NOTE TO USERS

DISSERTATION

TRANSCRIPTIONAL REGULATION OF OVINE PLACENTAL LACTOGEN

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY KIMBERLY M. JECKEL ENTITLED TRANSCRIPTIONAL REGULATION OF OVINE PLACENTAL LACTOGEN BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

TRANSCRIPTIONAL REGULATION OF OVINE PLACENTAL LACTOGEN

The placenta is a transitory organ, produced only during gestation, with the sole purpose of maintaining and promoting fetal growth. During gestation the growth and development of the fetus is regulated by a variety of hormones and growth factors, many of which are produced by the placenta. The placental lactogens are among the hormones produced during this unique period and they function to maintain fetal growth through repartitioning of nutrients to the fetus. During gestation there are many abnormalities which can occur, many of which result from aberrations in the production of these hormones. Intrauterine growth restriction (IUGR) is one such gestational abnormality thought to result from placental insufficiency. With IUGR pregnancies the risk of death increases with gestational age. In addition, the long term consequences of IUGR include a predisposition to diseases such as cardiovascular disease, hypertension, diabetes and coronary artery disease. Placental lactogen is also decreased in these IUGR pregnancies, indicating its importance in fetal development. The sheep provides a good model by which to study the hormones produced during gestation. Therefore, understanding the regulation of ovine placental lactogen (oPL) may lead to potential genetic therapies for IUGR pregnancies.

The ovine placental lactogen gene has been characterized and its transcriptional regulation examined to identify *cis* elements which bind nuclear proteins that function as transactivators. Within 1.1 kb of the oPL gene proximal promoter, 19 footprints were identified via DNase I protection analysis. Within -383 bp of the proximal promoter region, relative to the transcriptional start site, six footprints resided. Three of these, FP1, FP2 and FP3 were within -124/+16 bp, the minimal promoter region. This region was responsible for trophoblast-specific transactivation in human (BeWo) and rodent (Rcho-1) choricarcinoma cell lines. Various *trans*-acting factors were found to interact with these footprints. Footprint 1 contained an initiator element, FP2 contained a GATA site and an AP-2α site, FP3 contained another GATA element and a Purα element and FP4 contained an E-Box. Two footprints, FP5 and FP6, reside within the -383/-217 region responsible for full *in vitro* activation of the oPL promoter, containing a direct repeat of a GAGGAG sequence. This GAGGAG sequence was found to be functional through mutation analysis.

The region between -383/-217 contains the FP6 sequence found to be necessary for trophoblast-specific transactivation through block mutation analysis. Potential binding sites for CEBP-α and the Sp proteins were identified within the FP6 sequence (-319/-349) through a transcription factor analysis program. This coincided with the GAGGAG sequence within FP6 previously found to be functional through mutation analysis. Transfection analysis utilizing two-base pair transversion mutations along the length of FP6 showed significant ($p \leq 0.05$) reductions in transactivation with mutations 2, 4-6, 8-10 and 12 in human (BeWo) choriocarcinoma cells compared to the wild type -380 promoter construct indicating these base-pairs were necessary for transactivation. Co-

transfection analysis with over-expression constructs for the CEBP-α, -β and -δ proteins did not significantly increase activation of the oPL promoter. Additionally, dominant negative co-transfections with a CEBP-α specific and a general CEBP construct did not result in significant reductions in transactivation. In conjunction with supershift analysis, in which a CEBP-α antibody did not produce a supershift in BeWo and binucleate cell nuclear extracts, these results suggest that the CEBP proteins are not interacting with FP6 to activate transcription.

Co-transfection analysis with over-expression constructs for both Sp1 and Sp3 significantly ($p \leq 0.05$) increased transactivation in BeWo cells compared to the wild type -380 promoter construct. Additionally, co-transfections with the FP6 sequence in front of a minimal prolactin promoter construct, and Sp1 and Sp3 expression constructs also significantly ($p \leq 0.01$) increased transactivation. When these proteins were inhibited through siRNA co-transfections both resulted in significant ($p \leq 0.01$) decreases in transcriptional activation, indicating that they are important for regulation of the oPL promoter. Sp1 increased transcription to a greater degree than Sp3 in the over-expression co-transfections, while Sp3 resulted in a greater inhibition in the siRNA co-transfections, indicating that perhaps Sp3 is more important and already being expressed at a higher concentration. Supershift analysis showed that both Sp1 and Sp3 were in binucleate cells (BNC) and were binding to the FP6 region. Western analysis showed that Sp3 was in BNC and further Southwestern analysis revealed that this protein was specifically binding to the FP6 region. However, Sp1 did not show the same migration pattern in Western or Southwestern analysis, indicating that Sp1 may not be binding to the FP6

region in order to stimulate transcription. Together, these data indicate that Sp3 may be the *trans*-acting factor binding to the FP6 region and functioning to stimulate transcription of the oPL gene.

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TRANSCRIPTIONAL REGULATION OF OVINE PLACENTAL LACTOGEN

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Chapter I.

