression library with a triplicate concatamer of the *cis*-acting element identified Purα, a single-strand DNA binding factor, as the nuclear protein interacting with this element. Binding studies confirmed the Purα:o110 interaction, but represents a new Purα *cis*-acting element.

The PUR element, $(GGN)_n$, was defined as a purine-rich single-strand element in the human *c-myc* gene and hamster *dhfr* gene at zones of initiation for DNA replication (Bergemann and Johnson 1992). A Purα clone encoding a 322 amino acid protein was found to contain three consensus repeat motifs of 23 amino acids (class I) separated by two repeats of a consensus motif of 26 amino acids (class II) (Bergemann *et al.* 1993). The class I repeats contain 3 conserved aromatic phenylalanine or tyrosine residues, which are proposed to intercalate single stranded DNA. These regions also have several basic residues, which may interact with DNA by electrostatic forces (Bergemann *et al.* 1993). Amino- and carboxy-terminal truncation of Purα revealed that each of the class I motifs interact with DNA (Chen and Khalili 1995). Furthermore, the class II motifs, which are leucine-rich, have been found to provide protein-protein interactions with Purα to form homodimers, as well as with other proteins to form heterodimers (Chepenik *et al.* 1998; Gallia *et al.* 1999a; Safak *et al.* 1999). Purα contains a glutamine/glutamate-rich (Gln^{282} to Glu^{322}) region at the carboxyl-terminus, and these acidic residues have been

categorized as activation domains in other transcription factors (Darnell 1982; Ptashne 1989; Courey *et al.* 1990). A "psycho" domain between residues Pro^{251} to Met^{278} interacts with the retinoblastoma protein (Rb) (Johnson *et al.* 1996), and this motif was identified in several other viral (SV40 T antigen and the human papilloma virus E7 protein) and mammalian proteins (Erg1 and Erg2, of the Ets family) (Ma *et al.* 1995). The final motif identified in this protein includes a glycine-rich region at the amino-terminus of Purα, which has been identified in helix destabilizing proteins and RNA-binding proteins (Haynes *et al.* 1987; Haynes *et al.* 1990; Herault *et al.* 1995a; Kelm *et al.* 1999). The identification of these regions suggested that Purα, originally described as an initiator of replication, may possess many different functions.

One important function of Purα is its ability to stimulate transcription by interacting with a specific DNA element in eukaryotic cells. Transactivation by Purα has been shown in JC virus early and late promoters (Safak *et al.* 1999), myelin basic protein (Haas *et al.* 1995; Morasso *et al.* 1999), neuron-specific Fe65 promoter (Zambrano *et al.* 1998), HIV-1 LTR (Chepenik *et al.* 1998), neuropeptide Y (Kuo *et al.* 1999) and the avian clusterin gene (Herault *et al.* 1995b). Stimulation of these promoters required a specific *cis*-acting element. Another action of Purα is its ability to form protein-protein interactions with other transcription factors or intercellular signaling factors to promote transactivation. Associated proteins of Purα appear to add an additional hierarchy of enhancement to the transactivator, by stimulating DNA interaction or facilitating transcriptional stimulation. Subsequent studies have identified or purified activator proteins associated with Purα transcriptional enhancement (Ding *et al.* 1997; Kuo *et al.* 1999; Tretiakova *et al.* 1999; Safak *et al.* 1999). One of the activator proteins purified

was calmodulin, which may implicate that intracellular signaling mechanisms are able to mediate Purα transactivation (Kuo *et al.* 1999). Purα has been described to associate with phosphorylated Sp1 during myelination of the mouse brain and synergistically stimulate transactivation (Tretiakova *et al.* 1999). However, the effects of Purα are not always stimulatory, since Rb (Johnson *et al.* 1996) and E2F-1 (Darbinian *et al.* 2000) association negatively affect transcription either by inhibiting Purα-DNA interactions or E2F-1-DNA interactions.

The *cis*-acting element that was previously defined $(GGN)_n$ to interact with Purα, and that was used to identify Purα, has very little identity to the *cis*-acting element described in the oPL gene. Binding studies suggest that Purα interacts with a double stranded sequence of CCAGCA (Figure 20), but addition of the o110 sense strand competed for binding (Figure 24). Additionally the double-stranded o110 oligonucleotide was able to displace binding of the putative PUR element in the MF0677 oligonucleotide (Figure 25). However, the binding affinities of these interactions may vary. Protein-DNA interaction with o110 required 25 μg of BeWo or oBNC nuclear extracts, whereas the binding studies with radiolabeled MF0677 were performed with 5 μg of nuclear extract. Binding reactions containing a high concentration of nuclear extract and either radiolabeled o110 or MF0677, exhibited no specific inhibition of complex formation when 100-fold molar excess of unlabeled MF0677 was added. These data suggest that Purα is a predominant protein in the BeWo cell and oBNC nuclear extracts, and adding 25 μg of nuclear extracts reduces the mobility of the majority of radiolabeled MF0677, even in the presence of 100-fold molar excess unlabeled competitor. However, reducing the nuclear extracts to a fifth of it original concentration,

allows the specific interaction with the radiolabeled MF0677 to be studied. Three possible conclusions can be drawn for these experiments: 1) Purα binding to its element in the oPL minimal promoter requires additional transacting factors by protein-protein interaction to facilitate binding; 2) Purα requires posttranslational modification to interact with the oPL element; or 3) Purα has low affinity for the CCAGCA binding site, and a high concentration of Purα in required to distinguish specific binding. The first hypothesis is difficult to perceive, since Southwestern analysis detected Purα, but posttranslationally modified Purα maybe the resultant signal. However, different affinities for the two elements seem the most plausible explanation, in light of another low-affinity interaction proposed for Purα found when a double-stranded CRE element (TGACGTCA) was used as a competitor (Osugi *et al.* 1994; Kuo *et al.* 1999).

Another interesting finding was the location of this element in the human PL and GH genes. The proximal 500 bp of promoter region for these human genes were described to share greater than 90 % identity (Walker *et al.* 1991). Additionally, the location of this element in the human genes (-147 bp) is closely related to its location in the oPL gene (-110 bp), in respect to the transcriptional start site. Fitzpatrick *et al.* (1990) analyzed the promoter region of the hPL genes and indicated that a region between -152/-142 bp contributed to the transactivation of the hPL gene. A subsequent deletion from -142/-129 bp had a much more dramatic effect on transactivation, and was identified to contain a non-conical Sp1 site (Fitzpatrick *et al.* 1990). The functionality of the hPL promoter elements in human trophoblast cells is thought to provide a basal region to nucleate the preinitiation complex. Enhancement of hPL gene transcription is attributed to elements that reside about 2.2 kb 3' of the hPL/hGH gene locus that stimulates transcription in a

trophoblast-specific fashion (Rogers *et al.* 1986; Jacquemin *et al.* 1994; Jiang and Eberhardt 1994; Lytras and Cattini 1994). Therefore, general transcription factors such as Sp1 at –137 bp has been shown to play a critical role in enhancing hPL gene transcription by recruiting the TATA-binding protein. A Purα-Sp1 interaction has been shown to synergistically stimulate expression of the myelin basic protein gene (Tretiakova *et al.* 1999), and addition of Sp1 to purified Purα has been shown to increase Purα binding to DNA. Furthermore Purα and Sp1 have been shown to interact in the absence of DNA, which may indicate that a heterodimer may form at either of these sites to augement transactivation.

Mutation of the Purα element in the oPL minimal promoter significantly reduces transactivation in BeWo cells, and overexpression of Purα in BeWo cells augments the expression of a reporter vector containing the –109/-102 bp region (Figure 17 and 26). Therefore, Purα's function is mediated through a protein-DNA interaction at CCAGCA, but binding of Purα to this element appears to be at a lower affinity than the previously identified site. The data of Sp1 stimulating a Purα-DNA interaction to the myelin basic protein gene (Tretiakova *et al.* 1999) may indicate that other transacting factors facilitate Purα-DNA interactions. Calmodulin has been shown to enhance Purα-DNA interaction to a single stranded CRE element, as well as the MB1 site in the myelin basic protein (Kuo *et al.* 1999). Other transacting factors that interact with Purα include: T-antigens from polyomaviruses (JC virus; Gallia *et al.* 1999b); E2F-1 (Darbinian *et al.* 2000); and YB-1 (Safak *et al.* 1999). Possible Purα interactions with GATA-2 and AP-2α, which have been shown to enhance the oPL minimal promoter in BeWo cells, may provide other factors that interact with Purα. The proximity of the GATA element (-102/-99) and

Purα element (-110/-105) in FP3 may imply that these two factors cooperated to augment transcription, but no interactions with these transacting factors have been investigated.

In conclusion the data presented here identified a novel *cis*-acting element, distinct from the GATA element, which is required for transactivation of the oPL minimal promoter in human choriocarcinoma cells. The transacting factor associated with this element was found to be ovine Purα, which proved to be functional in BeWo cells. Although the protein-DNA interaction at this *cis*-acting element exhibited a lower affinity for Purα then a previously described *cis*-acting element, the interaction appears to be specific and may suggest that an additional activator protein is required to stimulate Purα and CCAGCA interaction. Further studies on potential protein-protein interactions and enhancement of the oPL gene minimal promoter will be required to fully understand the role of Purα.

Summary

Placental lactogen (PL) is thought to alter maternal metabolism to increase the pool of nutrients available for the fetus. The ovine (o) PL gene is expressed in chorionic binucleate cells (BNC) and has *cis*-acting elements located within the proximal 124 base pair (bp) promoter region capable of regulating trophoblast-cell specific activity in a human choriocarcinoma cell line (BeWo). DNase I protection assays identified protein-DNA interactions with ovine BNC nuclear extracts between -95/-123 bp, and mutational analysis in BeWo cells revealed a novel *cis*-element that was functional. Characterization of this *cis*-element in BeWo and BNC nuclear extracts identified a specific interaction with the nucleotide sequence CCAGCA (-110/-105; o110) and

southwestern analysis identified a nuclear protein in BeWo cells with an apparent Mr of 41,000. Screening an ovine placental expression library identified cDNAs encoding a DNA binding protein, which interacts with this site. Six of the eleven clones purified were homologous to each other by southern hybridization. The nucleotide sequence of these clones share similarity with the human (97%) and mouse (95%) Purα single-stranded DNA binding protein. The Purα-o110 interaction was confirmed by electrophoretic mobility-supershift assays with BNC and BeWo nuclear extracts. Transactivation of the –124 to +16 region of the oPL gene was enhanced by the ovine Purα expression vector. These data are the first to identify Purα as a placental *trans*-acting factor, which stimulates expression of the oPL promoter, through a novel *cis*-element.

Chapter VI

IDENTIFICATION OF *CIS*-ACTING ENHANCER ELEMENTS IN THE OVINE PLACENTAL LACTOGEN GENE

Introduction

During gestation a transient endocrine organ, the placenta, influences both maternal and fetal tissues to promote fetal growth and development. A hormone expressed exclusively by the placenta, placental lactogen (PL), is a member of the growth hormone-prolactin gene family. Placental lactogen is thought to alter intermediary metabolism in the mother to increase nutrient pools for the fetus, whereas in fetal tissues PL has anabolic actions (Talamantes and Ogren 1988; Handwerger 1991; Anthony *et al.* 1995a; Anthony *et al.* 1995b). Ovine chorionic binucleate cells (oBNC) synthesize ovine (o) PL, and secrete a non-glycosylated 198 amino acid polypeptide (M_r of ≈22,000) into both the maternal and fetal vasculature (Colosi *et al.* 1989; Warren *et al.* 1990; Kappes *et al.* 1992). The oPL gene has been structurally characterized and contains 5 exons and 4 introns, which span 11.2 kb of genomic sequence (Liang *et al.* 1999). Additionally, 4.5 kb of 5'-flanking sequence has been sequenced and used to study transcriptional regulation of the oPL gene (Liang *et al.* 1999). Transient transfections in human and rat

choriocarcinoma or non-choriocarcinoma cell lines with various lengths of oPL 5'-flanking sequence linked to a reporter gene, was used to identify trophoblast-specific promoter regions. The sequence between –383/+16 bp, relative to the transcriptional start site, is capable of stimulating maximal trophoblast-specific expression in choriocarcinoma cell lines (Liang *et al.* 1999). A subsequent deletion to –217 bp resulted in a loss of trophoblast-specific activity in Rcho-1 (rat) choriocarcinoma cells, and a significant decrease in activity was observed in BeWo (human) choriocarcinoma cells. Furthermore, a deletion construct containing -124/+16 bp of promoter sequence possessed trophoblast-specific expression in both choriocarcinoma cell lines, but activity was significantly reduced from that achieved with 383 bp of proximal 5'-flanking sequence.

DNase I protection assays were performed with oBNC nuclear extracts to locate protein-DNA interactions within the cells endogenously expressing oPL. The functionality of these protected regions was identified by the resulting transactivation obtained in choriocarcinoma cell lines. Six protected regions were identified within the -383/+16 bp region and are identified as footprint (FP) 1-6, starting with the protected region encompassing the transcriptional start site (Liang *et al.* 1999). Three FP were observed in the minimal promoter region (-124/+16) and 4 functional *cis*-acting elements were identified: an AP-2 element at -58 bp (FP2; Chapter IV); two GATA elements at -67 bp and –102 bp (FP 2 and FP3; Chapter III); and a novel *cis*-acting element interacting with Purα at –110 bp (FP3; Chapter V). An E-box was identified in FP4 at –163 bp, but the functionality of this element was not evident by deletion analysis. However, mutational analysis indicated that this element was functional and may play a role in a

context dependent manner (Chapter III). Two additional FP were identified with oBNC nuclear extracts at -284/-246 bp (FP5) and -349/-319 bp (FP6). These protected regions reside within the 166 bp (-383/-217 bp) that was identified to stimulate maximal activity in both choriocarcinoma cell lines and were suggested to bind transcriptional enhancing factors (Liang *et al.* 1999). A direct repeat (DR-1), 5'-GAGGAG-3', at –338 bp and -283 bp was identified in each of these protected regions and mutational analysis of these sites in Rcho-1 and BeWo choriocarcinoma cell lines significantly reduced transactivation (Liang *et al.* 1999). Other placental promoters, hPL (Fitzpatrick *et al.* 1990) and CYP19 (Kamat *et al.* 1998) genes, have an identical 6 bp site in the 5'-flanking sequence. Functionality of these *cis*-acting elements in the oPL gene indicates that they are involved in stimulating transcription, but further examination is required to specifically identify the functional element and its transacting factor.

In the present study the *cis*-acting elements that reside in FP5 and FP6, located within the enhancer region of the oPL gene 5'-flanking sequence, are characterized. Mutational analysis across these protected regions was performed to identify their functionality. Electrophoretic mobility shift assays and competition studies were used to identify the specific nucleotides interacting with transacting factors, and the apparent molecular weight of the factors was determined by Southwestern analysis. Finally, the enhancer function of the elements was determined by transient transfections with a triplicate concatamer of the protected region.

Materials And Methods

Mutation Constructs

A dual PCR amplification was employed to generate block mutations within the DNase I protected regions (FP5 and FP6). Over-lapping oligonucleotide primers containing a Not I restriction endonuclease recognition site at the 5' end were designed for each mutation and are: FP5Δ1F, 5'-CAT AGC GGC CGC AAG AGC TGA ATA CG-3'; FP5Δ1R, 5'-CTT GCG GCC GCT ATG GAC TGT AGC AA-3'; FP5Δ2F, 5'-CTA GCG GCC GCA GGC TCT CAA AGA G-3'; FP5Δ2R, 5'-CCT GCG GCC GCT AGC AAC CAG GCT-3'; FP5Δ3F, 5'-CCG CGG CCG CAC AGT CCA TAG GCT-3'; FP5Δ3R, 5'-TGT GCG GCC GCG GCT CCT CTG TC-3'; FP5Δ4F, 5'-GAC GCG GCC GCC TGG TTG CTA CAG-3'; FP5Δ4R, 5'-CAG GCG GCC GCG TCC AGG AGG ATT C-3'; FP6Δ1F, 5'-GCA GCG GCC GCC ATT CCA GCA TTC TTG-3'; FP6Δ1R, 5'-ATG GCG GCC GCT GCC CTC CTC CA-3'; FP6Δ2F, 5'-CTG GCG GCC GCC ATG GCA ACC CAT-3'; FP6Δ2R, 5'-ATG GCG GCC GCC AGG GGT CTT CCC T-3'; FP6Δ3F, 5'-GGA GCG GCC GCG GAG GAG GGC AT-3'; and FP6Δ3R, 5'-TCC GCG GCC GCT CCC TAC TCA GGG AT-3'. These primers, in conjunction with either the pGL3 forward or reverse primers (Promega, Madison, WI), were used to amplify the 5' or 3' DNA fragment of the proximal 380 bp of the oPL gene. The first PCR reaction contained 0.2 μM of forward and reverse oligonucleotides, 6.6 fmol 380pGL3, 0.1 μM of each dNTP, 1 U Taq DNA polymerase (Gibco BRL, Gaitherburg, MD), in a 1X reaction buffer with 1.5 mM $MgCl_2$. PCR amplification parameters, following a 5 min incubation at 95°C, consisted of a denaturation step at 94°C for 1 min, a 2 min annealing step at 48°C, and an extension step at 72°C for 1 min. After 10 cycles the annealing temperature

was increased to 60°C for an additional 30 cycles. DNA fragments generated were purified from an agarose gel with DEAE membrane (Ausubel *et al.* 1995), and added in equal molar ratios to a second PCR reaction as the template DNA with the pGL2 forward and reverse oligonucleotides. The reconstructed DNA fragments were digested with Kpn I and Hind III restriction endonucleases, agarose gel purified, and ligated into the pGL3-Basic vector. Southern blot and nucleotide sequencing confirmed the mutations. Covalently closed circle DNA was obtained from *E. coli* by alkaline-lysis procedure (Ausubel *et al.* 1995) and purified through CsCl equilibrium gradient centrifugation prior to transient transfection in mammalian cells.

Cell Culture and Transfection

A rat choriocarcinoma cell line, Rcho-1, was a generous gift from Dr. M. J. Soares, (University of Kansas, Kansas City, KS). BeWo cells from a human choriocarcinoma, Hela cells derived from a cervical carcinoma, and C127 cells, which originated from a murine mammary tumor, were obtained from American Type Culture Collection (Rockville, MD). Rcho-1 cells were maintained in NCTC-135 medium (Gibco BRL) supplemented with 1mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO) and 20% heat-inactivated fetal bovine serum (FBS; Gemini Bio-Products, Inc., Calabasas, CA). BeWo cells were maintained in F12K medium (Mediatech, Inc., Herndon, VA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10 % FBS. Non-choriocarcinoma-derived cell lines, Hela and C127, were maintained in Dulbecco's modified Eagle's medium (Mediatech, Inc.) supplemented with 100 U/ml penicillin, 100 μg/ml

streptomycin, 5% heat-inactivated horse serum and 5% FBS. All cells were maintained in monolayer cultures at 37°C in 5% CO_2, 95% air with 100% humidity.

Transient transfections were performed in 6-well plates at a density of 0.5×10^6 cells/well. The polycationic lipid/DNA complex reaction was carried out at room temperature for 20 min, with 6-10 µl LipofectAmine reagent (Gibco BRL), 5 µg pGL3 plasmid, 0.25 µg of pβgal control plasmid (RSV promoter and enhancer; ClonTech Laboratories, Inc., Palo Alto, CA) and culture medium without serum and antibiotics in a total volume of 100 µl. The cells were washed twice in serum-free medium, the lipid/DNA mixture was diluted to 1 ml with serum-free medium, added dropwise to the cells and incubated at 37°C for 24 h with BeWo and Rcho-1 cell lines, or 6 h for Hela and C127 cell lines. At this time the transfection mixture was replaced with 4 ml of serum-containing antibiotic-free medium and cultured for an additional 18-54 h.

After the second incubation, the cells were harvested, lysed, and luciferase and β-galactosidase activities of the cell lysate were analyzed using a Luciferase Assay System (Promega) or LumiGAL™ Chemiluminescent Assay kit (ClonTech). The light emission generated by both activities was measured using a Turner TD-20e luminometer with an integration time of 10 seconds. The number of light units generated by luciferase activity was normalized to β-galactosidase activity for each transfection to control for intra-assay variation. Relative luciferase activity for each construct is expressed as percent activity of the wild-type -380pGL3. Data are presented as mean percent activity±SEM (n>3), and analyzed by least square analysis of variance using the Statistical Analysis System (SAS Institute, 1993). In general linear model procedures (Snedecor and Cochran 1976), plasmid DNAs were used as the dependent variable and cell line as the independent

variable to compare differences in activity between plasmids. When cell line was used as the dependent variable, plasmids were used as the independent variable to compare differences of activity between cell lines. Means were separated using the protected LSD test (deletion constructs) or Dunnett's T test (mutation constructs), and statistical significance was accepted for $P<0.05$ (Steel and Torrie 1960).