INTRODUCTION

The regulation of fetal growth is complex and controlled by a variety of factors including hormones, growth factors, and nutrient availability. The maternal role in providing nutrients to the fetus depends not only on nutrient intake, but also on the partitioning of these substrates to the fetus. The placenta, a transient organ present only during gestation, exerts its effects on both maternal and fetal tissue. The role of the placenta, and the hormones and factors produced by this organ, is to provide an environment conducive to optimal fetal growth. This occurs mainly through directing substrates to the fetus to provide the nutrients required for proper growth and development in the intrauterine environment.

Pregnancy is a transitory state regulated by hormones and growth factors both unique to gestation and present in the post-natal mammal. Placental hormones produced during gestation promote fetal growth both directly and through repartitioning of maternal nutrients. Among the placental hormones, placental lactogen (PL) plays a major role in the regulation of fetal and maternal metabolism. This hormone, a member of the prolactin/growth hormone (PRL/GH) gene family, is found in most mammalian species and is expressed by the trophectoderm of the placenta solely during gestation. While the exact function of placental lactogen remains to be defined, it has been suggested that this

hormone may redirect the flow of nutrients to the fetus, making it indispensable to fetal development.

Abnormal development *in utero* may lead to the onset of adult diseases such as diabetes, hypertension, cardiovascular disease and obesity. Growth retardation of the fetus may reprogram the metabolic system, such that the fetus adapts to a limited nutrient supply, causing permanent changes in fetal physiology. These changes, in turn, predispose the adult to acquiring the diseases mentioned above. As intrauterine growth restriction (IUGR) affects nearly 8% of all pregnancies, it presents a significant problem in today's society. The Barker hypothesis suggests that fetal insults such a poor nutrition, hypoxia and growth restriction may lead to the onset of adult diseases. Thus, understanding the regulation of fetal growth, and the many factors underlying its control, may provide insight into preventing the myriad of diseases associated with compromised health in later adulthood.

Placental lactogen is decreased in growth restricted pregnancies, suggesting that it plays a role in maintaining proper fetal growth. While the exact function of placental lactogen remains to be defined, in the fetus it acts to increase IGF-II secretion, stimulate glycogen synthesis and ornithine decarboxylase activity. In addition, acute fasting increases placental lactogen concentrations in both fetal and maternal serum. Placental lactogen appears early in pregnancy and is present until the end of gestation, suggesting it may function to regulate fetal growth until postnatal hormones begin their control.

In order to understand the complex network regulating fetal growth and development, the study of pregnancy specific hormones such as placental lactogen is essential. Transcriptional regulation of the genes unique to gestation allows their products to be

produced solely during this transient state. The transcriptional control of placental lactogen is governed by a variety of *trans*-acting factors and the identification of these transcription factors will lead to a more complete understanding of how the gene is regulated thus, providing a means to study the control of fetal growth. The PL's are produced by ruminants, primates, and rodents however, the placental structure and length of gestation is more similar between humans and ruminants than rodents therefore, the sheep provides a good model by which to study pregnancy and the hormones produced during this period. Previous analysis of the ovine placental lactogen (oPL) promoter has identified specific regions responsible for activation of transcription. Various footprints were identified in the oPL promoter using DNase I footprint analysis. Two of these, footprint 5 (FP5) and footprint 6 (FP6), lie within the region responsible for maximal trophoblast-specific transcriptional activation. However, the *trans*-acting factors binding to FP6 have yet to be identified. The focus of this dissertation is to identify and analyze the transcription factors interacting with the FP6 region of the oPL promoter and to determine their functional role in regulating transcription.

Chapter II.

LITERATURE REVIEW

Placental hormones and growth factors act specifically to aid in fetal growth during gestation. Any aberration in the production of these factors could affect fetal growth, resulting in intrauterine growth restriction (IUGR). Therefore, nutrition alone is not responsible for the growth of the fetus. Studying the hormones directly responsible for controlling nutrient supply to the fetus will provide insight into the means by which fetal growth is regulated. One of these hormones, placental lactogen, is thought to play an essential role in maintaining the intrauterine environment through its actions on nutrient availability to the fetus. The main function of placental lactogen is believed to be the repartitioning of substrates to maternal and fetal metabolism, such that nutrients are directed to the fetus (Anthony et al., 1995a).

During intrauterine growth restricted pregnancies, the fetus is compromised such that growth is restricted and the risk of death increases with gestational age. This condition is caused by a variety of factors including decreased maternal nutrient intake and restricted nutrient flow to the fetus. The hormones and growth factors involved in the regulation of fetal development may also be altered in growth-restricted pregnancies. Early detection of this condition may prevent fetal death, and identification of the hormones specifically altered in IUGR pregnancies may target a route for possible therapeutic interventions.

Among the hormones responsible for directing nutrient flow to the fetus, the placental lactogens appear to be necessary for optimal fetal growth (Anthony et al., 1995b). Thus, focusing on the regulation of these genes may give insight into how these compromised pregnancies could be treated.

The regulation of a gene involves a large number of specific and general transcription factors. To further identify the tissue-specific expression of a gene the regulatory factors controlling transcription must be defined. The *trans*-acting factors that confer tissue specificity and transcriptional control of the ovine placental lactogen (oPL) gene have only recently begun to be described (Limesand et al., 2004; Limesand and Anthony, 2001). A further understanding of this gene and the control of its expression will provide insight into this pregnancy specific hormone and its role in the maintenance and regulation of fetal growth. Therefore, the focus of this research is to identify and define the *trans*-acting factors involved in the transcriptional regulation of the oPL gene.