Nuclear Extracts and Electrophoretic Mobility Shift Assays

Binucleate cells were isolated from 100 day post coitus cotyledonary tissue (Reimers *et al.* 1985; Morgan *et al.* 1990; Liang *et al.* 1999). Nuclear proteins from ovine binucleate cells, BeWo, Hela, and differentiated Rcho-1 nuclei were purified (Dignam *et al.* 1983). Nuclear proteins were dialyzed against Dignam D buffer (20 mM HEPES pH 7.9, 100mM KCl, 20 % glycerol (v/v), 0.2 mM EDTA, 0.2 mM phenylmethylsulphonyl fluoide (PMSF), and 0.5 mM dithiothreitol (DTT)) in dialysis tubing with a molecular weight cut off of 25,000 (Pharmacia), snap frozen, and stored at -70°C (Dignam *et al.* 1983; Ausubel *et al.* 1995).

The DNA binding assays were performed using T4 polynucleotide kinase (United States Biochemicals, Cleveland, OH) and [γ-^{32}P] ATP (ICN, Inc.) end-labeled antisense synthetic oligonucleotides that were subsequently annealed to their sense strand. The binding reactions were performed in 20 mM HEPES (pH 7.9), 20 % glycerol (v/v), 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.25 mM DTT at 4°C. In addition, spermidine (1 mM), 0.5% (w/v) acetylated bovine serum albumin and 0.5 μg/μl poly (dI-dC)-Poly (dI-dC) were added to the reactions. The reaction buffer, unlabeled competitors and nuclear proteins (15 μg) were gently mixed and placed on ice for 5 min. Five fmoles of

labeled oligonucleotide (30,000 cpm) was added to the reaction and subsequently incubated at 35°C for 20 min. The reaction mixture was cooled on ice for 5 min and electrophoresed through a pre-run 5% nondenaturing polyacrylamide gel at 4°C for 2-3 hr. The gel was then dried and exposed to x-ray film. Oligonucleotides generated by Gibco BRL for the EMSA are: FP5(-246/-284), 5'-ACA GAG GAG CCT GGT TGC TAC AGT CCA TAG GCT CTC AAA G-3'; FP5(-246/-266)F, 5'-ACA GTC CAT AGG CTC TCA AAG-3'; FP5(-253/-270), 5'-TGC TAC AGT CCA TAG GCT-3'; FP5(-267/-284)F, 5'-CAG AGG AGC CTG GTT GCT-3'; FP6(-327/-342), 5'-CCT GGA GGA GGG CAT G-3'; FP6(-321/-341)F, 5'-CTG GAG GAG GGC ATG GCA ACC-3'; Sp1, 5'-ATT CGA TCG GGG CGG GGC GAG C-3'; CYP19I.1(Hex)F, 5'-ATT CCA GAG GAG GTC ATG C-3'; hPL(Sp1), TGT GTG GGA GGA GCT TCC AAA-3'; and o99, 5'-AAGAGAGAAGAATGCGGTA-3' (sense strands represented). Single bp transversions bp across the FP5(-246/-267) between –249 and –260 bp, and two bp transversions across FP6(-321/-341) at –338/9 (A) to –322/3 (I) were created in synthetic oligonucleotide for EMSA competition studies. EMSA were exposed to a phosphorimager screen and analysis was performed with ImageQuant (Molecular Dynamics, Sunnyvale CA).

Southwestern Analysis

An SDS-PAGE gel (10%) with 100 μg of nuclear extract was transferred to 0.22 μm nitrocellulose membrane (Micron Separation, Inc., Westborough, MA). The proteins were renatured in 10mM HEPES (pH 7.9), 50 mM KCl, 10% glycerol, 0.1% Nonidet P-40, 0.5 mM DTT, and 0.5 mM spermidine for 16 hr at 4°C with a buffer change after the

first hr. Nonspecific binding was blocked for 3 hr at 4°C, with 10 mM HEPES (pH 7.9), 50 mM KCl, 10% glycerol, 0.1% Nonidet P-40, 0.5 mM DTT, 1 mM spermidine, 5 μg/ml sonicated herring sperm DNA, 25 μg/ml yeast tRNA, 100 μg/ml BSA and 5% non-fat dry milk. The binding reaction contained 10 mM HEPES (pH 7.9), 50 mM KCl, 10% glycerol, 0.1% Nonidet P-40, 0.5 mM DTT, 1 mM spermidine, 5 μg/ml sonicated Herring Sperm DNA, 2.5 μg/ml yeast tRNA, 10 μg/ml BSA, 0.5% non-fat dry milk and $1X10^6$ cpm/ml radiolabeled double stranded oligonucleotide. The double stranded oligonucleotides were generated by annealing the sense and antisense synthetic oligonucleotide concatamers (Gibco BRL) of the protected regions which are: FP5(3X)F, 5'-CGA GCC TGG TTG CTA CAG TCC ATA GGC TCT CAG AGC CTG GTT GCT ACA GTC CAT AGG CTC TCA GAG CCT GGT TGC TAC AGT CCA TAG GCT CTC ATG TAC-3', FP6(-328/-339)3X, 5'-CTG GAG GAG GGC ATG GAG GAG GGC ATG GAG GAG GGC AGG TAC-3', and FP6(3X)F, 5'-AAG ACC CCT GGA GGA GGG CAT GGC AAC CAG ACC CCT GGA GGA GGG CAT GGC AAC CAG ACC CCT GGA GGA GGG CAT GGC AAC CGG TAC-3' (sense strand represented). Binding reactions were carried out at 4°C for 4 hr. Following the binding reaction, the membranes were washed in ice cold 20 mM HEPES (pH 7.9), 100 mM KCl, 10% glycerol, 0.5 mM DTT and 1 mM spermidine three times for 5-7 min and exposed to x-ray film.

Construction and Transfection of Concatamers

The concatamers generated for Southwestern analysis were also designed for ligation into the pGL3 vector by creating a Kpn I restriction endonuclease 3' overhang end on the

termini of the double stranded products. The sense and antisense strands of FP5 or FP6 concatamers were phosphorylated with T4 polynucleotide kinase in 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 5% polyethyleneglycol-8000 and annealed. The pGL3 Basic, 124pGL3 (oPL gene –124 to +16), and PRLpGL3 (rat prolactin promoter -33 to +13) vectors were digested with Kpn I and dephosphorylated with shrimp alkaline phosphotase (United States Biochemicals). FP5 or FP6 concatamers were ligated into each of the vectors with T4 DNA Ligase (Gibco BRL), transformed into DH5α *E. coli* and screened for orientation. Orientation of the positive clones was confirmed by nucleotide sequencing. Plasmids were purified and transiently transfected as previously described into Rcho-1, BeWo, Hela and C127 cells.

Results

Mutational Analysis of DNase I Protected Regions

Interaction of nuclear protein from ovine binucleate cells (oBNC) with the 5'-flanking sequence of the oPL gene was demonstrated by DNase I protection assays in a region that stimulates maximal expression in choriocarcinoma cell lines (Liang *et al.* 1999). To analyze the functionality of these regions in trophoblast cells, mutations across the protected regions were created. Not I restriction endonuclease sites were created in the -380pGL3 vector (-380 to +16 bp) at specific areas within FP5 and FP6 (Figure 27). These vectors were transiently transfected into human (BeWo) and rat (Rcho-1) choriocarcinoma cell lines that produce hPL and rPL-I / rPL-II, respectively. A mutation in any portion of FP5 significantly ($P<0.05$) reduced activity of the oPL promoter by 48% to 61% in BeWo cells (Figure 28A). Furthermore, mutations in FP6 decreased

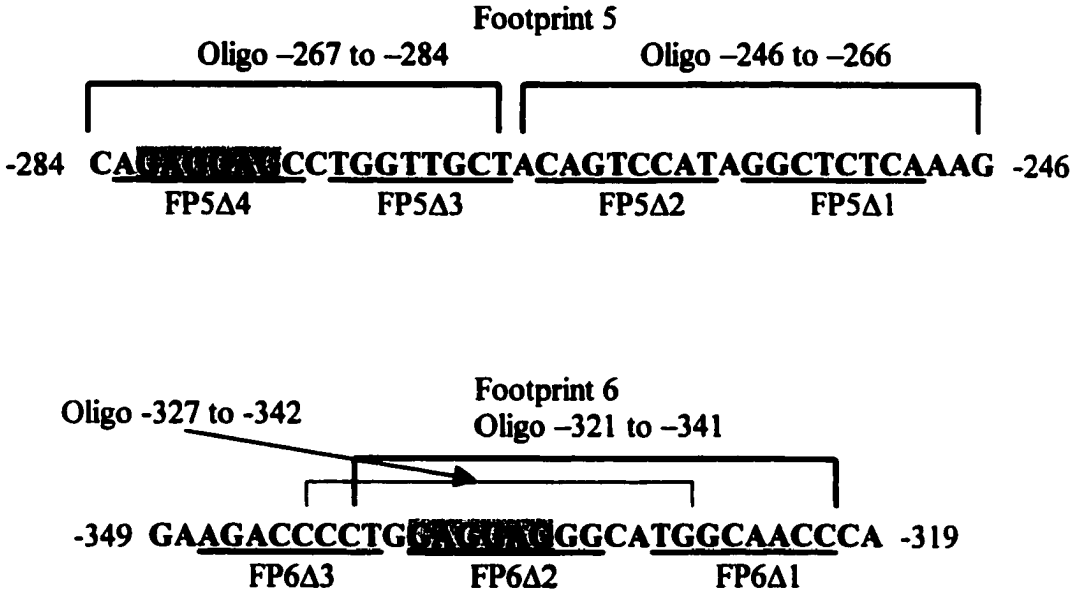
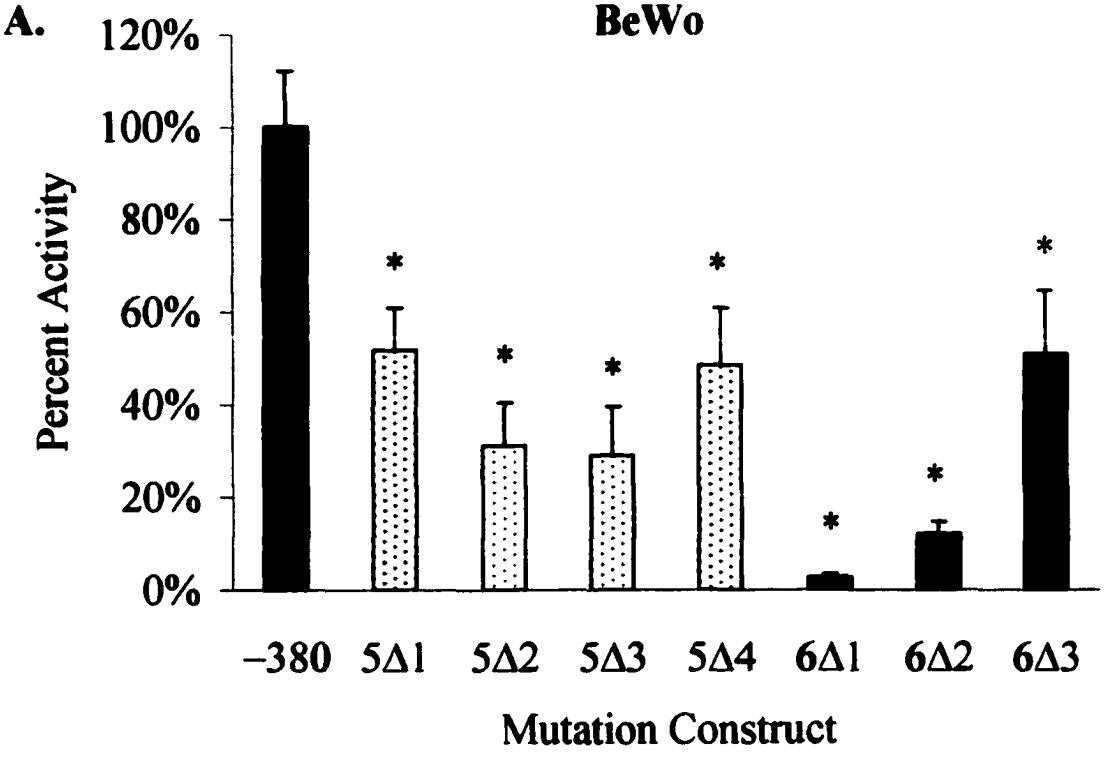
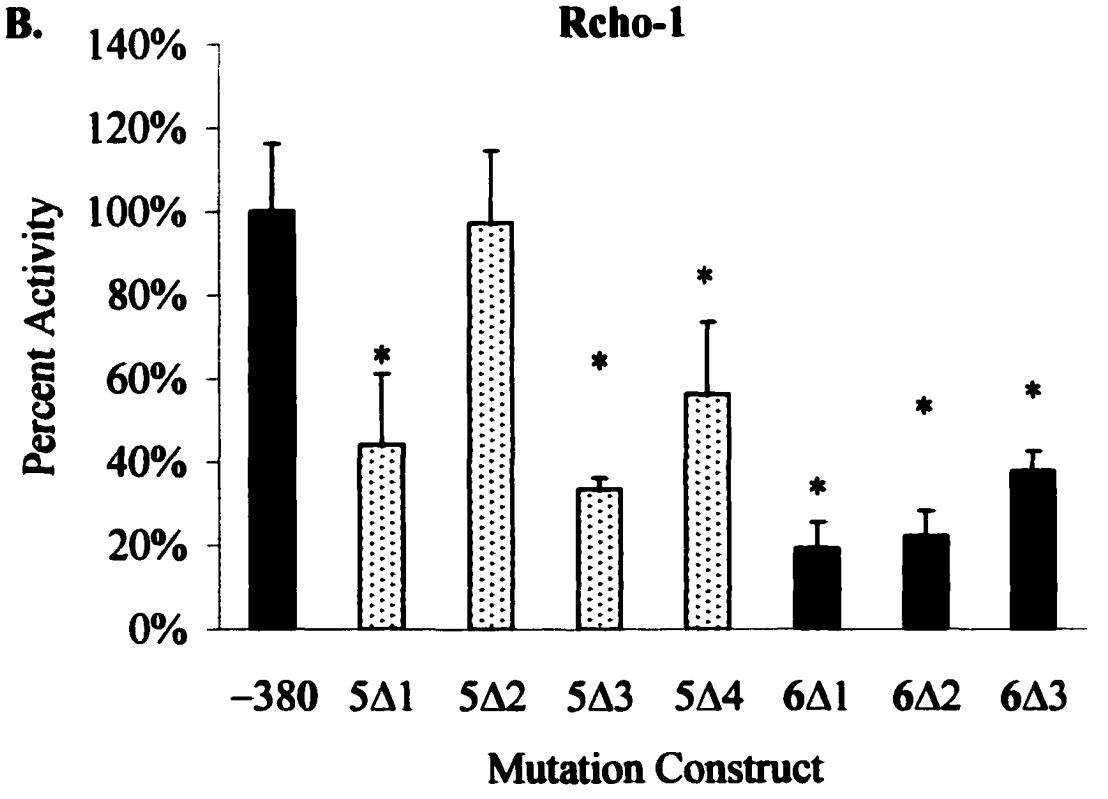


Figure 27. Footprint 5 and 6 sequence, oligonucleotides, and mutations. The sequence of DNase I protection sites 5 and 6 are presented in this figure. Footprint 5 encompasses the 5'-flanking sequence of the oPL gene between –246 bp to –284 bp. Four Not I restriction endonuclease site mutations generated for transient transfection analysis are underlined and labeled below the sequence. Footprint 6 was the region protected from -319 bp to -349 bp, and 3 mutations were created with Not I sites. In addition bars above the sequences indicate the synthetic oligonucleotides encompassing these footprints, which are FP5(-246/-266), FP5(-267/-284), FP6(-327/-342) and FP6(-321/-341).

Figure 28. Mutational analysis of Footprint 5 and 6. Transient transfection analysis of the mutations disrupting the DNase I protected regions (FP5 and FP6) for human (A) and rat (B) choriocarcinoma cell lines are shown. On the ordinate axis the footprints and the mutations are labeled and the y-axis shows the percent activity. The bars represent the mean percent activity±SEM for each mutant –380pGL3 construct. An asterisk above the bar illustrates a significant decrease in activity ($P<0.05$) from the wild type (-380) vector, separated by a Dunnett's T test.





($P<0.05$) the transactivation of the promoter by 97%, 88% and 49% for FP6Δ1, FP6Δ2 and FP6Δ3, respectively (Figure 28A). In Rcho-1 cells, FP5 mutations suggest that two regions (-249/-256 bp; 5Δ1 and -268/-283 bp; 5Δ3 and 5Δ4) are critical areas for transactivation of the oPL promoter, since reductions of 56%, 66% and 44% were observed with these mutations (Figure 28B). The FP5Δ2 –380pGL3 vector did not significantly differ in activity from that observed with the wild type. Additionally, an identical pattern of reduced activity as was seen in BeWo cells for FP6 mutations was demonstrated in Rcho-1 cells, with a reduction of 81%, 78% and 62% (Figure 28B). These data confirm that protected regions identified with oBNC nuclear proteins are critical areas for transactivation of the oPL promoter in heterologous trophoblast cells. Subsequent studies were designed to define the *cis*-acting element and analyze the nuclear protein interacting with the elements within FP5 and FP6.

Characterization of Footprint 5

The protein-DNA interaction within footprint 5 (FP5) was studied *in vitro* by electrophoretic mobility shift assays (EMSA). A synthetic oligonucleotide encompassing the entire region of FP5 (-246/-284 bp; Figure 27) was found to specifically interact with proteins in oBNC nuclear extracts to form two complexes (Figure 29). Unlabeled oligonucleotides spanning portions of FP5 were used as competitors in EMSA binding reactions to identify the sequence interacting with the oBNC nuclear proteins. The region between -246/-266 bp within FP5 was able to inhibit the protein-DNA complex formation similar to the FP5(-246/-284), whereas FP5(-253/-270) or FP5(-267/-284) were unable to compete equally (Figure 29). Furthermore, a region of FP6 (-327/-342) containing the

Figure 29. Footprint 5 EMSA. An EMSA with radiolabeled oligonucleotide encompassing the entire region of Footprint 5 (FP5(-246/-286)) is shown. The first lane contains no nuclear extracts (F) and subsequent lanes to the left all contain 20 μg of oBNC nuclear extracts. The competitors added to the binding reaction are labeled above each lane, and are: no competitor (No) or 250-fold molar excess of FP5(-246/-284), FP5(-246/-266), FP5(-253/-270), FP5(-267/-284), and FP6(-327/-342).

GAGGAG site was also used as a heterologous unlabeled competitor, but no competition was observed with this oligonucleotide in EMSA (Figure 29). Therefore, the initial hypothesis that transactivation through a direct repeat (DR-1; GAGGAG) sequence (Liang *et al.* 1999) was not substantiated by binding studies with an oligonucleotide encompassing FP5, and a sequence downstream of the FP5 DR-1 appears to be responsible for protein-DNA interaction.

To confirm the protein-DNA interaction observed with radiolabeled FP5(-246/-284), truncated portions of FP5 were radiolabeled and analyzed for protein-DNA interactions. EMSA studies with radiolabeled FP5(-253/-270) or FP5(-266/-284) oligonucleotides were unable to specifically interact with nuclear proteins from oBNC (data not shown). However, specific protein-DNA complexes with radiolabeled FP5(-246/-266) were identified by EMSA. Binding studies with radiolabeled FP5(-246/-266) revealed that competition with FP5(-246/-266) inhibited the formation of protein-DNA complexes, but complex formation was unaffected by a heterologous oligonucleotide (Figure 30). Deletion analysis of this region has suggested that transacting factors interacting within -383 to -217 bp stimulate expression in a trophoblast specific fashion. Therefore, binding studies were performed with trophoblast nuclear extracts from oBNC, BeWo cells and Rcho-1 cells and with a nontrophoblast cell nuclear extract from Hela cells (Figure 30). These binding reactions confirmed that the radiolabeled FP5(-246/-266) oligonucleotide specifically interacts with a nuclear protein from trophoblast and nontrophoblast cells (Figure 30). However, several interactions have been identified by variation in mobility of complexes, which may indicate multiple transacting factors interacting with this oligonucleotide, or the formation of a multimeric complex.