Placenta

Embryonic development in mammalian species depends on a complex network of regulatory factors devoted to providing and maintaining a specialized environment optimal for the growth of the fetus. This unique environment requires the coordination of regulatory factors stemming from a variety of sources, including maternal, fetal and extraembryonic tissues. In the rodent, human and ovine placenta, placental lactogen synthesis and secretion occurs in the cells that form at the maternal-fetal interface, the fully differentiated trophoblast cells (Anthony et al., 1995b; Handwerger, 1991; Robertson et al., 1982). The function is similar in all three species in that they secrete

hormones, transport nutrients between the maternal and fetal circulation and are involved in attachment of the placenta (Talamantes F and Ogren L, 1988).

The maturation of the fetus is dependent upon the placenta, an organ unique to gestation and arising from extraembryonic tissues. This specialized organ develops in coordination with the fetus and performs a myriad of functions upon which fetal development depends. A significant function of the placenta involves regulating the maternal environment during gestation. The placenta provides immunological protection, transports fetal waste, acts to transduce maternal and fetal signals and regulates nutrient flow to the fetus (Soares et al., 1991). In order to provide the conditions necessary for optimal fetal growth, the internal environment, as well as maternal metabolism, must be organized to promote fetal development. The repartitioning of metabolic substrates from maternal nutrient supplies to the developing fetus is important for the growth of the fetus. The specific endocrine functions of the placenta provide a venue for nutrient uptake and adequate supplies to be directed to the developing fetus (Anthony et al., 1995b; Talamantes F and Ogren L, 1988). This necessitates the production of hormones designed to ensure fetal maturation within the uterus. These hormones arise from the anterior pituitary, the ovary, the uterus and the placenta (Soares et al., 1991).

The placenta is derived from two specific cell lineages. The trophoectoderm of the blastocyst gives rise to the trophoblast cells which form the epithelial portion of the placenta (Cross et al., 1994; Cross, 2000). The generation of distinct trophoblast cell types within the placenta is necessary for implantation, vascular connections, immunological adaptation and fetal nutrition (Loregger et al., 2003). The trophoblast cells arise from the outermost epithelial layer of the blastocyst, the trophoectoderm. The

syncytium and the trophoblast are the specialized cell types found in human and ruminant placenta. The syncytium arises by the cell fusion of cytotrophoblasts and acts to transport nutrients to the fetus (Steven, 1975). The multinuclear epithelium produces the hormones associated with gestation, the placental lactogens and the chorionic gonadotropins (Handwerger and Freemark, 2000; Lacroix et al., 2002; Morrish et al., 1998; Soares et al., 1991). The trophoblast modification of maternal vessels is thought to be necessary for optimal gestation as a lack of endovascular trophoblasts is associated with pre-eclampsia and fetal growth restriction in humans (Loregger et al., 2003; McFadyen et al., 1986; Pijnenborg et al., 1991).

Placental Classifications

The placental type most common among mammals is the chorio-allantoic placenta. Early in gestation, before the chorio-allantoic placenta forms, a transient structure is responsible for the support of the embryo. This structure, the chorio-vitelline placenta, forms from the apposition of the yolk sac and the chorion. In the chorio-allantoic placenta, the outer membrane, the chorion, forms a vascular bridge between the fetus and the chorion by fusing with the outer allantoic mesoderm (Amoroso, 1952; Steven, 1975). The placenta arises from this fusion, which subsequently attaches to the uterus. This utero-placental attachment brings the maternal and fetal vasculature into proximity thus, allowing for the exchange of nutrients (Steven, 1975).

There are four categories of morphological shapes which describe the chorio-allantoic placental structure. They are: discoid, zonary, diffuse and cotyledonary. The discoid placenta, found in rodents and humans, presents as villi which are arranged in a circular

plate (Steven, 1975). Carnivores, such as the dog and cat, exhibit a zonary placenta, in which a chorionic villus area encircles the equatorial region of the chorion (Amoroso, 1952). In the diffuse placenta, found in the mare and pig, small villi or folds cover the chorion and directly contact the sulci of the uterine epithelium (Amoroso, 1952). The final placental type, the cotyledonary placenta, is found in ruminants. In this placental type the villous outgrowth is restricted to regions within the uterus called caruncles, which in turn are separated by smooth chroion (Amoroso, 1952). Together the placental unit, comprised of the maternal caruncle and the fetal cotyledon, is called the placentome.

In addition to morphological shapes, placentas have also been classified according to the intervening tissue layers between fetal and maternal blood. The tissue layers are: maternal endothelium, maternal connective tissue, maternal epithelium, fetal epithelium, fetal connective tissue and fetal endothelium. Based upon these tissue layers, the placental types are: hemochorial, in which only the fetal layers are present, while the maternal blood bathes the fetal epithelial layer; endothelialchorial, containing three fetal layers plus the maternal endothelial layer; syndesmochorial, in which there is a discontinuous uterine epithelium; and epitheliochorial, where all six layers are present (Amoroso, 1952; Steven, 1975). Together, both the structure and the morphological shape of the placenta are used to define placental types found in different species.

Species with a discoid-hemochorial placenta include human and rodent. In this placental type, the fetal epithelium and maternal blood are in direct contact and the placenta is discoid in shape. Originally, the ruminant placenta was classified as cotyledonary-syndesmochorial, as the maternal connective tissue and fetal epithelium were adjacent (Steven, 1975). However, in the ruminant, the uterine epithelium is

present as a syncytium and the binucleate cells (chorionic epithelial cells) fuse with the syncytial layer after migrating to the uterine epithelium, leaving a maternal epithelium that is no longer a finite layer. In view of these findings, the classification of synepitheliochorial placenta was adopted to describe the ruminant placenta (Amoroso, 1952; Ramsey, 1982; Steven, 1983; Wooding, 1992). Although, there is diversity between species with regards to placental structures, the function of the mammalian placenta is quite similar between species.

Placental Components

Although the cellular architecture of the placenta differs between species, there are specific differentiated trophoblast cells expressing placental lactogen, which are similar in location and function in primates, rodents and ruminants. The polar trophectoderm in the human is the site for invasion of the blastocyst, from which arise two categories of trophobast cells: the multinuclear syncytiotrophoblast and the mononuclear cytotrophoblast cells (Aplin, 1991; Ringler and Strauss, III, 1990). The multinuclear syncytiotrophoblast cells form an interface with the underlying cytotrophoblast cell layer. In addition there is an extravillous trophoblast (EVT) cell that migrates in and invades the myometrium. These EVT's migrate into the myometrium through the uterine stroma, subsequently eroding the maternal endothelial layers, after which they are in direct contact with the maternal blood (Aplin, 1991; Ohlsson, 1993). Progenitor cytotrophoblast cells eventually produce the syncytiotrophoblast cells, which form the endocrine cells of the placenta (Page, 1993; Talamantes F and Ogren L, 1988). The

cytotrophoblast cells continue to proliferate and grow thus, expanding the surface area directly contacting the maternal blood (Pierce, Jr. and Midgley, Jr., 1963).

During attachment, in the rodent, the decidual cells surround the blastocyst. The portion of the rodent uterus closest to the lumen which surrounds the embryo is the deciduas capsularis, while adjacent to the embryo lies the deciduas basalis (Soares et al., 1991). In the rodent chorio-vitelline placenta, the yolk sac expands and forms an anastomoses with the trophoblastic giant cells (TGC) forming the chorio-vitelline placenta, a single layer of differentiated trophoblast cells, which appear from implantation through mid-gestation (Soares et al., 1996). After day 14 of pregnancy, the chorio-vitelline placenta is displaced by the chorio-allantoic placenta which subsequently becomes responsible for the fetus. At mid-gestation the chorio-allantoic placenta forms two distinct zones at the deciduas basalis, the labyrinth and the junctional zones (Soares, 1987; Soares et al., 1991; Soares et al., 1996; Soares et al., 1998). In the rodent placenta there are four trophoblast cell phenotypes; the TGC, the spongiotrophoblast cells, the glycogen cells and the syncytiotrophpblast cells. The TGC, which appear at the maternal-placental border, are within the labyrinth and junctional zones (Soares et al., 1998). The TGC possess endocrine and invasive activity and they arise by endoreduplication (Ilgren, 1983; Varmuza et al., 1988). The cellular components of the junctional zone, underlying the trophoblastic giant cells, include the spongiotrophoblast and glycogen cells. The spongiotrophoblast cells, although morphologically distinct from TGC, may have arisen from trophoblastic stem cells. These spongiotrophoblast cells secrete other prolactin (PRL) family members thus, possess endocrine properties (Soares et al., 1996). The glycogen cells appear to function as energy reservoirs (Davies and

Glasser, 1968). The syncytiotrophoblast cells, which reside at the placental-fetal interface within the labyrinth zone, are involved in waste and nutrient exchange (Sibley, 1994; Soares et al., 1998).

In the ruminant, due to the lack of uterine response and trophectoderm invasion, there are differences in placentation compared to the human and rodent. In the ruminant, blastocyst hatching occurs at day 8 of gestation, while elongation takes place on day 12-14 (Rowson and Moor, 1966). By day 15 in the sheep, contact formation begins, while apposition and attachment throughout the uterine surface occurs by day 20 post coitus, at which point it appears similar to the diffuse placenta (Davies and Wimsatt, 1966). The formation of placentomes occurs by the fourth week, as the chorio-allantoic placenta develops cotyledons at the uterine caruncular epithelium (Boshier, 1968; King et al., 1982). By day 16 in sheep, and day 19 in cattle, binucleate cells can be found in the chorion of the conceptus, (Boshier, 1969; Wathes and Wooding, 1980). The ruminant binucleate cells migrate into the maternal uterine epithelium and fuse with the uterine syncytium and maternal epithelial cells, where this migration and fusion causes the displacement of a finite maternal epithelium layer (King et al., 1982; Wooding, 1982). At day 28 in the cow, the growth of the uterine epithelial cells, and cessation of multinucleate cell formation with uterine epithelium, results in a distinct layer of maternal columnar epithelial cells (Wathes and Wooding, 1980). In the ewe, the isolated regions of maternal-fetal syncytium continue throughout gestation due to a greater rate of binucleate cell migration out of the chorion for the entire gestational period (Wimsatt, 1951; Wooding, 1992). The ruminant binucleate cells reside in the chorionic epithelium and the migrating binucleate cells account for 15-20% of the total population of