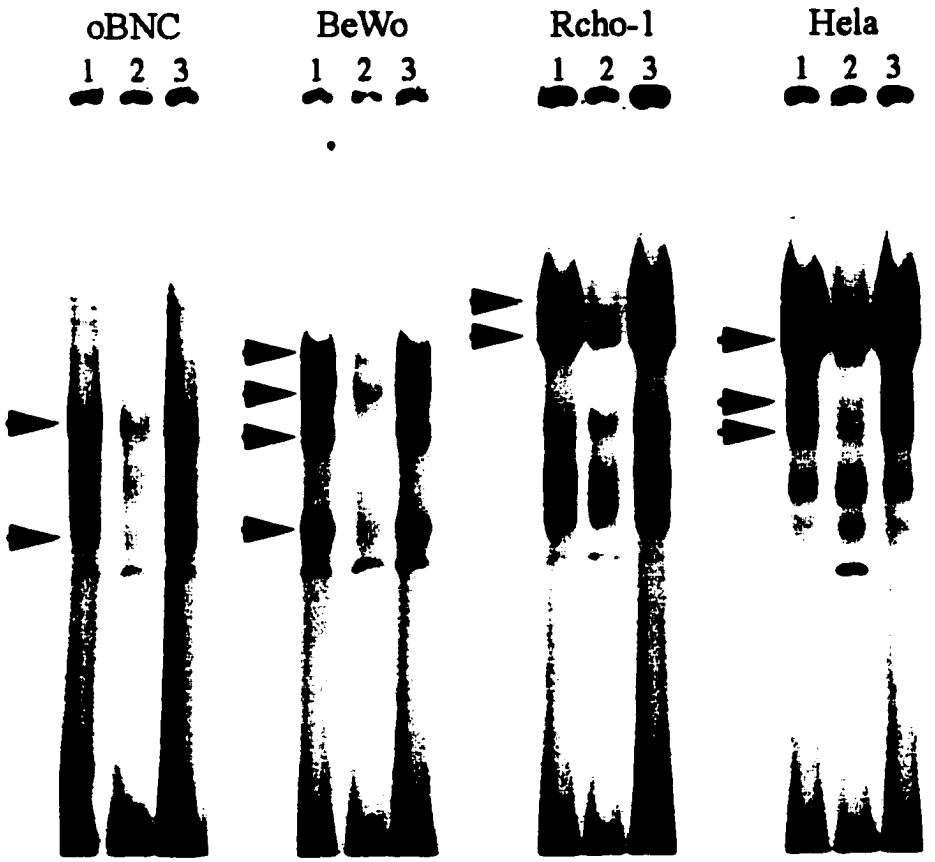
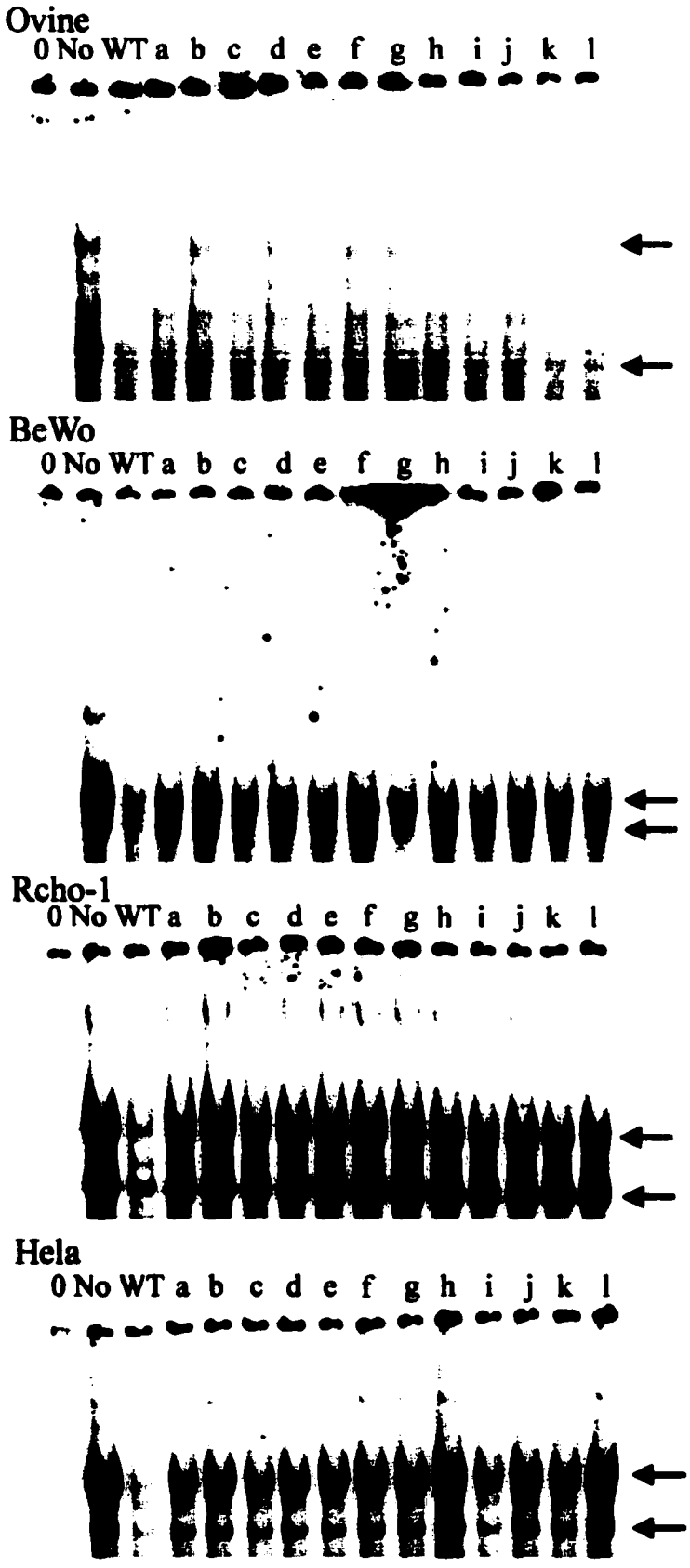


Figure 30. EMSA of FP5 between –246 bp to –266 bp with trophoblast and non-trophoblast nuclear extracts. Binding studies using radiolabeled FP5(-246 to -266) in the presence of ovine binucleate cell (oBNC), BeWo cell, Rcho-1 cell or Hela cell nuclear extracts (20 μg) are presented. Specific protein-DNA complexes were identified for each nuclear extract (arrows) by adding no, homologous or heterologous unlabeled competitor oligonucleotides to the binding reactions. The first lane (1) of each nuclear extract tested contains no unlabeled competitors. The second and third lanes have 200-fold molar excess of homologous, FP5(-246 to -266) or heterologous competitors (o99) added.

Competition EMSA studies with single base pair transversions mutating the FP5(-246/-266) oligonucleotide were performed to identify the binding site. The transversions spanned an area from –260 (a) to –249 (l), and 200 fold molar excess of each unlabeled mutant oligonucleotide was incubated in the binding reaction with radiolabeled FP5(-246/-266) and oBNC nuclear extract (Figure 31). The ability of the complex to form with the addition of the mutant unlabeled oligonucleotides was assessed and the percent binding was determined (Figure 32). These data indicate that the protein-DNA interaction occurs at -260 CATAGGCT –253 for the faster migrating complex labeled in figure 31. Important base pairs for the faster migrating band on the EMSA were also crucial for the slower migrating complex in EMSA (Figure 31), which identified –259 ANAGGC –254, indicating that the nuclear factor(s) interacts with identical nucleotides to form both complexes. Therefore, alteration in complex mobility suggests: 1) a reduction in mobility is the result of different nuclear proteins interacting at this *cis*-acting element; 2) multimeric complexes are formed at this element to reduce mobility; or 3) mobility is increased by degradation of the nuclear factor. Similar results were obtained in competition EMSA experiments with BeWo, Rcho-1, and Hela cell nuclear extracts, which identified an identical *cis*-acting element (Figure 31). Database searches with the defined element or the entire FP5 did not reveal any previously defined *cis*-acting elements.

Southwestern analysis was used to elucidate information on the transacting factors involved in the protein-DNA interaction within FP5. Nuclear proteins from Hela, BeWo and Rcho-1 cells were separated on a 10% SDS-polyacrylamide reducing (5% β-mercaptoethanol) gel and a radiolabeled triplicate concatamer of FP5 (FP5(3X)) was used

Figure 31. Competition EMSA with mutant FP5(-246/-266) oligonucleotides. Specific nucleotide interactions with the nuclear factors analyzed by the ability of single bp transversions created across FP5(-246 to -266), at –260 bp (a) to –249 bp (l), and analyzed with a phosphorimager to determine the mutant oligonucleotides ability to inhibit complex formation. The data presented above is a representative EMSA for the oBNC, BeWo, Rcho-1 and Hela nuclear extracts. The arrows indicate the specific interactions for each of cell type.



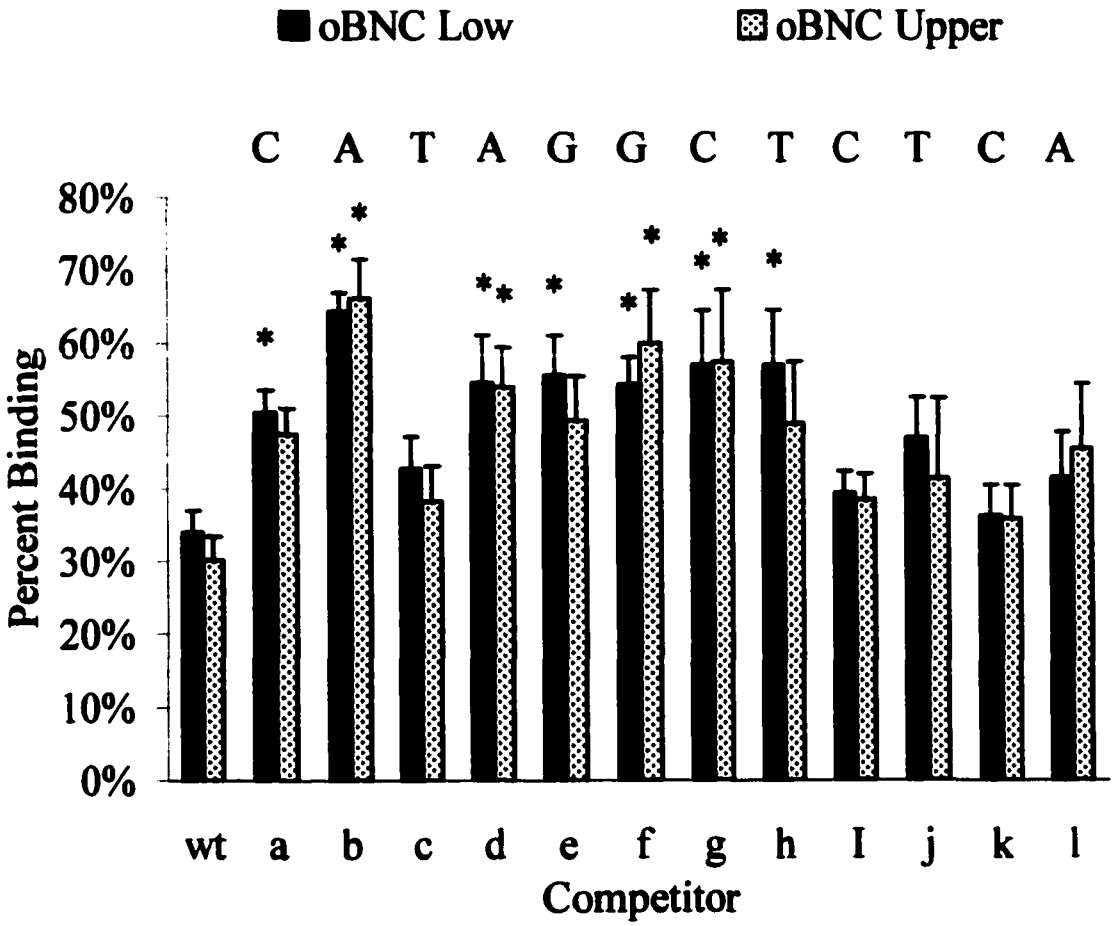


Figure 32. Graphic summary of the EMSA competition analysis of FP5. A summary of four EMSA competition experiments identifies the *cis*-acting element in FP5 with oBNC nuclear extracts. The bars represent the mean±SEM binding (y-axis) of the competitor lanes (x-axis), divided by the lane with no competitor, for both the upper and lower bands indicated in Figure 31. The means statistically different from the homologous (wt) competitor are indicated with an asterisk. Additionally, the base pairs that were altered with the transversion are depicted above the bars.

to probe the separated proteins. Specific protein-DNA interactions were identified for each nuclear extract by comparion to an adjacent lane with 500-fold molar excess of unlabeled FP5(3X) (Figure 33). Three interactions were identified in Hela cell nuclear extracts and the nuclear proteins had apparent M_r of ≅43,000, ≅38,000 and ≅30,000 (Figure 33). BeWo cells specifically interacted with 3 proteins as well, with apparent M_r of ≅45,000, 39,000 and ≅27,000 (Figure 33). However, only 2 proteins were identified in Rcho-1 cell nuclear extracts with apparent M_r of ≅42,000 and 25,000 (Figure 33). The nuclear proteins identified with human cell nuclear extracts (BeWo and Hela) share a similar pattern and may be homologs to one another or degradation products of the largest protein. Only two proteins were identified in the rat choriocarcinoma cell lines, but the M_r of these proteins are similar to the larger and small nuclear proteins in the human nuclear extracts. Therefore, these nuclear proteins may be similar to each other, but possess different posttranslational processing, or are variant forms of a transactivator superfamily. The identification of multiple nuclear proteins in these nuclear extracts may explain the mobilities of the protein-DNA interactions identified in the EMSA study, and suggest that the nuclear proteins identified interact with DNA as homodimers or heterodimers.

Characterization of Footprint 6

Footprint 6 (FP6) located within the enhancer region of the oPL gene 5'-flanking sequence was found to be functionally active in heterologous trophoblast cell lines (Liang *et al.* 1999). Initial binding studies revealed that an oligonucleotide encompassing the GAGGAG sequence, FP6(-327/-342), was able to interact with a nuclear protein from

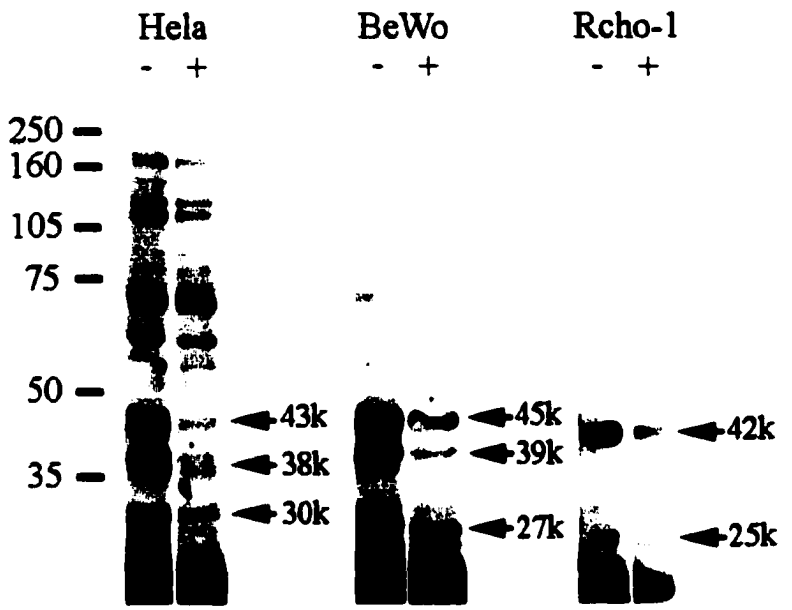


Figure 33. Southwestern analysis of FP5. Southwestern analysis was used to identify the molecular weights of nuclear proteins within the various cell lines that interacted with the *cis*-acting element in Footprint 5. A triplicate concatamer of FP5(-246/-284) was used to probe the membrane of proteins that were previously separated by 10% SDS-PAGE. Molecular weight standards are indicated on the left and the nuclear extracts from Hela, BeWo and Rcho-1 are labeled above their respective lanes. The addition of 500-fold molar excess unlabeled homologous competitor is indicated by the plus symbol above the lanes. Arrows are used to indicate the specific interactions observed for each nuclear extract, and the apparent molecular weight for each protein is indicated to the right of the arrow.

oBNC (Figure 34). Subsequent EMSA analysis, using 2 bp transversion mutations in the FP6(-327/-342) oligonucleotide as unlabeled competitors, indicated that the *cis*-acting element interacting with the nuclear protein in oBNCs was –339 GGAGGAGGGC -330' (data not shown). However, further evaluation of FP6 using mutations spanning the entire protected region suggested additions sequence located downstream of this region is required for transactivation (Figure 28). Therefore, an oligonucleotide encompassing -321/-341 bp (FP6(-321/-341)) was generated that includes the sequence disrupted by FP6Δ1 and FP6Δ2 mutations (Figure 28).

Competition EMSA analysis with mutated FP6(-321 to –341) oligonucleotides was used to identify the *cis*-acting element. Two bp transversions across this oligonucleotide were created and used as competitors in binding reactions (Figure 35). A composite of 4 individual replicates of this experiment were used to analyze the mean percent binding for each mutant competitor (Figure 36). Specific nucleotide mutations that did not inhibit complex formation identified the *cis*-acting element as -338 GGAGGAGGGCATGGC AAC -322. The *cis*-acting element identified in FP6(-321/-341) overlapped the element previously identified in FP6(-327/-342). In EMSA, the formation of the protein-DNA complex with either radiolabeled oligonucleotide was inhibited by the addition of unlabeled FP6(-327/-342) or FP6(-321/-341), indicating that the nucleotides (- 336 AGGANNGC -330) are critical for the interaction. However, in light of the mutational analysis other nucleotides (-327 GGNNAC -322) may be required to interact with the transacting factor to allow it to stimulate transactivation.

The upstream portion of this site in FP6 was recognized as a possible non-conical Sp1 element, which was identified in the hPL promoter at -139 GTGGGAGGAGC –129

Figure 34. Footprint 6 EMSA with FP6(-327/-342). Electrophoretic mobility shift assays for radiolabeled FP6(-327/-342) indicate that the protein-DNA interaction was specific. The radiolabeled FP6(-327/-342) was incubated with 15 μg oBNC nuclear extract in the presence of no (-), 50-fold (+) or 100-fold (++) molar excess unlabeled homologous FP6(-327/-342) or heterologous (o580, oAP2, & o110) competitor. The arrow to the left indicates the specific protein-DNA complex formed.

Footprint 6 EMSA

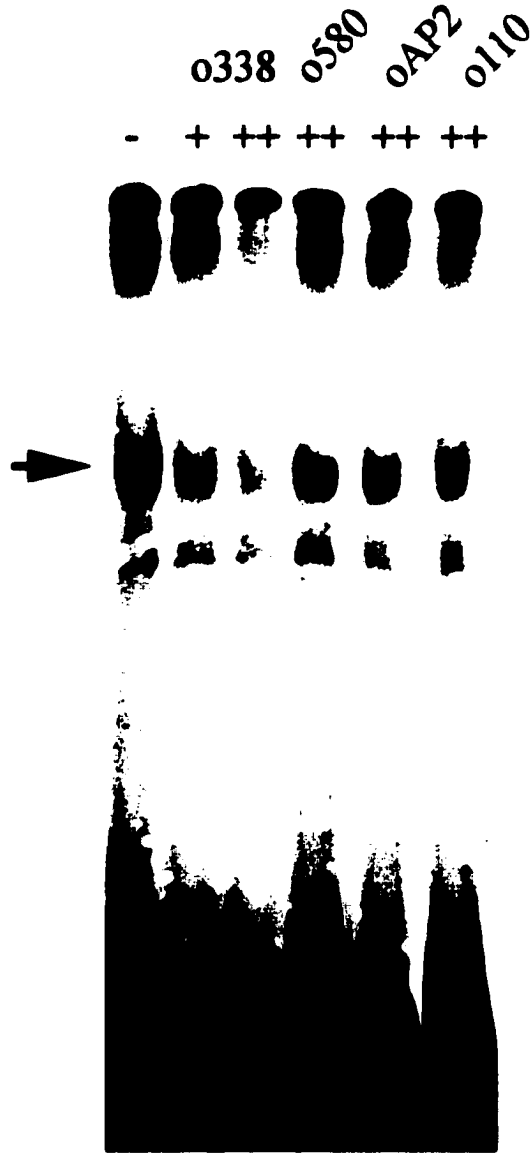


Figure 35. Footprint 6 EMSA with FP6(-321/-341). EMSA with radiolabeled FP6(-321/-341) was tested in the presence of mutant FP6(-321/-341) competitors. Two base pair transversions (A-I) were generated across the FP6(-321/–341) oligonucleotide to define the *cis*-acting element. The lane on the left (0) contains no oBNC nuclear extracts and the subsequent lanes have 15 μg of oBNC nuclear extracts. The unlabeled competitors (200-fold molar excess) are labeled above the lanes, and include no competitor, homologous (wt) and mutant competitors (A-I). Arrows to the right indicate the specific protein-DNA complexes that were formed.