binucleate cells. The binucleate cells account for approximately 20% of the chorionic epithelium which covers the villous surface of the ruminant placenta (Wooding, 1983; Wooding et al., 1986; Wooding, 1992). The ruminant binucleate cells, which are derived from differentiating cytotrophoblast cells, secrete protein and steroid hormones and play an important role in placentation (King et al., 1982; Wooding, 1992).

Intrauterine Growth Restriction

Abnormalities occurring during pregnancy increase the risk of fetal mortality, pre-eclampsia and fetal growth restriction, and may lead to increased incidence of heart disease, hypertension, stroke and diabetes in later life (Barker et al., 1990; Barker et al., 1993). IUGR is a significant cause of fetal morbidity and mortality and can affect up to 8% of all pregnancies (Brar and Rutherford, 1988; Pollack and Divon, 1992). Intrauterine growth restriction manifests itself as either symmetrical or asymmetrical growth. Symmetrical growth restriction often arises due to genetic abnormalities or infections affecting the fetus, and the insult occurs during the first trimester (Anthony et al., 2001). Asymmetrical growth restriction is more common, occurs during late gestation and is a result of placental or maternal factors which affect the flow of nutrients to the fetus (Anthony et al., 2003). Growth restricted pregnancies often exhibit abnormal placentation, altered placental transfer and use of amino acids, placental structural abnormalities, and changes in placental proteins, all of which contribute to the adverse impact on fetal development (Anthony et al., 2003; Bersinger and Odegard, 2004).

Aberrations in fetal nutrition during pregnancy can negatively affect fetal development resulting in growth restriction. Undernutrition during various stages of

gestation adversely affects the growth of the fetus. During early embryogenesis, suboptimum nutrition may retard development of the fetus. Both hypoglycemia and hyperglycemia may be associated with the low birth weight observed with IUGR pregnancies (Leese, 1990). During mid-gestation, when the placenta is growing faster than the fetus, nutrient deprivations may negatively affect fetal growth by altering interactions between the fetus, placenta and mother. While severe maternal undernutrition results in lowered fetal and placental growth, mild nutrient deprivation may cause increased placental size, potentially affecting fetal growth (McCrabb et al., 1991). Undernutrition during late gestation slows fetal growth, as the fetus is sacrificed to maintain placental development, and fetal-placental metabolic interactions are negatively affected (Cetin et al., 1990; Soothill et al., 1987). Fetal catabolism occurs during periods of lowered intake and sustained undernutrition may result in irreversible fetal growth restriction (Divon et al., 1986; Harding et al., 1992). Thus, undernutrition during any stage of pregnancy negatively impacts fetal growth. During early pregnancy, embryonic growth may be retarded resulting in small babies. Mid-gestational undernutrition alters the interactions between the fetus and placenta causing the placenta to become small or hypertrophied, while poor nutrition during late gestation diverts the available nutrient supply to the placenta at the expense of the fetus, resulting in fetal wasting.

During IUGR pregnancies, the placenta, and the hormones and growth factors produced by this organ, as well as the fetus, may be adversely affected. The metabolic adaptations observed during growth restricted pregnancies are linked to the changes in fetal and placental hormone concentrations. The placental lactogens are among the

hormones affected by growth restriction (Bersinger and Odegard, 2004). During studies in which IUGR was induced in sheep during active placental development, via maternal hyperthermia, placental lactogen and progesterone concentrations were significantly decreased (Regnault et al., 1999). Impaired PL secretion is implicated in the altered maternal and fetal metabolism observed in growth restricted pregnancies as PL is an important marker for normal placental function and integrity (Freemark and Handwerger, 1989; Handwerger, 1991). In the hyperthermic ewes differences in oPL and progesterone concentrations do not appear to be based on changes in transcription or translation, suggesting the observed effects may be due to impaired trophoblast development or migration (Regnault et al., 1999). Thus, PL is one of the placental hormones adversely affected during growth restricted pregnancies, implicating it in the abnormal development of the fetus.

During intrauterine growth restricted pregnancies, the fetus is compromised such that growth is restricted and the risk of death increases with gestational age. This condition is caused by a variety of factors including decreased maternal nutrient intake and restricted nutrient flow to the fetus (Owens, 1991). The hormones and growth factors involved in the regulation of fetal development may also be altered in growth-restricted pregnancies. All of the abnormalities associated with growth restriction can adversely affect placental function, subsequently depriving the fetus of the nutrients necessary for optimal growth (Regnault et al., 2002). Early detection of this condition may prevent fetal death, and identification of the hormones specifically altered in IUGR pregnancies may target a route for possible therapeutic interventions. Among the hormones responsible for directing nutrient flow to the fetus, the placental lactogens appear to be necessary for

optimal fetal growth. As these hormones are also altered during IUGR pregnancies, they appear to play an important role in the maintenance and development of the fetus. Thus, focusing on the regulation of these genes may give insight into how these compromised pregnancies could be treated.