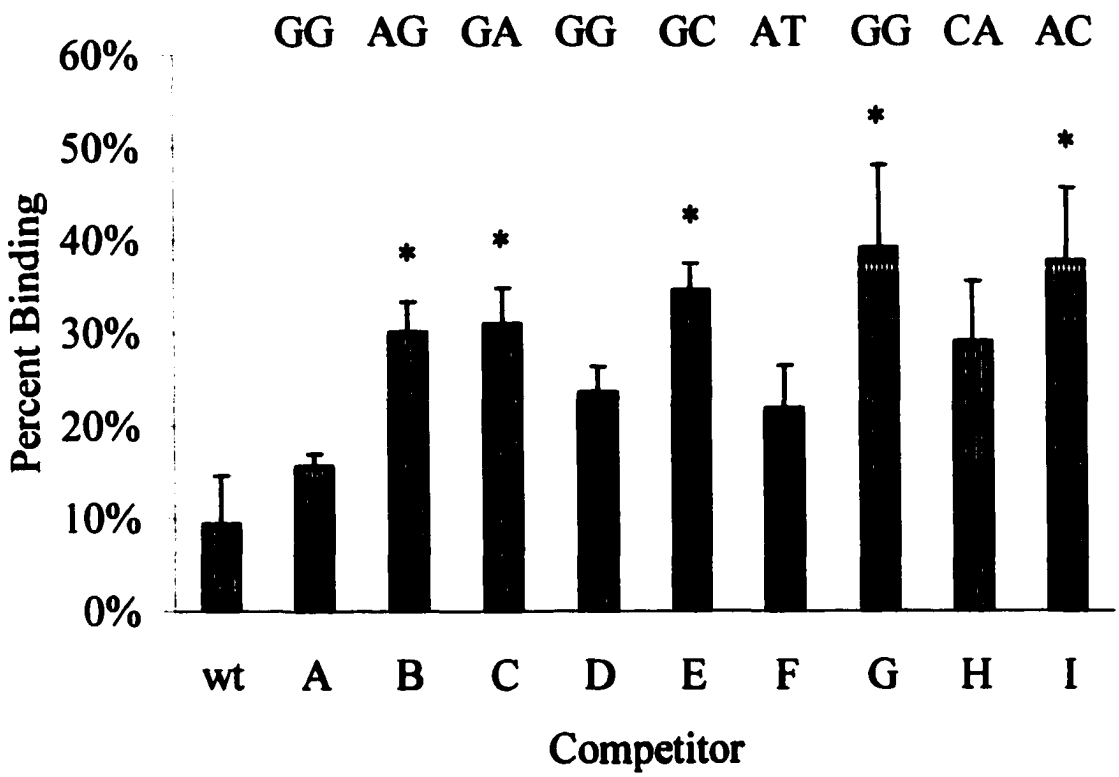
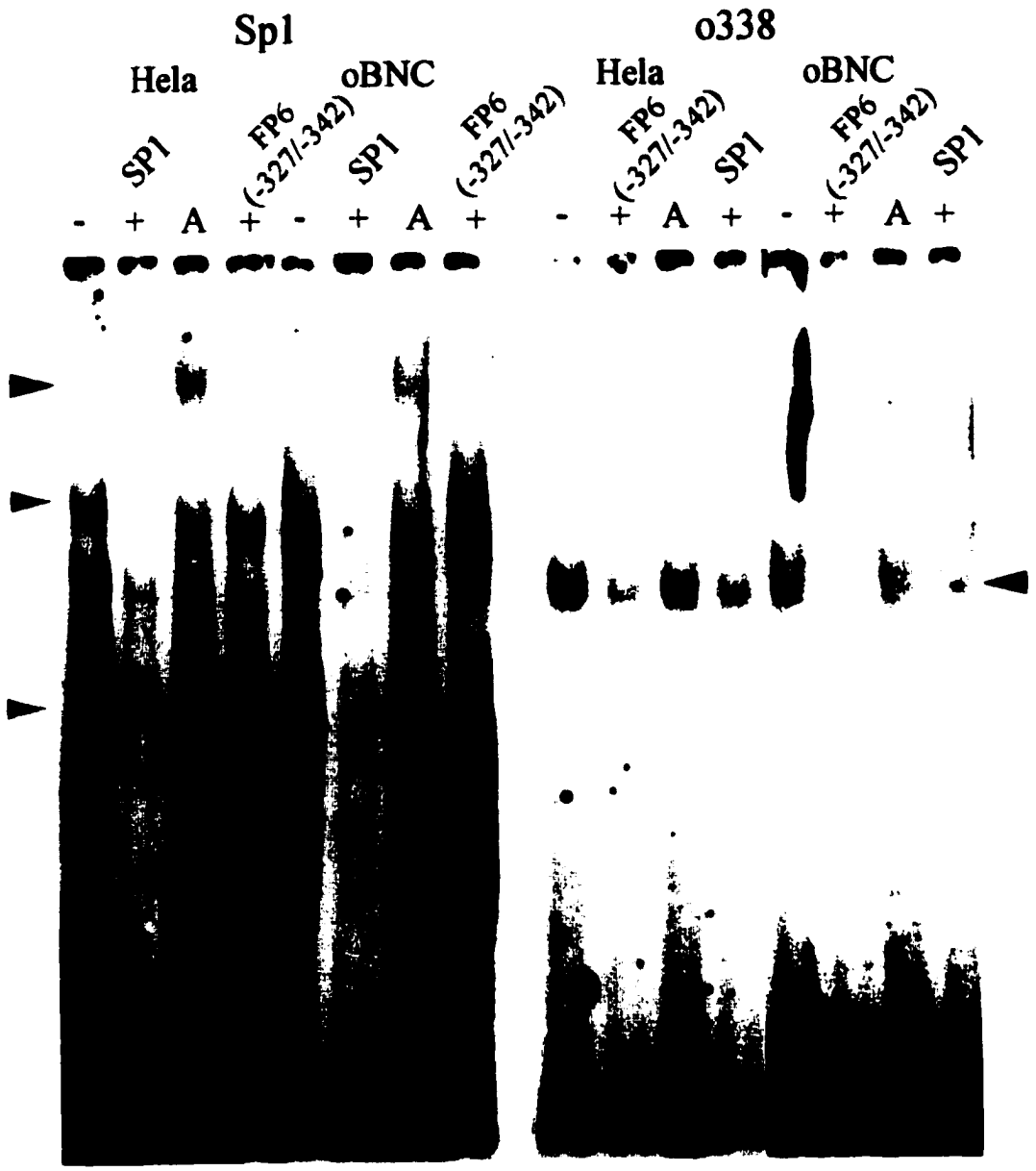


Figure 36. Identification of the *cis*-acting element in FP6. The specific nucleotide interactions in FP6(-321/–341) were identified with four replications of the EMSA shown in Figure 35. A phosphorimager was used to assess the intensities of the protein-DNA complex, and the percent binding with the addition of competitors was calculated. The bars for each competitor (x-axis) added represent the mean percent binding±SEM. The two base pairs mutated are indicated above the respective bar. A Dunnett's T test was used to separate the means from the wild type competitor mean percent binding, and a significance of $P<0.05$ (*) was accepted.

(Fitzpatrick *et al.* 1990). This report indicates that all of the guanosine residues in this sequence are responsible for protein-DNA interactions by methylation interference (Fitzpatrick *et al.* 1990). Due to the similarity of the hPL Sp1 site and the *cis*-acting element defined in the oPL gene, experiments were designed to identify if this element interacts with Sp1. A putative Sp1 element was generated and tested in Hela and oBNC nuclear extracts. Two specific protein-DNA complexes were identified in both cell types and the mobility of the slower migrating complex was further reduced with the addition of antiserum against human Sp1 (Figure 37). Studies on the rhesus monkey GH-variant gene promoter, which is identical to the hPL gene promoter at this site, indicated this element binds to Sp1 and Sp3 in JEG-3 choriocarcinoma cells (Schanke *et al.* 1998). Therefore, the faster migrating complex in the binding studies may be Sp3, because Sp1 antiserum only affected the complex with the slower migration. FP6(-327/-342) was used in the EMSA because the *cis*-acting element identified in this oligonucleotide overlapped with the Sp1 sequence identified in the primate promoters. Specific binding occurred in these reactions, but the Sp1 antiserum did not reduce the mobility of the protein-DNA complex in either oBNC or Hela cell nuclear extracts. However, the addition of the purine-rich oligonucleotide did affect complex formation, but the inhibition was not equivalent to that observed with the unlabeled homologous competitor. Furthermore, competition with the Sp1 element identified in the hPL promoter (hPL:Sp1) did not inhibit the formation of the protein-DNA interaction to FP6(-321/-341) (data not shown). Therefore, the interaction described in FP6 does not appear to be Sp1, and the formation of the protein-DNA complex does not seem to be trophoblast specific.

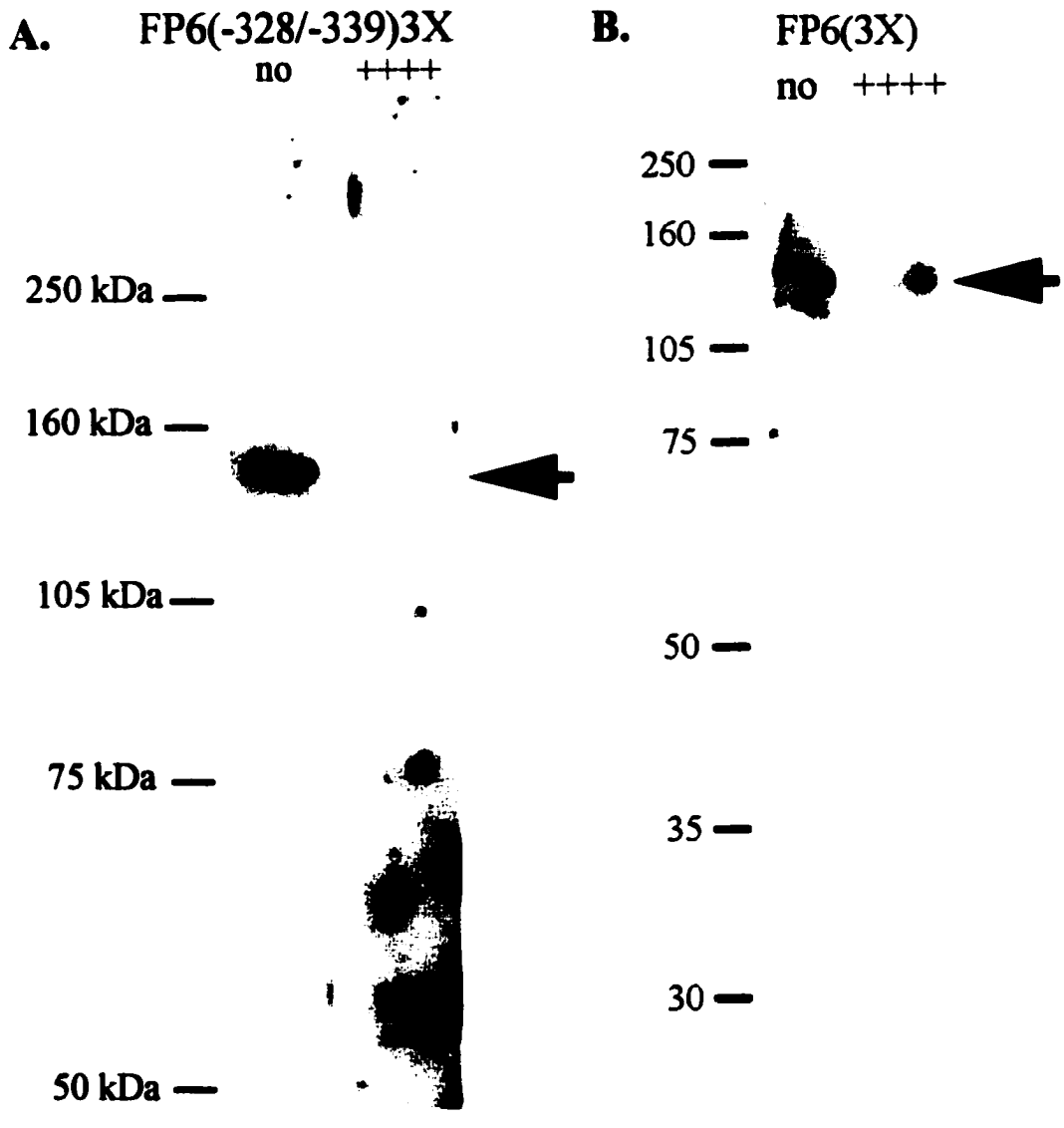
Figure 37. SP1 and FP6(-327/-342) binding to oBNC and Hela nuclear extracts. The eight lanes on the left represent an EMSA with a radiolabeled Sp1 oligonucleotide, and the lanes on the right have radiolabeled FP6(-327/-342). The first four lanes for each set contain Hela nuclear extract (15 μg) and subsequent lanes contain oBNC nuclear extract (15 μg). Binding specificity of the complexes were tested in the absence of unlabeled competitors (-) and compared to addition of 250-fold molar excess of homologous or heterologous competitor (+, as labeled), or 1 μg of Sp1 antiserum (A). Specific Sp1 protein-DNA interactions are indicated with grey arrows, and reduced mobility of the Sp1 complex in the presence of Sp1 antiserum is indicated with the black arrow. Radiolabeled FP6(-327/-342) formed a specific protein-DNA complex with both Hela and oBNC nuclear extracts, and is identified with the arrow on the right.



Another *cis*-acting element identified in the CYP19 gene, which harbors a GAGGAG sequence in the trophoblast-specific promoter region of exon I.1, was functionally active in human syncytiotrophoblast cells (Kamat *et al.* 1998). The CYP19 I.1 promoter initiates transcription of P450 aromatase in the human placenta. Two hexameric sequences (Hex sites) between -194 ATTTCCAGAGGAGGTCATGC -175 were functionally identified in this region, but all mutations performed to functionally define these elements disrupted the central region containing GAGGAG. Binding studies with an oligonucleotide encompassing the CYP19 I.1 Hex sites (CYP19I.1(Hex)) as the unlabeled competitor did not show specific competition with the protein-DNA interaction formed by FP6(-321/-341) (data not shown). Experimental analysis of the oPL *cis*-acting element in FP6 indicates that more sequence is needed downstream of the GAGGAG, since hPL and CYPI.1 elements do not share similarity to the sequence located 3' of the GAGGAG sequence.

The apparent molecular weight of the transacting factor interacting with FP6 was determined by Southwestern analysis. Ovine BNC nuclear extracts were separated on an SDS-polyacrylamide reducing gel (5% β-mercaptoethanol), transferred to a nitrocellulose membrane and renatured. Synthetic double stranded oligonucleotides containing three adjacent regions from –328 to –339 bp (FP6(-328/-339)3X) or a triplicate of the entire FP6 protected region from –321 bp to –348 bp (FP6(3X)) were generated to identify the nuclear protein interacting with FP6. The transacting factor specifically interacting at FP6 has an apparent Mr of ~140,000, when compared to an adjacent lane with excess homologous competitor (Figure 38). Furthermore, the apparent M_r of the nuclear proteins identified with either FP6 concatamer were indistinguishable from each other.

Figure 38. Southwestern analysis of FP6. Southwestern analysis was utilized to identify the molecular weight of the transacting factor interacting with a triplicate concatamer of FP6. The molecular weight standards are indicated on the left of each southwestern experiment. **Panel A** represent ovine binucleate cell nuclear proteins separated by 10% SDS-PAGE, transferred to nitrocellulose and hybridized with a triplicate FP6(-328/-339) concatamer. The addition of 250-fold molar excess of homologous competitor is indicated above the lanes (++++). **Panel B** is an identical southwestern analysis except the radiolabeled triplicate concatamer of the full-length FP6 was used to identify the protein of interest. The arrows to the right identify the specific interaction in the Southwestern analysis, which is a nuclear factor with the apparent $M_r \approx$ 140,000.



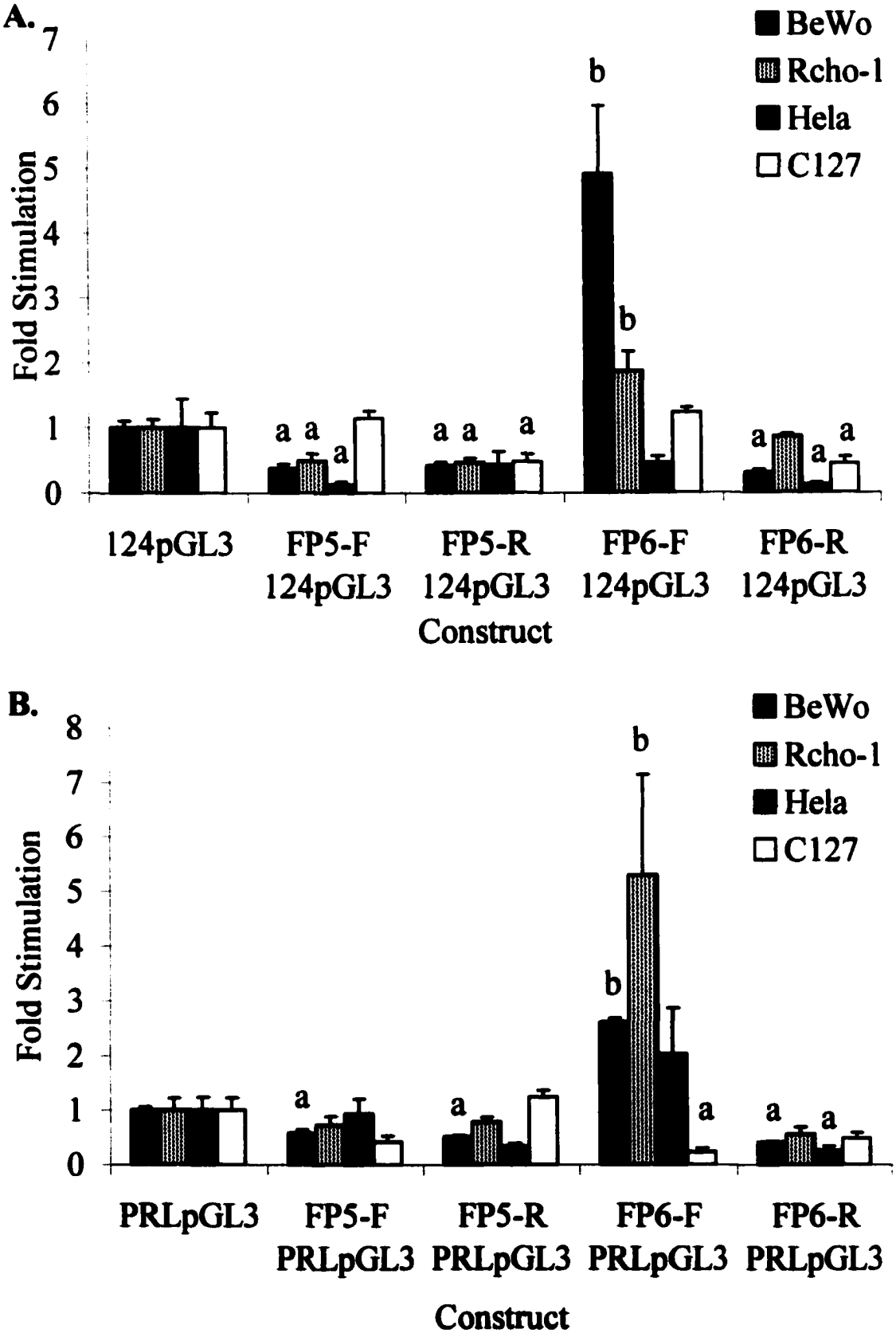
These data suggest that an identical nuclear protein in oBNC nuclear extracts interacts with the overlapping *cis*-acting element between –338 bp to –330 bp. These data provide further evidence that FP6 is not an Sp1 site, since Sp1 has a M_r of 97,400 (Kadonaga et al. 1988).

Transient Transfection Analysis of Concatamers

A trophoblast-specific enhancer region has been identified within a 138 bp region located 2 kb downstream of the hPL-3 gene (Rogers *et al.* 1986; Walker *et al.* 1990). Enhancer elements stimulate transcription independent of orientation, location and minimal promoter. Therefore, to classify the elements within the oPL gene, concatamers of FP5 or FP6 identical to those used in Southwestern analysis were ligated into the 124pGL3 (-124/+16 bp; homologous minimal promoter) and PRLpGL3 (rat prolactin gene, -33bp/+13 bp; heterologous promoter) vectors in either a forward or reverse orientation. These vectors were transiently transfected into two choriocarcinoma cell lines and two non-choriocarcinoma cell lines to determine the ability of these elements to stimulate transcription.

Transient transfections with the FP5 concatamer did not enhance activity in choriocarcinoma cell lines. In fact a significant reduction in transactivation was observed in BeWo cells with FP5 for both the oPL and PRL-minimal promoters (Figure 39). In Rcho-1 cells a reduction in activity was also found with FP5 in both orientations for the oPL minimal promoter, but no change in activity was observed with the PRLpGL3 promoter (Figure 39). Non-choriocarcinoma cell lines exhibited orientation-dependent reduction in transactivation with the oPL minimal promoter. The FP5 in a sense

Figure 39. Enhancer analysis of FP5 and FP6. Transient transfection data of concatamers of FP5 or FP6 in choriocarcinoma and non-choriocarcinoma cell lines are presented. The concatamers were constructed ahead of the oPL minimal promoter (-124pGL3; A) or the rat prolactin gene minimal promoter (PRLpGL3; B) in both orientations (F or R), and are labeled along the ordinate axis. The fold stimulation over the vectors with out enhancer elements is indicated on the y-axis. The bars represent the mean fold stimulation (n>3) ± SEM. Means were separated by a Tukey's multiple comparison test and statistical difference between construct within a cell line are indicated by letters above the respective bars. The "a" represent an inhabition with the synthetic promoter/enhancer vector, and "b" identifies an enhancment.



orientation reduced activity (P<0.05) in Hela cells, but not in C127 cells. The antisense orientation of the FP5 concatamer only reduced activity significantly in the C127 cell lines. Activation of the PRL minimal promoter in the non-choriocarcinoma cells was unaltered with either orientation of the FP5 concatamer. These data suggest that the FP5 element interacts with an orientation independent repressor in trophoblast cells. However, these data with the FP5 concatamer do not coincide with the previous data collected with the mutational analysis of FP5. The inhibitory action may be an artifact of the chimeric promoter constructed with the concatamer of FP5, and may indicate that FP5 is important for transactivation in a context-dependent manner.

On the other hand, a concatamer of the full length FP6 (FP6(3X)) in a sense orientation has a stimulatory effect on transactivation in choriocarcinoma cell lines with both the homologous and heterologous minimal promoters (Figure 39). However, no stimulation in non-choriocarcinoma cell lines was found with these vectors (FP6-F-124pGL3 and FP6-f PRLpGL3; Figure 39). In the reverse orientation FP6(3X) has an inhibitory effect in BeWo cells ($P<0.05$), but no significant reduction was identified in Rcho-1 cells with either minimal promoter vector. The reverse orientation of FP6(3X) in the oPL minimal promoter vector inhibited transactivation in both non-choriocarcinoma cell lines, Hela and C127, but only a significant reduction in Hela cells was demonstrated with the PRL minimal promoter. These data indicate that FP6 stimulates transcription in a trophoblast-specific, orientation dependent manner. Additionally, a triplicate concatamer of FP6 encompassing -328/-339 bp was unable to stimulate transactivation in BeWo cells with either the homologous or heterologous promoter sequences (data not shown). In fact, the data from these transient transfection

demonstrated a significant reduction in transactivation with out regards to orientation. Although these concatamers interacted with an identical transacting factor by Southwestern and EMSA analysis, the sequence identified 3' of the AGGAGGGC region appear to be critical for transactivation. These results may also explain the 97% reduction observed with the FP6Δ1, since mutating this region may create an inhibitory action with an identical transacting factor.

Discussion

The present study begins to critically evaluate the DNase I protected regions identified by oBNC nuclear extracts that are located within the enhancer region of the oPL 5'-flanking sequence (Liang *et al.* 1999). Initially, a direct repeat (DR-1) was demonstrated to be functional element within the oPL promoter in heterologous human and rat choriocarcinoma cell lines, but the present study indicates that additional sequence in both footprints is required to stimulate transactivation. Mutational analysis within FP5 and FP6 suggest that the entire protected region may contribute to enhancing oPL promoter expression, but specific elements were described in both protected regions. Identification of these *cis*-acting elements in FP5 and FP6 revealed novel elements that mediate expression of the oPL gene.

Footprint 5

The *cis*-acting element defined for FP5 was -260 CATAGGCTC -252 (Figure 32). The mutation in FP5Δ1 disrupts the GGCT of this sequence and reduces transactivation in both choriocarcinoma cell lines (Figure 28). However, a disruption of the 5' portion of this element (CAN) by FP5Δ2 significantly inhibited oPL transactivation only in BeWo

cells. Therefore, the CA at -260/-259 bp, which is located in FP5Δ2, may play a critical role in transactivation for BeWo cells, but may be less influential for Rcho-1 cell transactivation of the oPL promoter. The importance of these nucleotides in binding Rcho-1 cell nuclear factors was confirmed by EMSA competition studies with the transversion mutations. Therefore, only species differences between the human and rat choriocarcinoma cell lines in the mutational analysis were observed for the interaction of this protein at the 5' portion of the defined element. A purine to pyrimidine alteration at -259 (EMSA analysis) appears to have more influence on binding than an adenosine to guanosine mutation (mutational analysis) for Rcho-1 cells, whereas both of these mutations disrupt an interaction in BeWo cells. Southwestern analysis of nuclear proteins from these two choriocarcinoma cell lines indicated two specific-protein interactions, which may be homolog of one another, but identified a third protein in BeWo cell (Figure 33). The additional protein identified in the BeWo cell nuclear extracts may be interacting specifically with the CA sequence in BeWo cells, whereas the absence of this protein in Rcho-1 cells may allow the interaction of the other 2 factors to recognize a CA or CG element.

An identical AGGCTC sequence is located on the antisense strand of this footprint between -274 bp and -279 bp, in the proper 5' to 3' orientation seen between -257 and -253 bp, indicating that an inverted palindrome, -279 GAGCCT$(N)_{16}$AGGCTC -252, is located within FP5. Evidence for the functionality of this palindromic sequence is provided by disruptions at FP5Δ1 and FP5Δ3 or FP5Δ4 (Figure 27 and 28). However, EMSA competition studies with truncated portions of FP5 did not confirm an interaction with the 5' sequence of the inverted palindrome (Figure 29). The upstream region lacks

the CA identified as critical nucleotides in the binding of nuclear factors to the downstream region of the inverted palindrome. Therefore, binding to the upstream element may be initiated by downstream binding.

Additionally, data from the Southwestern analysis may implicate the formation of a hierarchy complex that is mediated at the downstream site of the inverted palindrome. Our findings suggest that at least two or three proteins from Rcho-1, BeWo and Hela specifically interact with FP5 (Figure 33). These nuclear factors may be splice variants for a single gene, which create proteins of different molecular weights, as described for AP-2 (Chapter IV). They may represent different family members for a group of transcription factors, or a superfamily of nuclear proteins that recognize an identical element. In either case the protein-DNA interactions formed at this site may compete for binding between the proteins identified or form a multimeric complex at the *cis*-acting element defined.

Although no previously defined element was identified by database searches, a similar sequence resides in the leptin gene. Deletion analysis of the leptin gene 5'-flanking sequence identified a trophoblast specific region within –1946/-1887 bp, and EMSA binding studies identified three protein-DNA interactions (Bi *et al.* 1997). The PLE1 element was identified by methylation interference analysis to be GTACCCTCAGGCT, which is similar to the element described in FP5 (Bi *et al.* 1997). Only a single bp difference is observed between the two *cis*-acting elements, an A at -259 in the oPL promoter versus a C in the leptin promoter. Although, this nucleotide was important for binding, some divergence was noted between human and rat choriocarcinoma cell lines. Bi *et al.* (1997) indicated that mutation of this element in the

leptin gene reduced activity 56%, but alone the element was unable to enhance transactivation in trophoblast cells. These data are similar to FP5 analysis where a mutation in the palindrome sequence reduces activity between 44-66% in either trophoblast cell line (Figure 28), yet a triplicate concatamer of FP5 was unable to stimulate transactivation of a minimal promoter (Figure 39). Moreover, the transacting factor interacting with this element was not tissue specific, and they identified a nuclear protein in Hela cells that interacted with PLE1 (Bi *et al.* 1997). Therefore, a similar factor may be mediating transactivation of the oPL gene and the placental expression of the leptin gene in a context dependent manner. Yamada *et al.* (1999) purified a protein that interacted with the CYP19 I.1 gene between –205 to –184 bp, which was a homolog of the *Drosophila* glial cell missing gene (GCM) (Akiyama *et al.* 1996). This transacting factor interacted with a region in the P450 aromatase gene as well as the PLE1 element of the leptin gene (Yamada *et al.* 2000). However, the protein-DNA interaction was predicted at the 5' end of the PLE1 site, since the consensus sequence for the GMC DNA-binding motif is (G/A)CCCGCAT (Akiyama *et al.* 1996). Therefore, a transacting factor with the GCM DNA-binding motif does not appear to be binding the oPL FP5 element, and the similarity with PLE1 in the leptin gene may indicate that an additional transacting factor interacts with the oPL FP5 element.

Footprint 5 appears to play a significant role in the transactivation of the oPL gene in trophoblast cells, since it was identified with oBNC nuclear extracts and shown to be functional by mutational analysis in choriocarcinoma cell lines. However, a triplicate concatamer of the entire FP5 sequence failed to stimulate transactivation of homologous or heterologous promoters. This type of analysis has been used to assess the function of

other *cis*-acting elements to determine if the element is orientation independent, location independent and promoter independent in its ability to stimulate transactivation. A 138 bp enhancer region of the hPL gene, which is located 2.2 kb downstream of the hPL-3 gene, possesses these qualities (Rogers *et al.* 1986; Lytras and Cattini 1994; Jacquemin *et al.* 1994; Jiang and Eberhardt 1994). The PLE3 region (-1887/-1892) in the leptin gene was also shown to synergistically enhance activity in a trophoblast specific fashion (Bi *et al.* 1997). However, a concatamer of FP5 was not able to augment transactivation, and suppression of the promoters tested was noted in some instances. These data indicate that the transacting factors interacting with the FP5 element are position dependent or context-dependent relative to other factors required. Fry and Farnham (1999) reviewed the five mechanisms by which a transcriptional activator can be context dependent. Three of mechanisms could be occurring with the factors interacting at the FP5 element. First, factors could induce DNA bending, which is observed with YY1, to narrow the proximity of enhancer elements (Fry and Farnham 2000). The factor could have specific protein-protein interactions with additional DNA-binding proteins or recruit non-DNA binding proteins, which enhance transcription (Fry and Farnham 2000). The third possible function for nuclear proteins acting in a context-dependent fashion could be to remodel chromatin to allow efficient transcription (Struhl 1999; Brown *et al.* 2000). Therefore, the lack of FP5 enhancer activity in the synthetic promoter, contrasts its importance in the full-length oPL promoter, indicating that it may possess one of these three activities in the provision of context-dependent transactivation.

Footprint 6

Mutational analysis of FP6, within the –380 to +16 bp promoter region of the oPL gene, indicated the presence of a functional protein-DNA interaction that stimulated transcription in a heterologous system. Binding studies were performed and overlapping nucleotides in both FP6(-327/-342) and FP6(-321/-341) were identified, which included DR-1 (Figure 34 and 35). Although different nucleotides were critical for the protein-DNA interaction, concatamers of both elements interacted with a nuclear protein with the apparent M_r of 140,000 (Figure 38). Sequence homology for this site and other *cis*-acting elements that are important in placental promoters was reported (Liang *et al.* 1999). A non-conical Sp1 site (GGGAGG) was suggested to stimulate transcription of the hPL gene promoter, and interactions at the guanosine nucleotides were confirmed by methylation interference studies (Fitzpatrick *et al.* 1990). Similar GA or GC boxes for Sp1 binding have been described in other placental promoters; the rhesus monkey GH-V promoter (Schanke *et al.* 1998), human CYP19 I.1 (aromatase P450) promoter (Kamat *et al.* 1998), human CYP11A (cholesterol side chain cleavage P450) promoter (Guo *et al.* 1994; Moore *et al.* 1993), HSD11K gene (Agarwal and White 1997), leptin gene (Bi *et al.* 1997) and human luteinizing hormone receptor gene promoter (Klisch *et al.* 1999). EMSA and supershift studies do not support Sp1 interactions with FP6, since competition with an oligonucleotide of the hPL gene Sp1 site or the addition of Sp1 antiserum do not significantly alter complex formation with human or oBNC nuclear proteins (Figure 37). Furthermore, the molecular mass of the transacting factor in oBNC (Figure 37) was not equivalent to the Sp1 factor purified from Hela cells (Briggs *et al.* 1986).

The CYP19 I.1 gene (P450 aromatase) promoter stimulates expression in human syncytiotrophoblast cells and an element that contains a GAGGAG was reported (Kamat *et al.* 1998; Sun *et al.* 1998). This element was recognized as an imperfect palindromic sequence (RRAGGTCA) and designated hexametric sequences (Hex), which were responsive for basal and retinoid-induced reporter activity of this gene (Sun *et al.* 1998). The Hex sites surround the GAGGAG sequence (Sun *et al.* 1998). Hex sites were suggested to interact with an Ac4BP/SF-1 protein, or another member of the nuclear receptor superfamily (Kamat *et al.* 1998). Mutational analysis of Hex also disrupted the central GAGGAG sequence, so competition studies did not rule out the functionality of GAGGAG. Therefore, EMSA with radiolabled oPL FP6(-321/-341) were performed, and no deleterious affects were observed on protein-DNA complex formation with an unlabeled oligonucleotide encompassing the Hex sites, indicating that a different DNA-binding proteins interacts with these two oligonucleotides. The binding data for these GAGGAG sequences, in the hPL and CYP19 I.1 genes, and oPL FP6 suggest that FP6 is interacting with a novel transacting factor to stimulate transcription in trophoblast cells, but additional nucleotides are responsible for the protein-DNA interaction.

In addition to the DNA-binding analysis, functional data is provided that indicates the region of FP6 downstream of the GAGGAG element, is critical for transactivation. The FP6Δ2 mutation, which disrupts GAGGAG (Figure 27), was found to significantly reduce the transactivation of the oPL promoter in heterologous choriocarcinoma cell lines. The FP6Δ1 mutation was found to reduce transactivation more than FP6Δ2, suggesting an additional factor interacting with this region. However, the same nuclear protein was shown to interact (Figure 38) with different overlapping nucleotides within

this region (Figure 36). Furthermore, the results from the FP6 concatamer transfection analysis reveal a paradoxical effect between FP6(-328/-339)X3 and FP6(3X). In BeWo cells, FP6(-328/-339)3X in the sense orientation significantly inhibited the transactivation of homologous and heterologous minimal promoters, whereas the full-length FP6 concatamer enhanced activity of the two minimal promoters (Figure 39). Combined these data suggest that the nuclear factor interacting at FP6 requires nucleotides in FP6Δ1 for enhancer function, but it interacts with FP6Δ2.

The full-length FP6 concatamer was found to contain a trophoblast-specific enhancer element that was stimulatory in a promoter independent/orientation dependent fashion (Figure 39). An orientation, position and promoter independent enhancer region has been identified downstream of the hPL/hGH gene locus (Rogers *et al.* 1986). Characterization of this region localized enhancer activity to a 138 bp region (Walker *et al.* 1990) and DNase I protection analysis revealed 2 specific protein-DNA interactions at +3928/+3967 (DF-3) and +3983/+4018 (DF-4) that were functional (Walker *et al.* 1990; Lytras and Cattini 1994; Jacquemin *et al.* 1994). Concatamers of these individual elements stimulated transcription synergistically in JEG-3 choriocarcinoma cells (Jacquemin *et al.* 1994). Transactional enhancing factor-5 (TEF-5) was reported to interact with TEA/ATTS elements located within the footprinted regions to stimulate transcription in JEG-3 choriocarcinoma cells (Jacquemin *et al.* 1996; Jacquemin *et al.* 1997). However, the full potential of the 138 bp enhancer region was not recapitulated with the individual elements, indicating that additional factors may be required (Lytras and Cattini 1994; Jacquemin *et al.* 1994; Jiang and Eberhardt 1995). Lytras and Cattini, (1994) identified an Ets element, AGGA at +3891, that interacted with a protein in JEG-3 cell nuclear

extracts and mutation of this element resulted in a 97.5% loss in enhancer activity. Ets elements have been identified in other placentally expressed genes, rat PL-II (Sun and Duckworth 1998) and interferon-tau (Ezashi *et al.* 1999) and transforming growth factor-β type II receptor (Chio *et al.* 1998). Additionally, a targeted disruption of Ets2 in the mouse genome resulted in trophoblast dysfunction, specifically with metalloproteinase-9 gene expression and ectoplacental cone proliferation (Yamamoto *et al.* 1998).

Ets family members have been shown to specifically interact with the major groove of GGA and form contacts with the minor groove of a 10 bp region surrounding this core sequence (Nye *et al.* 1992; Wasylyk *et al.* 1992; Li *et al.* 2000). The selectivity of Ets DNA-binding domain motifs for Ets family members was defined by the combined surrounding nucleotides and not just individual base pairs (Wasylyk *et al.* 1992; Wasylyk *et al.* 1993). Therefore, the element defined in FP6 may interact with a novel Ets protein, but the functionality of this interaction requires the base pairs located 3' of the core element. Interestingly, a putative Ets *cis*-acting element was located in a trophoblast-specific enhancer region of the rat PL-II gene (Sun and Duckworth 1998). In this report, by Sun and Duckworth (1998), the effectiveness of the enhancer region was stimulatory in both orientations, but greater stimulation was observed with the sense fragment. The data did not assess the stimulatory effects of the elements defined individually, and the presence of an AP-1 element may mar the effects of the Ets site on orientation dependence, since Ets proteins have been shown to interact with Jun and Fos (Wasylyk *et al.* 1993; Sun and Duckworth 1998). These data coincide with the element identified within FP6, since both oligonucleotides have a GGA element, but the nucleotides located 3' of the core element appear to be required for transactivation.

The data presented in this study further define the two DNase I protected regions, which are located in an area of the oPL gene responsible for transcriptional stimulation. Novel *cis*-acting elements were defined in both protected regions, and the apparent molecular weight of the proteins interacting with each element was identified. The data collected provide important groundwork for purifying the transacting factors responsible for mediating an effect on the oPL promoter in trophoblast cells. The functionality and enhancer capabilities of these elements were also evaluated. FP6 data suggest that the transacting factor interacting with this element functions as an orientation dependent, promoter independent trophoblast-specific enhancer.

Summary

Transcriptional regulation of the oPL gene has been localized to 383 bp of 5'-flanking sequence. The proximal 124 bp of the oPL gene promoter appears to stimulate transcription in a trophoblast specific fashion, but at a reduced state. An enhancer region between –383 bp and –217 bp was identified by transient transfections of deletion constructs in human (BeWo) and rat (Rcho-1) choriocarcinoma cell lines. Two DNase I protected regions were identified in this region with ovine binucleate cell nuclear extracts. Footprint 5 (FP5) encompasses the region between –246 bp to –284 bp and footprint 6 (FP6) spans nucleotides –319 to –349. Mutations were created across these footprints and they were transiently transfected into both choriocarcinoma cell lines and found to be functional. Specific protein-DNA interactions were demonstrated for both footprint regions in oBNC, human and rat choriocarcinoma cell lines and non-choriocarcinoma cell lines. These interactions were examined to define the *cis*-acting

element. The *cis*-acting element for footprint 5 was identified as -260 CANAGGCT -253, and shown to interact with homologous nuclear proteins with an apparent molecular weight of 42-45,000, 38-39,000 and 25-30,000. EMSA competition analysis with the region between –321 to –341 (FP6) identified a specific protein-DNA interaction with -336 AGGAggGCatGGcaAC -322. Southwestern analysis with oBNC nuclear extracts indicated that the transacting factor interacting with FP6 has a molecular weight of ~140,000. Additionally, enhancer properties were tested with homologous and heterologous promoters in choriocarcinoma and non-choriocarcinoma cell lines, and FP6 was demonstrated to stimulate trophoblast specific transactivation in a promoter independent, orientation dependent fashion. However, the in ability of FP5 to stimulate transactivation through a minimal promoter suggests that this element may require other factors located within the oPL gene promoter to be functional.

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Appendix A

DEVELOPMENT OF AN *IN VIVO* MODEL TO STUDY OVINE PLACENTAL LACTOGEN GENE TRANSCRIPTIONAL REGULATION

Introduction

One of the major responsibilities of the placenta is to provide a conduit for nutrients, and placenta endocrine activities increase the nutrient pool in the mother and facilitate uptake in fetal tissues (Talamantes and Ogren 1988; Handwerger 1991; Anthony *et al.* 1995; Anthony *et al.* 1998). Placental lactogen (PL), a member of the growth hormone (GH) / prolactin (PRL) gene family, has been implicated to modulate intermediary metabolism in both maternal and fetal tissues (Talamantes and Ogren 1988; Handwerger 1991; Anthony *et al.* 1995; Anthony *et al.* 1998). Fully differentiated trophoblast cells, located at the maternal-fetal junction secrete PL into both the maternal and fetal vasculature.

Significant interspecific divergence exists for PL gene evolution, biochemical characteristics and secretion profiles between human, rodent, and ruminant genes. Human (h) PL genes and other primate PL genes arose from a GH precursor gene, whereas nonprimate PL genes are derivatives of the PRL precursor gene (Walker *et al.*

1991; Wallis 1993). The ovine (o) PL gene shares 49% identity with PRL, 28% identity with GH and 67% identity with bovine (b) PL, which is strikingly lower than the interspecific divergence of ruminant GH or PRL (~99%), insinuating evolutionary pressures on ruminant PL genes (Colosi *et al.* 1989; Warren *et al.* 1990; Wallis 1993; Kappes *et al.* 1992). Ovine PL is secreted as a non-glycosylated 198 amino acid polypeptide, whereas bPL is secreted as a glycoprotein with both N- and O-linked oligosaccharide chains linked to a 200 amino acid polypeptide chain (Kessler and Schuler 1991; Anthony *et al.* 1995; Anthony *et al.* 1998). Secretion profiles into maternal circulation exhibits significant variation between oPL (~1000 ng/ml) and bPL (2-3 ng/ml) (Taylor *et al.* 1980; Beckers *et al.* 1982; Kappes *et al.* 1992). In contrast, the rodent temporally expresses two distinct PLs in trophoblast giant cells. PL-I is secreted as a glycosylated polypeptide and peaks at midgestation, whereas PL-II, a non-glycosylated protein, is not present until midgestation and plateaus prior to parturition (Soares and Talamantes 1982; Soares and Talamantes 1983; Colosi *et al.* 1986).