Placental Lactogen

Although placental lactogens vary greatly between species, they can be structurally characterized into two groups. Those that are similar to growth hormone (GH), which include the PL's produced by primates, and those that share greater similarity with prolactin (PRL), including rodent and ruminant PL's (Anthony et al., 1995a).

Rats, mice and rabbits have successfully been used as models for studying human disease. However, larger animals such as sheep and cattle are also proven models for human research. Sheep have anatomical and physiological characteristics similar to humans including their cardiovascular structure, multiparity, and size advantage. Sheep have proved especially useful in studying fetal, maternal and placental physiology during gestation. The sheep fetus has in fact become the principal model applicable to humans for the study of fetal physiology. The PL's are produced by ruminants, primates, and rodents, however the placental structure and length of gestation is more similar between humans and ruminants than rodents. Therefore, the ewe provides a good model by which to study pregnancy and the hormones produced during this period.

There are numerous studies in sheep indicating that exposure of the pregnant uterus to hormones such as the placental lactogens, growth hormone, progesterone and estrogen act to establish uterine growth, endometrial remodeling and secretory function occurring

during gestation. This indicates that these hormones are important for the developing fetus and may suggest potential targets for abnormal fetal growth. Although the exact function of placental lactogen is not known, PL has been shown to stimulate amino acid transport, glycogen synthesis, DNA synthesis and IGF production in fetal tissues (Anthony et al., 1995a). It is thought that this hormone acts to direct nutrients to the fetus thus, playing an essential role in fetal growth and development.

In order to understand the complex network regulating fetal growth and development, the study of pregnancy specific hormones such as placental lactogen is essential. Transcriptional regulation of the genes unique to gestation allows their products to be produced solely during this transient state. The transcriptional control of placental lactogen is governed by a myriad of factors and the identification of these transcription factors will lead to a more complete understanding of how the gene is regulated thus, providing a means to study the control of fetal growth.

Structural Characteristics of Placental Lactogen

Human

The human PL's have been more thoroughly studied than the rodent or ruminant PL's. Human PL (hPL) exists as a 191 amino acid, single chain, non-glycosylated polypeptide with a molecular weight of 22,279 (Talamantes F and Ogren L, 1988). There is an 87% amino acid sequence identity between hPL and hGH, while hPL shares only a 23% sequence identity with hPRL (Bewley et al., 1972; Shome and Parlow, 1977). In hPL two disulfide bridges between Cys^{53} and Cys^{164} and between Cys^{181} and Cys^{188} are hypothesized to stabilize the secondary and tertiary structure of hGH and hPL. These

bridges are required for full immunological activity, however they are not required for full lactogenic activity (Beck and Catt, 1971; Handwerger et al., 1972). The chemical composition of both hGH and hPL reveal strong secondary and tertiary interactions based on their resistance to denaturation by urea, acid or alkali (Aloj et al., 1972). Studies in rat tibia suggest that the growth-promoting activity of hGH requires these disulfide bridges in order to inhibit protein degradation. Additional reports indicate that growth-promoting activity decreased by 50-80% after cleavage of one disulfide bridge however, disruption of these bonds did not affect the lactogenic activity of hGH (Bewley et al., 1969; Mills and Wilhelmi, 1968). In contrast, disrupting the disulfide bonds of hPL caused an alteration in the secondary and tertiary structure of the protein and affected the immunological properties of the protein (Aloj et al., 1972). These results suggest that hGH possesses greater conformational stability than hPL (Aloj et al., 1972; Handwerger et al., 1972).

Human PL and GH are encoded by 5 genes located on chromosome 17, and human PL has two active forms, hPL-3 and hPL-4, both translated into an identical functional protein (Barrera-Saldana et al., 1983; Resendez-Perez et al., 1990). Human PL, produced in syncytiotrophoblast cells, is continuously secreted into both fetal and maternal circulation and is detected in maternal serum at three weeks of gestation (Grumbach et al., 1968).

Rodent

In contrast to hPL, rodent PL's show greater amino acid sequence identity to PRL than to GH (Talamantes F and Ogren L, 1988). Mouse PL-I (mPL-I) is an acidic, N-linked

glycoprotein appearing in two apparent M_r forms of 29-32,000 and 36-42,000, while rat PL-I has an apparent M_r of 40,000-50,000 (Colosi et al., 1987a; Robertson et al., 1982). Mouse and rat PL-I contain a 30 amino acid signal peptide, become mature proteins with 194 (mouse) and 200 (rat) amino acids and, unlike human PL, exhibit two sites for N-linked glycosylation (Colosi et al., 1987b; Robertson et al., 1990). The amino acid sequence identity between mPL-I and mPL-II is 44%, between mPL-I and mPRL it is 33% while mPL-I and mGH share only 21% amino acid sequence identity (Colosi et al., 1987b). Placental lactogen-II has been identified in the mouse, rat, and hamster (Duckworth et al., 1986; Jackson et al., 1986; Southard et al., 1986). The second PL found in rodents, PL-II, is a 191 amino acid peptide with no glycosylation sites and an apparent M_r between 20-25,000 (Colosi et al., 1982). There is an additional rat PL, PL-I variant (PL-Iv), with an apparent M_r of 33,000 and two N-linked glycosylation sites. Rat PL-Iv shows 85% amino acid sequence similarity to rPL-I (Deb et al., 1991; Robertson et al., 1991). Both PL-I and PL-II are produced by the trophoblast giant cells in the placenta, similar to the human syncytiotrophoblast cells (Soares et al., 1982; Soares et al., 1991).