Although interspecific variations are prevalent, comparisons between human and ovine PL secretion profiles and biological activities reveal similarities (Handwerger 1991). *In vitro* and *in vivo* studies suggest that oPL and hPL have somatogenic action on fetal tissues (Handwerger 1991; Freemark and Handwerger 1986; Schoknecht *et al.* 1996). A natural deletion ablating the placentally expressed GH-variant and PL genes in the human GH/PL locus resulted in no detectable levels of these hormones in one case report (Rygaard *et al.* 1998). The fetus in this case had severe intrauterine growth restriction, but postnatal growth was normal (Rygaard *et al.* 1998), providing evidence for the role of hPL in fetal growth. However, there have been reports of hGH/hPL gene

deletions that result in normal pregnancy outcomes (Wurzel *et al.* 1983; Goossens *et al.* 1986; Simon *et al.* 1987), and these contrasting reports underscore the need to examine the physiology of pregnancy in individuals or an animal model lacking PL, before its necessity can be ascertained. Classical gene ablation experiments are not applicable, and other means of reducing hormone secretion will be required.

Transcriptional regulation for human and mouse PL genes defined the best, but considerable variation exists amongst the species. Furthermore, these species do not lend themselves readily to *in vivo* fetal physiology studies. Trophoblast-specific enhancement of hPL gene transcription requires a 138 bp enhancer located ≈2.2 kb 3' of the GH/PL locus (Rogers *et al.* 1986; Walker *et al.* 1991; Jiang and Eberhardt 1994). This region contains a GT-IIC site which is thought to interact with TEF-5, but multiple *cis*-acting elements are required for trophoblast-specific expression (Jacquemin *et al.* 1994; Jiang and Eberhardt 1994; Lytras and Cattini 1994; Jiang and Eberhardt 1995; Jacquemin *et al.* 1997). In addition to the enhancer region, two orientation-dependent pituitary repressors, PSF sequences, are found ≈2 kb upstream of the placentally expressed genes (Nachtigal *et al.* 1993). Therefore, tissue-specific enhancers and silencers are required for hPL gene regulation. In contrast, the mouse (m) PL-I gene retains trophoblast-specific transactivation with 274 bp of 5'-flanking sequence (Shida *et al.* 1993). Analysis of this region indicates the involvement of three GATA sites and two activator protein-1 (AP-1) elements (Shida *et al.* 1993; Ng *et al.* 1994). Disruption of the GATA-2 or 3 genes of mice significantly reduced expression of the mPL-I gene, thus confirming the role of GATA factors in regulation of mPL-I gene expression *in vivo* (Ma *et al.* 1997). Basic helix-loop-helix (bHLH) transcription factors, like Hand1, have been described to

enhance mPL-1 promoter transactivation and are thought to interact with an E-box (Cross *et al.* 1995; Scott *et al.* 2000).

Investigations on oPL gene transcriptional regulation are being performed to divulge important *cis*-acting elements and their transacting factors, which mediate oPL expression. Maximal trophoblast-specific expression in human and rat choriocarcinoma cell lines for the oPL gene resides within the proximal 383 bp of 5'-flanking sequence (Liang *et al.* 1999). Elements binding AP-2α, Purα, GATA-2, and a potential basic helix-loop-helix (bHLH) protein have been defined (Limesand 1997; Liang *et al.* 1999). Additionally, two novel elements within -319/-349 (FP6) and -246/-286 (FP5) possess enhancer *cis*-acting elements that are crucial for oPL expression *in vitro* (Limesand 1997; Liang *et al.* 1999). These data define functional *cis*-acting elements *in vitro,* and provide evidence for important protein-DNA interaction, but heterologous cell lines may not recapitulate trophoblast-specific expression in chorionic binucleate cells. Variation in oPL promoter activity within the human and rat choriocarcinoma cell lines has been observed, but both trophoblast cell lines exhibited maximal activity with the –383 bp of 5'-flanking sequence (Liang *et al.* 1999). However, ovine trophoblast cell lines do not exist, and chorionic binucleate cells are not able to be transfected by conventional methods. Therefore, transgenic models may be required to fully understand *in vivo* trophoblast-specific transcriptional regulation.

Transgenic mice have been used to characterize transcriptional regulation of human and rodent genes that are expressed in placenta. The mouse adenosine deaminase gene (ADA) is expressed in the chorio-allantoic placenta, and a 770 bp region approximately 5.4 kb 5' of the transcriptional start site modulated trophoblast-specific transactivation

identical to endogenous ADA gene expression (Shi *et al.* 1997). Mutational analysis of this region in transgenic mice has identified an AP-2 element, GATA elements and multiple binding sites for bHLH proteins, as well as two previously undefined elements (Shi *et al.* 1997; Shi and Kellems 1998). In addition, rat PL-II and mouse PL-II 5'-flanking sequences were characterized for trophoblast-specific expression in transgenic mice (Shida *et al.* 1992; Shah *et al.* 1998). Human chorionic gonadotropin (hCG) α- and β-subunit genes have been analyzed in transgenic mice and expression in extraembryonic tissues was observed (Bokar *et al.* 1989; Strauss *et al.* 1994; Strauss and Boime 2000). The human CYP19 gene (P450 aromatase) is expressed in syncytiotrophoblast cells by exon I.1, located ≈40 kb 5' of exon II, and 500 bp of proximal promoter was shown to stimulate transactivation in the labyrinthine zone of transgenic mouse placenta, which contains syncytiotrophoblast cells.

Transcriptional regulation of oPL was analyzed *in vivo* with transgenic mice, since the definitive characterization of the oPL promoter will require *in vivo* transactivation within chorionic binucleate cells. Furthermore, procedures to generate a transgenic ruminant model to study transcriptional regulation are outlined. The development of a ruminant transgenic model could potentially lead to the ability to create targeted disruption of this and other genes.

Materials And Methods

Production of Transgenic Mice

Deletion constructs of the oPL gene 5'-flanking sequence with 3662 bp or 800 bp were cloned into pGL2 Basic vector (Promega, Madison WI) by Liang *et al.* (1999).

Cesium chloride centrifugation gradient purified plasmids were digested with restriction endonucleases, Bpu1102I (-3465 bp), Bsa I (-1616 bp) or Sac I (-800 bp) at the 5' end of the oPL promoter and a Bam HI restriction endonuclease at the 3' end of the luciferase gene poly-A tail. The linearized products (3.5/luciferase; 1.6/luciferase; and 0.8/luciferase) were purified from an agarose gel by electro-elution, and subjected to a phenol, 2 phenol:chloroform:isolamyl (25:24:1), and 2 chloroform:isolamyl extractions (Hogan *et al.* 1994; Ausubel *et al.* 1995). The DNA was ethanol precipitated twice and resuspended into 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Linear DNA concentration was estimated on an agarose gel with a pGEM DNA molecular weight ladder at known concentrations and diluted to 2000 copies / pl prior to pronuclear injection.

Mouse embryos were obtained from superovulated B6D2 females (F1). Donor females were given 7.5 U PMSG intraperitoneally (i.p.) at 1 pm, 50 hours later they received 7.5 U hCG i.p., mated to a fertile male and recipient females were mated to vasectomized males concurrently. Bred females were examined for vaginal plugs after 16 hr, and donor females were euthanized (Hogan *et al.* 1994). Embryos (20-60/ mouse) recovered from their oviducts had their cumulus cells removed with 300 μg/ml hyaluronidase in M16 media (Sigma, St. Louis, MO) supplemented with 1% fetal bovine serum (FBS; Gemini Bio-Products, Inc., Calabases, CA). The embryos were washed 3 X in M16/1% FBS and cultured in drops under mineral oil until micromanipulation. Linear DNA was injected into one pronuleus of each zygote, and 20-30 zygotes were surgically transferred into oviducts of recipient females (Hogan *et al.* 1994). Pregnancies were

carried to term, and after the offspring reached 2 weeks of age, transgenic founders were verified.

Tails were clipped and placed in TB buffer (50 mM Tris-HCl/pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS and 600 μg/ml proteinase K), incubated at 55°C for 16 hr and extracted with phenol:chloroform:isoamyl. Genomic DNA was ethanol precipitated and resuspended in TE buffer. Slot blot and Southern hybridizations were performed to identify mice carrying the reporter insert (luciferase gene). Founders were mated to non-transgenic mice, and Mendalian inheritance rates in the offspring were observed with all transgenic mice obtained by pronuclear injection. Subsequent, breeding regimes produced homozygous and/or heterozygous fetuses to analyze. Reporter activity was analyzed in tissue lysates in 25 mM Gly-Gly, 1mM DTT and 0.1% triton X100 with the Luciferase Assay System (Promega) and a Turner TD-20e luminometer at day 10 and 16 post coitus. Light units (luciferase activity) for each sample were normalized by the concentration of protein (mg/ml) in the lysate. Proteins were precicpated with 100X the volume of saturated ammonium sulfate, resuspended in a 0.9% saline and quantified by a Bradford Assay (BioRad). Mean activity of the transgenic animal was statistically analyzed by Students T test to separate the means from nontransgenic animals.

Preparation of Stable Transfection Construct

Ovine PL 5'-flanking sequences were constructed 5' of the eukaryotically enhanced green fluorescent protein (EGFP) gene, derived from the bioluminescent jellyfish, *Aequorea victoria*. The vector, pEGFP-1 (Clontech Laboratories, Inc), contains a

neomycin resistant gene for G418 selection, enabling isolation of stablely transfected OFF cells with the promoter region of interest. Reporter construct were generated by ligating -4.5/+59 bp of oPL promoter, excised with EcoRI and PvuII restriction endonucleases from the lambda Zap II clone 2 phage (Liang *et al.* 1999; Liang 1995) into a EcoRI/Sma I restriction endonuclease digested pEGFP-1. Promoter regions of -1616/+11 bp and -380/+11 bp were amplified by polymerase chain reaction (PCR) with SWL-1.6Eco, 5'-GGA ATT CAG TTG AAG CCC AGA-3'; SWL-380Kpn, 5'-GAG GTA CCA GAC ACT GGT TTG-3'; and SWL+11Bam, 5'-TGG GGA TCC GTA ATG GGG AAT-3'. PCR reactions containing 0.1 μM of forward primer and reverse primer, 15 fmoles of the lambda Zap II EcoRI purified DNA insert, 0.1 μM of each dNTP, 2 mM $MgCl_2$ and 0.5 U *Taq*™ DNA polymerase (Gibco BRL) in a 1X reaction buffer with a final volume of 50 μL. The products were gel purified (DEAE membrane), digested with restriction endonucleases (Eco RI/Bam HI, -1.6 kb; or Kpn I/BamHI, -380 bp) and ligated into pEGFP-1. The insertion of the oPL 5'-flanking sequence was confirmed by dideoxy nucleotide sequencing. Supercoiled DNA preparations were purified over CsCl gradient centrifugation, linearized, phenol:chloroform extracted and ethanol precipitated in preparation for electroporation.

Cell Preparation

Ovine Fetal fibroblast (OFF) cells from 54, 70 and 90 day post coitus ovine fetuses were collected. Breifly, skin was removed from the back of the fetus and minced to a slurry texture and rinsed 3X with phosphate buffered saline (PBS). After the final wash cells were resuspended in Dulbecco's Minimal Essential Medium (DMEM; Sigma)

supplemented with penicillin (100U/ml)-streptomycin (100 μg/ml) (Sigma), 5 % FBS, and 5% horse serum (HS), plated and cultured at 37°C in 5% CO_2 air atmosphere. Media was replaced daily, until the cells reached 80%confluency. The OFF cells were removed from the plate with calcium-free PBS and frozen in DMEM culture media supplemented with 10% DMSO and frozen.

OFF cells were cultured in DMEM supplemented with penicillin (100U/ml)-streptomycin (100 μg/ml), 5% FBS and 5% HS in tissue culture flasks (75 cm^2 surface area) incubated at 37°C in 5% CO_2 and air. The cells were passed every 4-5 days with Ca^{++} free PBS and divided 1:10. In preparation for electroporation, an 80% confluent monolayer of OFF cells was suspended, washed 2X sterile PBS and resuspended in ice cold PBS at 1 X 10^7 cells/ml. Linear DNA was resuspended in sterile PBS, quantified by spectrometry, and 50 μg was added to 0.4 ml of OFF cells. Electroporation of OFF cells was performed in disposable cuvettes (BTX) with a 2 mm gap (BTX) with two 450 V electrical pulses for 80 μsec generated by a BTX Electro Cell Manipulator®200. Cells (viability >60%) were plated at <2X10^4 cells/cm^2 in tissue culture plates with culture media for 24 hr. Subsequently selection media containing 600 μg/ml G418 was added, changed daily and single colonies present after 10 days of culture were transferred to single well of a 24 well plate. OFF cell colonies were expanded to generate enough cells from the single colony to freeze and harvest genomic DNA. The genomic DNA from the single colonies were digested with several different restriction endonucleases and EGFP insertion was confirmed by Southern blot hybridization (Ausubel *et al.* 1995). At least two independent OFF cell lines for each construct were obtained.

Oocyte enucleation and Nuclear Transfer

Ovaries obtained from the abattoir were transported to the laboratory in PBS with 2% PS at 30°C. Ovaries were rinsed 2X in 30°C PBS-2% PS and cumulus-oocyte complexes (COC) were aspirated from 1-5 mm follicles with an 18-gage needle. The COC were placed in Hepes-buffered Tissue Culture Media 199 (TC199) containing 10 μg/ml heparin and 0.4% Fatty acid-free bovine serum albumin (BSA). COC were morphologically assessed and selected for compact, nonatretic cumulus oophorus-corona radiata and homogeneous ooplasm (Wells *et al.* 1997). Selected COC were thoroughly washed in TC199 with 10% FBS, transferred to a drop (total volume 50 μl) of maturation media (TC199 supplemented with 10% FBS, 10 μg/ml ovine LH, 10 μg/ml ovine FSH and 1 μg/ml estradiol) and cultured 24 h at 38.5°C in 5% CO_2 under oil. Following maturation, expanded cumulous cells were removed with 0.1% hyaluronidase for 1 min and rinsed through 3 drops of TC199-10% FBS. Oocytes were enucleated by aspirating the 1st polar body and metaphase II plate with a 30 μm beveled glass pipette in PBS supplemented with 3% sucrose and 1% BSA. Concurrent with enucleation, a stablely transfected OFF cell was injected under the zona pellucida, adjacent to the enucleated oocyte (cytoplast) membrane. Cytoplast/OFF cell complexes were incubated in synthetic oviduct fluid (Gardner *et al.* 1994; Tervit *et al.* 1972) supplemented with 20 essential and nonessential amino acids (SOF20aa) for 18 hours at 38.5°C in 5% CO_2 under paraffin oil.

Embryo activation and Culture

Cytoplast/OFF cell complexes were equilibrated in fusion media (0.3 M mannitol, 0.2 mM calcium, 0.1 mM magnisium, 0.5 mM Hepes, and 0.05% BSA), aligned in the fusion chamber and subjected to a single electrical pulses (200 V/cm) for 15 μsec from the BTX Electro Cell Manipulator. Reconstituted embryos were reequilibrated in SOF20aa, and cultured for 1.5 h post fusion in SOF20aa. Embryos were then activated in SOF20aa supplemented with 5 μM ionomycin (Cal Biochem, San Diego CA) and 2mM 6-dimethylaminopurine (DMAP; Sigma) for 4 minutes, washed through 5 drops of SOF20aa/DMAP and incubated in SOF20aa/DMAP for 4 hours at 38.5°C in 5% CO_2 under oil. Following this incubation the embryos were rinsed through 5 drops of SOF20aa and cultured in SOF20aa supplemented with 10% FBS in the presence of mitomycin C treated OFF cells. These OFF cells, at 90% confluency, had been treated with culture media containing 10 μg/ml mitomycin C (ICN, Inc.), washed extensively with PBS and frozen in 1 ml aliquots with 1 X 10^7 cells/ml. Cells were thawed and washed twice in SOF20aa/FBS and plated into 24-well plates at $2X10^4$ cells/well prior to embryo co-culture. Reconstituted embryos cultured *in vitro* were assessed daily for viability and cell numbers of each embryo.

In vivo cultured embryos were transferred into ligated oviducts of ewes in diestrus for 6 days after which time the oviducts were collected. Reconstituted embryos were flushed from the oviducts with TC199/10%FBS and compacted morulae or blastocysts were transferred into a ewe in standing estrus 6 days prior (Rowson and Moor 1966a; Rowson and Moor 1966b). Briefly, ewes are anesthetized with sodium pentobarbital, prepared for aseptic surgery and their uterine horns exteriorized through a midline incision, cranial to the udder (Bowen *et al.* 1983). A blunt needle is used to puncture the proximal tip of each uterine horn, and the embryos were delivered into the lumen using a glass pipette.

Construction of Vector for a Targeted Disruption in oPL Gene

A vector was designed to create a disruption of exon 2 and 3 in the oPL gene by homologous recombination. The pKO Scrambler Series V918 and Selection vectors containing neomycin (Neo; V800) and thymidine kinase genes (TK; V830; Lexicon Genetics, Inc., The Woodlands, TX) were purchased to assist in vector construction. An Eco RI fragment containing 4.5 kb of 5'flanking sequence, exon 1 and ~1 kb of intron A was cloned into the pKO Scrambler multiple cloning site A. The Eco RI fragment from Zap II clone 2 (Liang, 1995) was agarose gel purified (DEAE membrane), and the fragment was ligated (T4 ligase; Gibco BRL) into pKO V918, previously digested with Eco RI restriction endonuclease and dephosphorylated with shrimp alkaline phosphatase (SAP; Boehringer Manhiem). The plasmid was transformed into ultracompetent SURE cells (Stratagene) and clones were screened with restriction endonucleases to indicate orientation and sequenced to confirm (Davis Sequencing, Davis CA). This vector was used in a second cloning step to insert exon 4 and 5 into the multiple cloning site B.

EMBL3 clone 16 (Liang *et al.* 1999; Liang 1995) was amplified in K803 *E. coli* (Ausubel *et al.* 1995). Phage particles were pelleted with a 132,000 x g centrifugation for 90 minutes and DNA was purified from the proteins with phenol:chloroform extractions. Phage DNA was digested with several restriction endonuclease sites (Bam HI, Hind III, and Eco RI) and subcloned into pBluescript. A Bam HI/Hind III fragment (1.8 kb) containing a portion of intron C, exon 4, intron D, and exon 5 was purified and ligated into multiple cloning site B. The ligated vector was transformed into ultracompetent XL-2 *E. coli* cells (Stratagene) and plasmid (pKO145) construction was confirmed by

sequencing. The pKO selectTK (V830) vector was digested with Rsr II restriction endonuclease (New England Biolabs), and the TK gene with the MC1 promoter was purified. The MC1-TK gene fragment was ligated into Rsr II digested-dephosphorylated pKO145, transformed into XL-2 *E. coli*, and screened for the presence of the insert (pKO145T). The pKO selectNeo V800 was digested from the plasmid with the Asc I restriction endonuclease and the Neo gene with a PGK promoter and bGH polyadenylation signal was agarose gel purified. The fragment was inserted into Asc I digested-dephosphorylated pKO145T located between multiple cloning site A and B. The resultant vector, pKO145TN#32, contains 2 oPL gene homologous arms (5.7 kb and 1.8 kb), a negative selection gene (TK), and a positive selection gene (Neo). Orientation of the Neo construct was confirmed by nucleotide sequencing with KONeoSelectFOR, 5'-TCGCATTGTCTGAGTAGGTGTC-3', and KONeoSelectREV, 5'-CATCTGCACGTGTCTAGTGAGA-3'.

The construct, pKO145TN#32, was linearized with a Not I restriction endonuclease and electroporated into OFF cells as previously described. Selection media contained 600 μg/ml G418 and 2μM ganciclovir, and was changed daily until colonies were picked. The colonies were cultured in 24-well plates and subdivided once into two identical 24-well plates. At 80% confluency the cells in one plate were suspended in 250 μl of PBS, and 2X freezing media was added to the well and the plates were frozen and stored at -80°C. The duplicate plate was washed with PBS, and 0.5 ml of 100 mM Tris-HCl (pH 8), 5 mM EDTA, 200 mM NaCl, 0.2% SDS, and 1 mg/ml Pronase (Boehringer Manhiem) was added. After a 16 hr incubation at 37°C, 0.5 ml isopropanal was added to each well and incubated at room temperature on an orbital shaker. The genomic DNA

was removed from the well, washed twice with 70% ethanol and resuspended in 50 µl TE buffer. The genomic DNA was analyzed by PCR with KONeoslectREV and oPLExon5-3'UTR (5'-CTT TCT CTC AGA GGT AAG GAT GGA-3') or oPLExon4FOR (5'-TTA AGG AAA AGG CCA AAG TAC T-3') and oPLExon5-3'UTR. The PCR reactions contained 0.1 mM of each primer, 10 ng of genomic DNA, 0.1mM of each dNTP, and 2.5 mM $MgCl_2$ in 1X buffer in a final volume of 50 µl under mineral oil. The mixture was heated at 95°C for 5 min, placed on ice and 2.5 U Platium Taq DNA polymerase (Gibco BRL) was added. The reactions were subjected to a 2 min incubation at 95°C, and 30 cycles of 1min at 95°C, 1 min at 55°C, and 2 min at 72°C. Products were separated on a 1% agarose gel an visualized with ethitium bromide intercalation and UV light.

Results And Disscussion

Transgenic Mice

Transgenic mice have been used to study expression of other placentally expressed genes from human and mice. The lack of a ruminant trophoblast cell line will require characterization of oPL transcriptional regulation *in vivo*, and transgenic mice may serve as an *in vivo* model to assess promoter activity. Three oPL promoter fragments, -3465/+16 bp, -1616/16 bp, or -800/+16 bp, ahead of the luciferase gene were injected into a pronucleus of mouse zygotes. A founder male with the 3.5 kb/luciferase fragment was generated and neither heterologous nor homologous carriers expressed luciferase in any fetal tissues. Offspring from a transgenic founder with the 0.8 kb/luciferase fragment stimulated activity 8.6 fold ($P<0.05$) over non-transgenic mice in the fetal brain (Figure 1). No other fetal tissues demonstrated an enhancement in transactivation with the 800

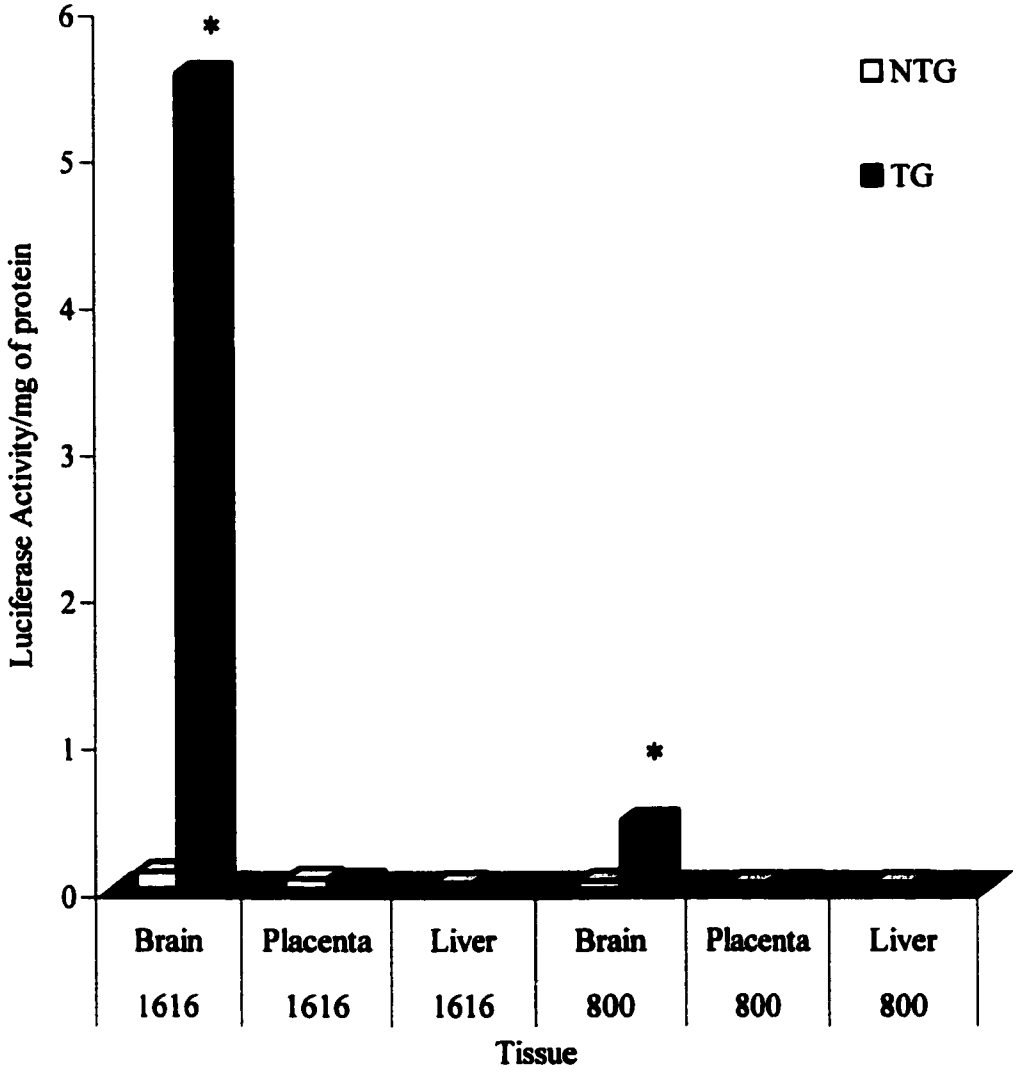


Figure 1. Ovine PL promoter activity in transgenic mice. Luciferase expression between transgenic (TG) and non-transgenic (NTG) mice at day 16 of gestation is compared above. The light units (AUI) were normalized on the protein concentration (mg/ml) of lysate. The tissue types and length of promoter are indicated under the pairs of bars. Student T-test comparisons between the tissues of TG and NTG mice were made and the asterisk indicates significant difference ($P<0.05$).

bp/luciferase. Two other founder lines for the 0.8 kb/luciferase fragment had no expression. A transgenic line with an additional 816 bp of 5'flanking sequence confirmed the expression in the fetal brain by stimulating activity 47.3 fold ($P<0.05$) (Figure 1). Again, a second founder carrying the 1.6 kb/luciferase fragment did not have detectable activity in any fetal tissues. Furthermore, reporter activity for 1.6 kb of the oPL promoter appears to be temporally regulated in the fetal brain and was found between 14-18 days post coitus. Cell-specific expression in the fetal brain was not characterized, due to the lack of reliable antiserum against luciferase. Although these data were unexpected, these observations may indicate the functionality of *cis*-acting elements previously reported within this dissertation. GATA-2, AP-2, and Purα are expressed in developing nervous tissues during this stage of development, and may influence expression. Differences within the oPL minimal promoter (-124/+16 bp) are prevalent between human (BeWo) and rat (Rcho-1) choriocarcinoma cells. In Rcho-1 cells, GATA elements (-102 bp and –67 bp) were found to be functional and a putative AP-1 element was suggested to be functional (Limesand 1997). In contrast, an AP-2 element, a novel Purα element, and the GATA elements were demonstrated to be functional in BeWo cells (Limesand 1997; Liang *et al.* 1999; Limesand and Anthony 1999). Therefore, these transacting factors may be enhancing transactivation in fetal mouse brains.

These data can be viewed in two ways. First the mouse may not be an adequate model to study oPL promoter activity, or the oPL promoter region tested does not contain trophoblast-specific elements required to stimulate activity *in vivo*. Since the data for the founder with 3.5 kb/luciferase region was negative, no conclusions can be drawn for the

region between –3465 bp and –1616 bp. A vector containing 4.5 kb of oPL 5'-flanking sequence was generated in pEGFP. Transgenic mice generated with the 4.5 kb/EGFP fragment were analyzed as founders at day 16 of gestation. Six positive founders were identified out of 30 fetuses from microinjected zygotes. Founder mice were cryosectioned (10 μm) through half of the fetus and placenta, were analyzed for EGFP expression with fluorescent microscopy, and counter stained with DAPI to identify tissue morphology. No detectable EGFP expression was observed in the fetus or placenta when compared to non-transgenic fetal-placental sections. These data suggest that elements located upstream of -1.6 kb region of the oPL promoter inhibit expression in the fetal brain. Conclusions on oPL promoter activity with –3.5 kb or –4.5 kb of promoter region in the transgenic mouse model must be taken with some caution because we cannot rule out the fact that these animals are simply incapable of transgene expression due to the insertion site within the mouse genome. Analysis of the equine chorionic gonadotropin α-subunit promoter (2.8 kb) resulted in a similar conclusion that placental expression resides outside of this promoter region (Farmerie *et al.* 1997). However, transgene expression with this promoter was identified in the pituitary, which rules out inactivation due to incorporation site. Transgenic mice with the rat growth hormone-releasing hormone (GHRH) gene promoter (7.5 kb) did not elicit placental expression, but the addition of intron sequence stimulated expression in the brain and spongiotrophoblast expression (Nogues *et al.* 1997). These data indicate that sequence outside of the promoter region is required for placental expression. However, the results of both studies were confirmed *in vitro* in choriocarcinoma cell lines (Farmerie *et al.* 1997; Nogues *et al.* 1997). The transgenic mice generated with the oPL promoter regions were do not

coincide with *in vitro* analysis of the oPL promoter in heterologous choriocarcinoma cell lines which included a rodent choriocarcinoma cell line, Rcho-1 (Liang 1995; Limesand 1997; Liang *et al.* 1999).

The inconsistencies observed might be due to species variations in trophoblast-specific expression of placental genes. Therefore, placental expression of the oPL gene may require analysis within the ovine placenta. Until recently, generating a transgenic sheep was a tremendous undertaking, but breakthroughs in somatic cell nuclear transfer greatly improved the ability to generate transgenic ruminants (Schnieke *et al.* 1997; Wilmut *et al.* 1997; Cibelli *et al.* 1998; Wells *et al.* 1998; Wakayama *et al.* 1998; Kato *et al.* 1999). Development of a transgenic sheep model to study oPL transactivation is our primary goal, but the application of this technique in other studies could provide a valuable technique. Preliminary work on procedures to establish a transgenic sheep model will be discussed.

Ovine Fetal Fibroblast Cells

Proliferation rate for various passages, days of gestation and freeze thaw techniques were analyzed for ovine fetal fibroblast (OFF) cells, and OFF cell cycle length was estimated to be 24-30 hr. Additionally, the chromosomes were karyotyped at various stages of culture including: passage 3, 8, 11 or 9-76 culture days. Chromosome spreads did not indicate any aberrations in modal chromosome number (54 chromosomes) during culture. The most limiting factor for culturing these cells was cell confluency. If OFF cells reached 90% or greater confluency, their ability to divide became impaired, and the morphology of the cells changed in culture from a spindle to a large oval shape. These

culture techniques are not described adequately in the literature, and may explain the early onset of senescence in cattle (<40 days; Cibelli *et al.* 1998), but longer duration (80 days) were observed in sheep (Schnieke *et al.* 1997).

The induction of quiescence (G_0 phase) has been deemed as a substantial ingredient for the formation of viable embryos (Schnieke *et al.* 1997; Wilmut *et al.* 1997; Kato *et al.* 1999). Serum deprivation at 0.5 to 1% slows cell proliferation (Figure 2). Schnieke *et al.* (1997) identified that serum reduction reduced the number of cells in S phase, since proliferating-cell nuclear factor, an indicator of active DNA replication, was not present in starved OFF cells. Another important feature of induced quiescence is the ability of OFF cells to regain function, so increasing serum (>5%) in previously starved cells reestablishes proliferation. The purpose for inhibiting proliferation of OFF cells is to allow the embryo to reactivate the diploid genome in the embryonic environment (Campbell *et al.* 1994).

Some dispute in the literature over the necessity of having nuclear donor cells at G_0 exists. Stice *et al.* (1998) indicate that nonquiescent cells are able to create a viable reconstituted bovine embryo. However, a greater number of reconstituted embryos were generated to yield the bovine blastocysts for transfer. Fibroblast cells have a long G_1 stage, and embryos generated may have been derived from cells in G_1 (Cibelli *et al.* 1998), but passage number or confluency of fibroblast cells was not provided (Wilmut and Campbell 1998). Additional studies on reconstituted bovine embryos identified a greater blastocyst formation for reconstituted embryos with quiescent bovine fetal fibroblast cells (Zakhartchenko *et al.* 1999b). Although, the mitotic state of the nuclear

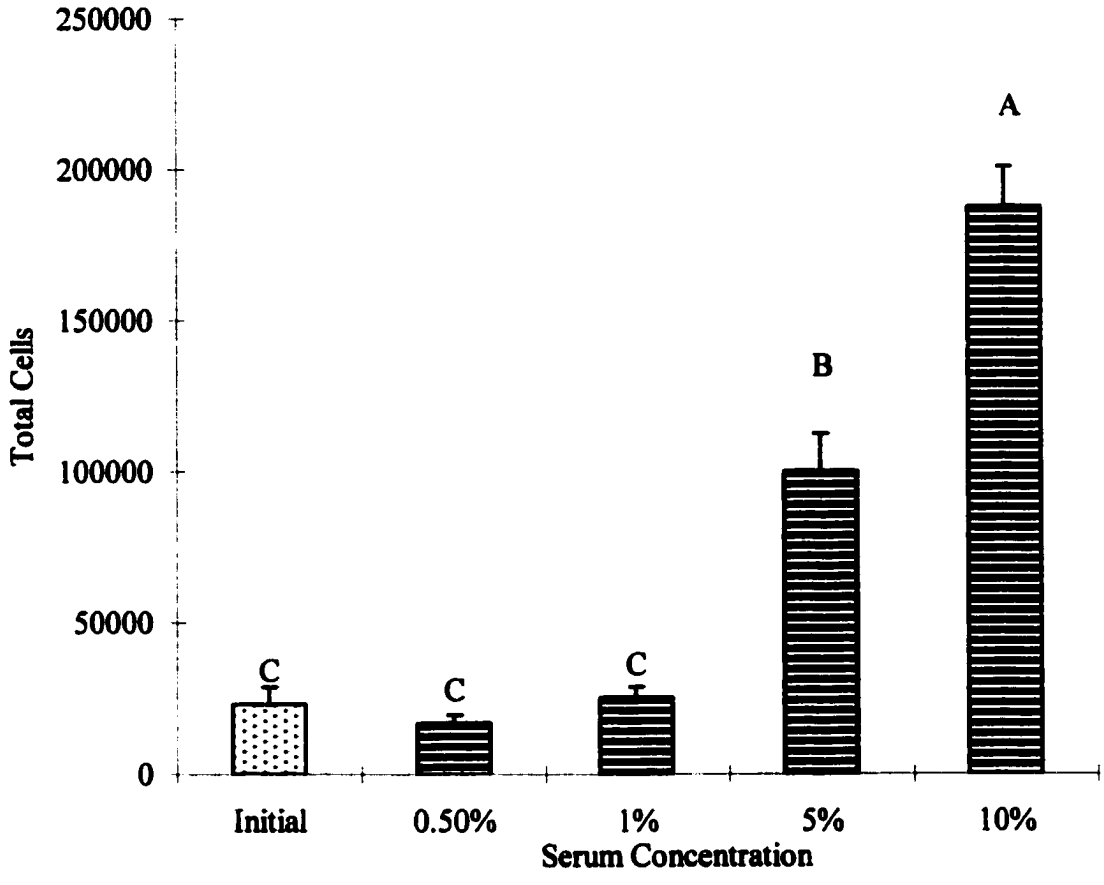


Figure 2. Serum deprivation of ovine fetal fibroblast cells. Serum deprivation was tested to drive the cells into a quiescent state. The bars indicate the mean number of cells and the error bars represent the SEM for 9 replicates. Labels above the bars represent a significant difference ($P<0.05$) in logarithmically transformed data using a Student-Newman-Keuls test to separate the means.

donor cells appears to influence the viability of the reconstituted embryos, subtle differences in the experimental design for producing nuclear transfer embryos may also influence development. Serum starved cells used to create Dolly (Wilmut *et al.* 1997) were transferred into *in vivo* matured oocytes 28-33 hours post ovulation. The micromanipulation was performed in 10% FBS and fusion/activation occurred 3-8 hr after manipulation. This methodology suggests that OFF cells will begin to enter a proliferative state (Wilmut *et al.* 1997). Bovine oocytes matured *in vitro* were manipulated in CR1 supplemented with 0.3% BSA, and immediately fused at 42 hr. In reality the reconstituted embryos generated by both these methodologies may be derived from a fibroblast cell in G_1, since the fetal fibroblast cells return to a proliferating state in the presence of 10% serum.

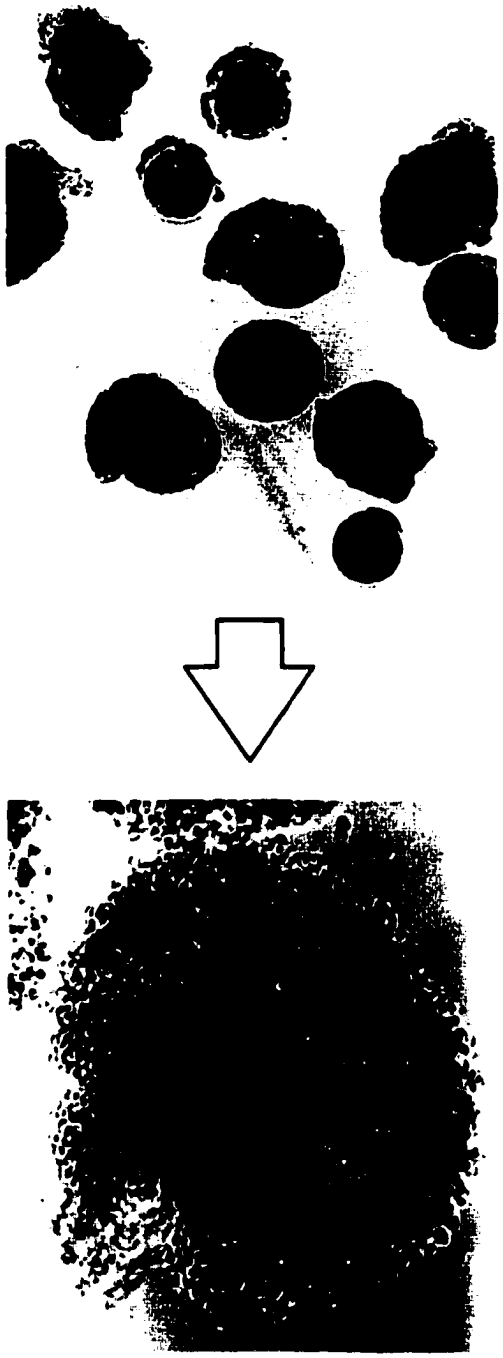
Methodologies for creating quiescent OFF cells were tested with our nuclear transfer protocol. Reconstituted embryos developing to ≥16 cells with normal morphology in respect to cell division were used for analysis, with each group containing >140 manipulated oocytes. Thirteen percent of serum starved OFF cells, as the nuclear donor, developed to the 16-cell stage, whereas cycloheximide treatment exhibited a 49% development rate. Development rate to the 16-cell stage for OFF cells that were cultured for >5 days and not treated to induce quiescence was 48%, whereas cells that were thawed within 48 hours prior to micromanipulation achieved a 46% rate. Therefore, creating quiescent OFF cell for nuclear donors with our procedure did not augment the production of reconstituted embryos developing to the 16-cell stage or greater *in vitro*.

Oocyte cumulus complexes were matured for 24 hours and cumulus cell expansion was observed (Figure 3). Analysis of maturation was performed on 20 oocytes from each batch of ovaries obtained from the abattoir by staining the metaphase plate with aceto-orcein (Figure 4). *In vitro* maturation rates of ovine oocytes aspirated from abattoir acquired ovaries was 82% (n=28) the first year and 77% (n=13) the second year, which mimic previous reports (Ledda *et al.* 1997). Additionally, the metaphase plate in the mature oocyte was located adjacent to the polar body. This observation allows for the removal of oocyte genetic material without using fluorescent dyes as an indicator. Enucleation rates achieved without Hoechst 33342 as an indicator averaged between 60-70%, and maximal rates of 90% were observed.

Fusion parameters to incorporate the OFF cell genetic material into the cytoplast were assessed (Figure 5). The lowest pulse that efficiently fused the OFF cell to the oocyte was 200 V/cm for 15 μsec, and is similar to the fusion-pulse described for generating bovine reconstituted embryos (Cibelli *et al.* 1998).

Embryo culture parameters were tested to assess the effect of culture media, inhibitors, or co-cultures with somatic cells on *in vitro* development. The action of kinase/protein synthesis inhibitors was suggested to decrease genomic abnormalities in reconstituted embryos that occur immediately after activation (Kato *et al.* 1999; Zakhartchenko *et al.* 1999a; Loi *et al.* 1998; Cibelli *et al.* 1998; Campbell *et al.* 1996; Szollosi *et al.* 1993; Wells *et al.* 1898). An experiment was designed (2X2 factorial) to analyze the effects of DMAP or cycloheximide in two types of culture media (SOF vs CR1) after activation. Reconstituted embryos were randomly assigned to one of the four

Figure 3. Ovine oocyte maturation *in vitro*. Early stage oocytes aspirated from the follicle are shown on the left. After 24 hours in TC199 supplemented with FBS, oLH, oFSH and estradiol, cumulus cell expansion occurs and is illustrated on the right for a single oocyte.