Ruminant

Placental lactogens are found in the placenta of goats, cattle and sheep (Currie et al., 1990; Martal and Djiane, 1975; Murthy et al., 1982). Ruminant PL's, like the rodent placental lactogens, show more sequence similarity to prolactin than to growth hormone, with a 47-50% amino acid identity with PRL and only a 22-25% identity to GH (Anthony et al., 1995a). However, unlike the human or rodent PL's, only a single polypeptide has been isolated in ruminants (Anthony et al., 1995a). Ovine PL (oPL) and caprine PL

(cPL) are non-glycosylated polypeptides with apparent molecular weights of 22,000 and 23,000 respectively (Sakal et al., 1998; Warren et al., 1990). In contrast, bovine PL (bPL) is glycosylated by N-linked and O-linked oligosaccharides and this polypeptide has multiple isoforms with an apparent M_r between 32-34,000 (Murthy et al., 1982). Bovine and ovine PL share 67% amino acid sequence identity, while that between ovine PL and caprine PL is 86% (Anthony et al., 1995a; Anthony, 1998). The structure of oPL is similar to that of oPRL as oPL contains three disulfide bonds which form three loops in the protein (Hurley et al., 1977). Binding activity to rat lactogenic and somatogenic receptors was diminished (22.6% and 28.7% respectively) after modification of Trp^{150}, which coincides with that described for oPRL (Cymes et al., 1993; Kawauchi et al., 1973). In rat liver, Tyr^{46} has been shown to be involved in the binding capacity of oPL to somatogenic and lactogenic receptors (Cymes and Wolfenstein-Todel, 1996), suggesting the secondary and tertiary structure of oPL is important for hormone binding.

Placental Lactogen Synthesis and Secretion

Human

After week 6 of gestation, and continuing until term, localization of human PL messenger RNA has been confirmed in syncytiotrophoblast cells (Boime et al., 1982; Hoshina et al., 1982; Maruo et al., 1992; McWilliams and Boime, 1980). It is believed that a developmental switch occurs early in gestation, as cellular localization of hPL synthesis was identified in the cytotrophoblast and undifferentiated stem cells prior to week six of gestation (Maruo et al., 1992). In humans, PL can be detected by day 18 of pregnancy and maternal serum concentrations continuously increase throughout

gestation, reaching maximal concentrations of 5-10 μg/ml by the third trimester (Beck, 1970; Biswas et al., 1972; Braunstein et al., 1980; McWilliams and Boime, 1980; Tyson, 1972). Human PL production reaches concentrations of 0.5-1 gram of protein per day by term, at which point approximately 5% of the total placental mRNA is hPL (Beck, 1970; Beck and Daughaday, 1967; Biswas et al., 1972; Braunstein et al., 1980; Chen et al., 1989; Kaplan et al., 1968; Tyson, 1972). In pregnancies with reduced placental weight or placental insufficiencies, maternal serum concentrations of hPL are decreased (Saxena et al., 1969). There is also a correlation between hPL concentrations and placental weight in blood samples obtained before parturition (Saxena et al., 1969; Tyson, 1972). The increases in hPL concentrations observed during pregnancy coincide with the increases in placental weight and in the number of syncytiotrophoblast cells found as gestation progresses (PIERCE, Jr. and MIDGLEY, Jr., 1963). Instances in which maternal serum PL concentrations decreased were associated with distress of the fetus (Saxena et al., 1969). This suggests that hPL continues to increase throughout gestation due to increased placental mass and syncytiotrophoblast cell number. In the human, placental lactogen is secreted into the fetal blood, albeit at considerable lower concentrations. Early in gestation fetal concentrations of hPL are low however, these concentrations rise to 20-30 ng/ml by the end of pregnancy (Crosignani et al., 1972; Hill et al., 1988). As PL is not able to cross the placental barrier, the syncytiotrophoblast cells secrete it into both the fetal and maternal circulation (Kaplan et al., 1968).