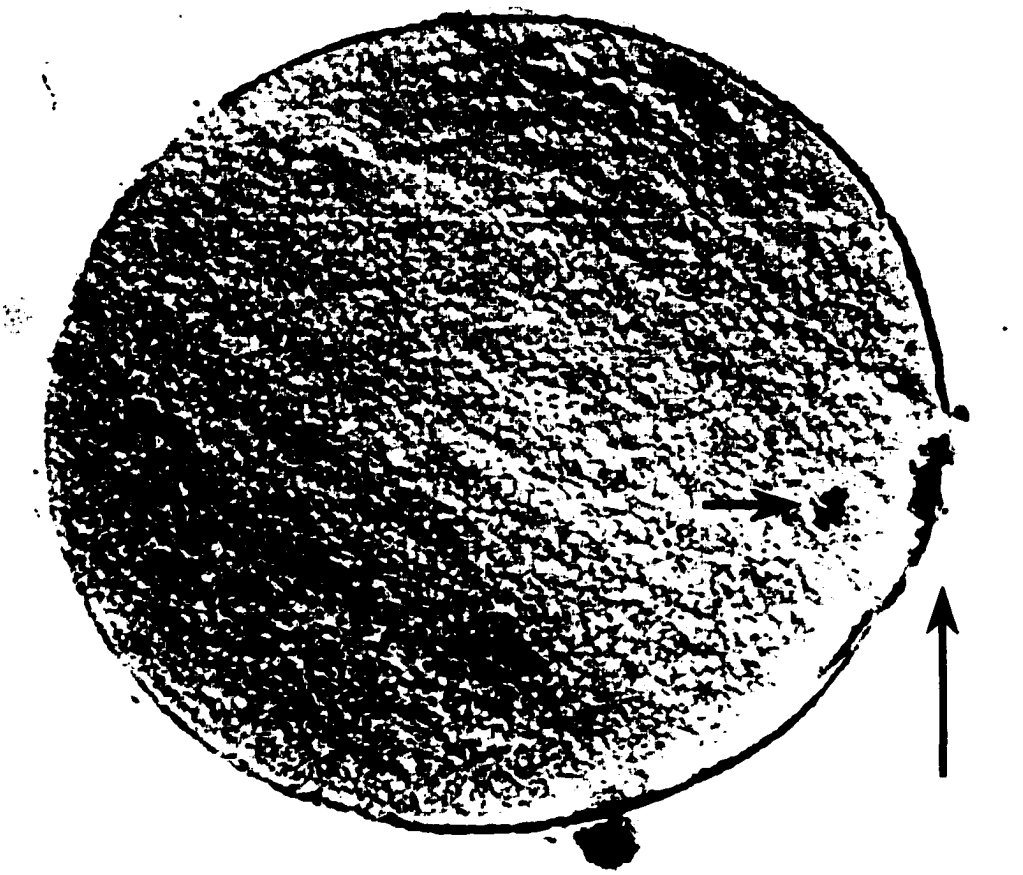


Figure 4. Matured oocyte. A maturated oocyte was fixed and stained with aceto-orcein to identify the genomic DNA. The horizontal arrow indicates the metaphase plate and the vertical arrow identifies the 1st polar body. This staining was performed on at least 20 oocytes from every batch of ovaries to obtain the percent maturation for the group of oocytes.

Figure 5. Reconstituted embryo. Aceto-orcein staining of a reconstituted embryo is illustrated in the photograph. The arrow represents the ovine fetal fibroblast incorporated DNA.

groups. The embryo culture data were analyzed for cleavage rates and embryonic cell numbers at 48 hr and 72 hr post activation. A mixed procedure (SAS Institute, 1993) was used to analyze the media and inhibitors as fixed variables and the group (n=3 separate times) as the random variable. Cleavage rates were analyzed for the two inhibitor treatments, and reconstituted embryo cleavage rates was 71.4% with DMAP treatment and 66.5% (P=0.46) cycloheximide treated embryos. The embryo culture media, SOF and CR1, were supplemented with 20 amino acids and 10% FBS, and the embryos were co-cultured on a fetal fibroblast cell feeder layer. Embryonic cell numbers (48hr) for each embryo that cleaved was visually assessed, and the number of embryos progressing to the compact morula stage was greater for SOF media (Table 1). The visual assessment was confirmed with actual embryonic cell number counts for embryos fixed at 72 hours, and the embryonic cell numbers were greater for SOF media ($P<0.01$).

Table 1. Embryonic culture conditions.

Cell Numbers	CR2-20aaFBS (n=84)	SOF20aaFBS (n=70)
≤ 16 cells	38%	17%
24 cells	25%	21%
32 cells	15%	20%
48 cells	11.5%	19%
≥ 60 cells	9.5%	23%

The utility of the co-culture system was studied next to determine its efficiency. The type of somatocyte cells used in as a feeder cell layer do not seem to be critical, but the presence of somatocyte cells does appear to be necessary in some embryo culture systems

to control nitrogen levels (Eyestone and First 1989; Yang *et al.* 1993; Watson *et al.* 1994; Holm *et al.* 1997). Embryos were culture in the presence or absence of mitotically inactivated OFF cells. Mean embryonic cell numbers for the embryos (n=70/group) did not differ at 48 hr post activation (P=0.13; Figure 6). However, a greater number (P=0.028) of cells at 72 hr was distinguished with the group cultured on a feeder cell layer (Figure 6). Combined, these data indicate that reconstituted ovine embryos treated with DMAP, cultured in SOF20aa 10% FBS in the presence of somatocyte cells develop more efficiently. However, reconstituted embryos develop at an enhanced rate and are unable to form a blastocoele cavity *in vitro*.

In vivo ovine embryos form a blastocoele cavity at 160 hr (6.7 days) after the onset of standing estrus, and 90% hatch after 224 hr (Rowson and Moor 1966a; Rowson and Moor 1966b). *In vitro*, ovine embryos cleave 24 hours after fertilization, become 4-cells at 48 hr and form a blastocoele cavity at 120 hr (4 to 6 days) (Walker *et al.* 1992; Dominko *et al.* 1999). Our *in vitro* culture experiments with reconstituted embryos indicate accelerated development through the fifth cell cycle, and 62% of the embryos generated have ≥32 cells at 48 hr (Table 1). Since embryos did not progress to the blastocyst stage *in vitro* they were tested *in vivo*, by transferring morula (16-64 cells at 24 hr) into a ewe at day 5 of the estrus cycle. Embryos reconstituted with an OFF cell line carrying CMV/EGFP were sacrificed at day 10, 5 days after transfer. Embryos recovered from the uterus revealed that development persisted, and blastocysts develop, hatch normally and elongate (Figure 7). Furthermore, expression of the reporter gene was identified in the hatched blastocysts (Figure 7). These data suggest that synchrony between the ewe and embryo was close, since 90% of the *in vivo* developed blastocysts

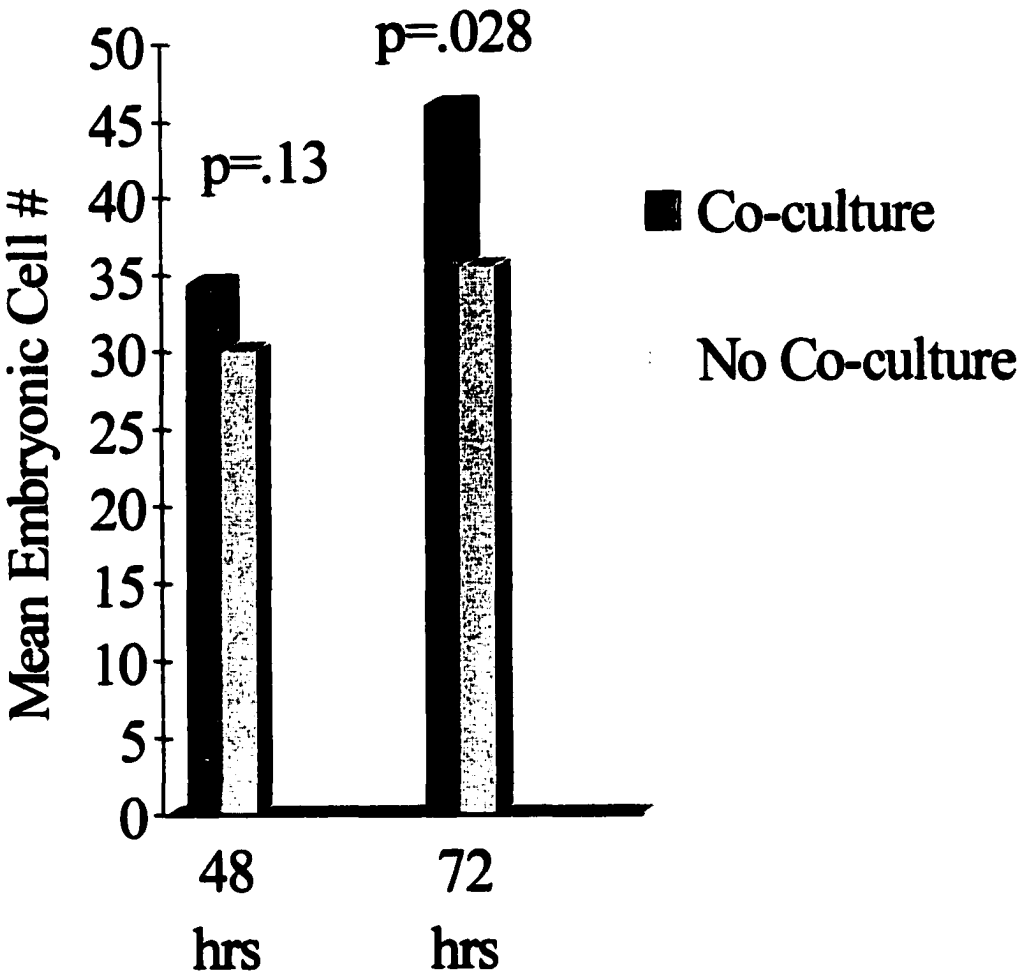
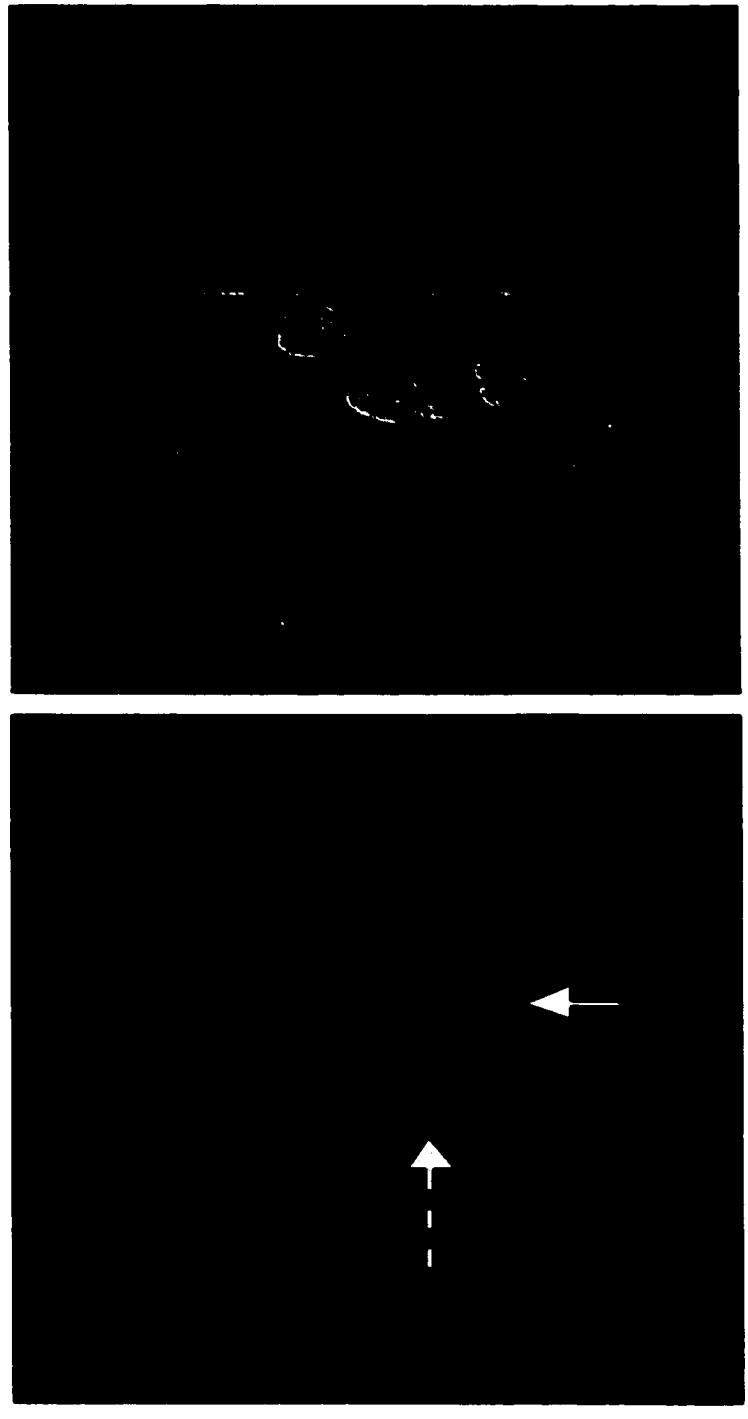


Figure 6. Utility of co-culture system on embryo development. Mean embryonic cell numbers for embryos cultured in the presence or absence of mitotically inactivated ovine fetal fibroblast cells was analyzed. The bars at 48 hr represent a visual assessment of the embryos, whereas the 72 hr time point indicated the actual cell counts on the embryos which were obtain by staining the nuclei. The p-values indicate the likelihood of the means being equal.

Figure 7. Nuclear transfer embryos generated with an OFF cell (CMV/EGFP). The micrographs depict embryos that were flushed from the uterus of a ewe 5 days after transfer. Bright-field micrograph shows 4-hatched blastocysts that collapsed during recovery and one unhatched blastocyst. The solid arrow marks the unhatched blastocyst and the broken line arrow identifies a embryo that appears to be elongating. Expression of the reporter gene was identified by fluorescent microscopy and pictured on the right. Only the hatched balstocysts appear to be expressing the transgene under the control of the viral promoter.



hatch by day 8.3. A reconstituted embryo with an OFF cell carrying -1.6 kb/EGFP was transferred into ewes (day 3) and assessed at day 16. An elongated embryo was recovered from the uterine flush and EGFP positive cells were identified within the embryonic tissue. The positive cells identified morphologically resemble chorionic binucleate cells, but exclusive expression remains to be determined (Figure 8). Subsequent transfers with reconstituted embryos in which the ewes were not sacrificed during the early stages of development indicate that embryos were capable of extending the estrus cycle. Nine of the 13 ewes exhibited an extended estrous cycle (18-35 days), when the herd averaged was 16-17 days. These data indicate the presence of trophectoderm at day 13, because normal developing embryos flushed from the uterus at day 13 extend the interval between standing estrus (19±1 days), whereas embryos flushed at day 17 extend the interval past day 30 (Hansen *et al.* 1985).

The inability to establish pregnancies with *in vitro* culture reconstituted embryos prompted an alteration in our procedure. Reconstituted embryos were transferred into the ligated oviducts of recipient ewes in diestrus (Wilmut *et al.* 1997). Embryos recovered (43%) from the ligated oviducts, 6 days after the initial transfer, were found to be compact morula or blastocysts. However, hatched zona pellucida's and undeveloped embryos were also identified. The compact morula and blastocysts recovered were transferred into synchronous recipient ewes, and 50% of these ewes demonstrated an extended estrous cycle. One of these ewes was sacrificed at day 40, and regressing trophoblast tissue was found in the uterus. A single embryo appeared to elongate and implant, the estimated age of the developing fetus was day 32-35. However, the necrotic

Figure 8. Reconstituted embryo with –1.6 kb/EGFP OFF cell at day 16. A portion of embryonic trophectoderm was recovered and positive cells for reporter activity were found. In the microphotographs, bright field (left) and fluorescent (right), the arrows identify cells that appear to be chorionic binucleate cells.

tissue was unable to be analyzed. These results are promising, and reconstituted embryos cultured *in vivo* were able to generate a pregnancy that survived to day 32 of gestation.

Targeted Disruption of the oPL Gene

Initial steps have been taken to generate a targeted disruption in sheep. A vector that will disrupt the oPL gene by removing a portion of intron A, all of exon 2, intron B, exon 3, and a portion of intron C by homolgous recombination was created. The region to be removed lies between the EcoRI site in intron A and the BamHI site in intron C, which contains ~ 9 kb of oPL genomic sequence. This disruption should severely alter oPL by removing 19 amino acids of leader sequence, and 77 amino acid residues from the N-terminus, and if exon 1 and 4 are spliced together a frame shift will be introduced (Warren *et al.* 1990; Liang *et al.* 1999).

The methodology for disrupting non-selectable genes, like oPL, has been enhanced by the inclusion of both positive and negative selection techniques (Mansour *et al.* 1988). The strategy behind dual selection is to separate random integration from homologous recombination. If the targeting vector is incorporated by homologous recombination into the proper site of the genome, neomycin resistance is conferred by resistance to the drug G418, and the thymidine kinase gene will not be present. However, if the insertion is random, the thymidine kinase gene will be inserted into the genome with the rest of the construct. The drug ganciclovir was designed to inhibit viral propagation of Herpes by selectively blocking metabolism of viral thymidine kinase, making nucleoside analogues are cytotoxic (Mansour *et al.* 1988). The ovine genomic DNA in the phage clones comes from an unknown source and may contain nucleotide polymorphisms. Two nucleotide

polymorphisms were observed between the genomic DNA sequence and the cDNA of the oPL gene (Liang *et al.* 1999). A report by Sedivy *et al.* (1999) indicates that isogenic DNA is not required. In practice, the reported (Mansour *et al.* 1988) efficiency of cells surviving G418 selection was 1.07%, and of those 0.05% survived ganciclovir selection. Target homologous recombination is estimated to occur in ≈5% of the cells surviving the double selection. Another report indicates a linear relationship with homologous DNA and recombination (Hasty *et al.* 1991), such that the best results are obtained with 6.8 kb (5.6 and 1.2 kb) of homologous sequence. Four electroporation have been performed with our construct (pKO145TN#32). One plate (one electroporation) was selected only with G418 and 210 colonies were present after 10 days of selection, whereas 3 plates selected with G418 and ganciclovir contained only 95 colonies after selection. Thereby confirming that the presence of the TK gene was cytotoxic to the OFF cells, but genomic DNA screening of the colonies has yet to identify targeted disruption of the oPL gene.

Summary

The generation of transgenic embryos by somatocyte nuclear transfer (NT) techniques provides the opportunity to generate transgenic ruminant animals at a greater efficiency than previously employed techniques. Placental expression of transgenes, or expression of transgenes used to alter fetal physiology, can be examined during gestation of the founder animal because the pregnancies are not chimeric. We have begun to establish the system for manipulating and culturing somatocyte NT embryos. Initial studies were established to analyze the in vitro culture conditions of NT embryos. Our *in*

vitro maturation for oocytes aspirated from abattoir acquired ovaries was 82% (year 1) and 77% (year 2). In addition to high maturation rates, we established an optimum fusion pulse of 200V/cm. With these criteria in place, the first culture conditions tested was the use of cycloheximide or 6-demethylaminopurine (DMAP) to inhibit extrusion of genetic material after reconstituted embryos were activated with ionomycin. No differences in the cleavage rates were observed for cyclohexamide (66.5%) or DMAP (71.4%) treatments. The second condition tested compared common embryo culture medium in co-culture with fetal fibroblast cells. Cell numbers from embryos cultured in CR2 and synthetic oviductal fluid (SOF) were visually assessed at 48 hr and fixed for cell counts at 72 hr post activation. At both time points, mean embryonic cell numbers for the SOF media group were greater that the CR2 media group ($P<0.01$). Finally, we evaluated the utility of co-culturing NT embryos on fetal fibroblast feeder layers. Cell counts at 72 hr after activation were significantly greater for the co-cultured embryos relative to those cultured without feeder cells ($P=0.028$). Additionally, NT embryos transferred into the proximal uterine horn developed to blastocysts, hatched normally and elongated, and many were positive for reporter expression. These data suggest that embryonic culture parameters tested are capable of supporting early NT embryos, which can be transferred into the uterus and form a viable fetus. However, *in vivo* culture techniques were employed to assess the efficacy of the in vitro culture. Although accelerated development was also observed, embryos developing at a rated similar to normal were observed in the *in vivo* cultured NT embryos. Subsequent transfer of these embryos into a recipient ewe resulted in the maintenance of pregnancy until day 32. Therefore, *in vivo* culture of NT embryos may be required to generate viable embryos that will implant.

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