Rodent

In the rodent chorio-vitelline and chorio-allantoic placenta, PL-I and PL-II are secreted by the trophoblastic giant cells at specific time points during gestation, with PL-I appearing early in gestation, followed by a decline in serum concentrations, after which PL-II concentrations increase (Colosi et al., 1987a; Robertson et al., 1982; Soares et al., 1983). In the mouse, PL-I can be detected at day 6 of gestation, with concentrations of 20 ng/ml, after which it increases until mid-gestation to peak concentrations of 8 μg/ml at day 9 in maternal serum (Colosi et al., 1987a; Soares et al., 1983). PL-I in the mouse declines by day 13, after which it is no longer detectable throughout the remainder of pregnancy (Colosi et al., 1987a). In contrast, mPL-II first appears at day 9 of pregnancy at concentrations of 1 ng/ml after which it increases until term to peak concentrations of 135-250 ng/ml (Soares et al., 1982; Soares and Talamantes, 1983). The observed differences in concentration range depend on the strain of mouse, as confirmed by genetic cross-breeding experiments, which indicate that the fetal-placental unit influences PL-II secretion more than the maternal environment (Soares et al., 1982; Soares and Talamantes, 1983). PL-II secretion is associated with conceptus number, with larger litters producing higher PL-II serum concentrations (Soares and Talamantes, 1983). In addition to being secreted into maternal serum, mPL-II is also found in fetal amniotic fluid at day 10, and in fetal blood at day 15 (Talamantes F and Ogren L, 1988).

Similar to the mouse, the trophoblastic giant cells of the rat placenta also secrete two PL's into the maternal serum at different stages of pregnancy. Rat PL-I is found in maternal serum at day 8 of gestation, peaks on day 12 at a concentration of 3 μg/ml, and declines by day 15 of pregnancy (Robertson et al., 1982). Rat PL-II appears at day 14

and increases until term with maximal concentrations of 500 ng/ml per day (Klindt et al., 1981; Robertson et al., 1982). A third PL, rat PL-I variant (rPL-Iv) is a product of the trophoblast giant cells and the cytotrophoblast cells, and is initially observed at gestation day 14, with peak serum concentrations of 2 μg/ml found on day 18 of gestation (Robertson et al., 1991; Robertson et al., 1996).

Rodent PL-II, unlike the PL's found in humans and ruminants, is affected by ovarian and pituitary hormones. Evidence of this can be found in rats and mice hypophysectomized at mid-gestation, which causes increases in PL-II concentrations (Blank and Dufau, 1983; Day et al., 1986; Voogt et al., 1985). In mice, these observed changes in PL-II concentrations did not affect placental mass, and the hormone concentrations in placental homogenates remained unaltered, indicating that PL-II synthesis and secretion were unaffected (Day et al., 1986). The removal of the hypothalamus results in increased maternal serum concentrations of mPL-II via reduced clearance of the hormone (Day et al., 1986; Pinon et al., 1988). Ovarian effects on PL-II appear to have contrasting effects as bilateral ovariectomy will increase PL-II secretion (Robertson et al., 1984b; Robertson et al., 1984a; Soares and Talamantes, 1985). Progesterone, when added to mouse placental explants has inhibitory actions on PL-II secretion (Soares and Talamantes, 1985). In addition to ovarian effects on PL-II, in the rat, the fetus has a stimulatory effect on PL-II levels. Removal of the fetus alone results in suppressed rPL-II while fetalectomy combined with ovariectomy increases PL-II concentrations. Based on these experiments, it is possible that fetal and ovarian factors influence PL-II secretion, while the pituitary does not affect regulation of PL-II (Robertson et al., 1984b).

Ruminant

Placental lactogen appears at day 17 in cattle and day 16 in sheep and is produced by the placental binucleate cells localized to the trophectoderm (Carnegie et al., 1982; Kappes et al., 1992; Kessler and Schuler, 1991; Martal et al., 1977; Milosavljevic et al., 1989; Wooding and Beckers, 1987). However, there are some conflicting reports on the exact location of trophoblast expression. PL is only found in the binucleate cells in sheep after day 28 and in cattle after day 22, and has been found as early as day 16 in ovine trophectoderm (Boshier, 1969; Carnegie et al., 1982; Wooding and Beckers, 1987). Although there are some reports of PL appearing in mononucleate cells, these may be due to assay conditions (Carnegie et al., 1982; Kessler and Schuler, 1991). In fact, after gestation day 50, PL expression exists solely in the binucleate cell population (Kappes et al., 1992; Martal et al., 1977; Milosavljevic et al., 1989). The ruminant placenta, unlike the human trophectoderm, does not undergo a physiological switch from cytotrophoblast to binucleate cell expression thus, providing further evidence that PL is produced by the binucleate cell population in ruminants.

The method by which PL is delivered to the maternal vasculature can be explained by the migration of binulceate cells into the maternal tissue. This occurs when the syncytium is formed after the chorionic binucleate cells migrate from the chorionic epithelium into the uterine epithelium (Wooding, 1992). In the sheep, binucleate cell number corresponds with the maternal serum concentrations of oPL, with gestation day 60 levels of 7.1 ng/ml increasing to greater than 1 μg/ml by day 135 (Kappes et al., 1992; Taylor et al., 1980). In contrast to this, bovine maternal serum levels of PL are much lower, due to the decreased binucleate cell migration out of the chorionic

epithelium observed in cattle, with PL detected by the fourth month of gestation, peaking at 3 ng/ml and remaining at this concentration until term (Wallace, 1993). In the sheep fetus, serum concentrations of PL increase from 11 ng/ml at gestation day 60 to 29 ng/ml by day 90 and remain constant until term (Kappes et al., 1992). Bovine PL concentrations in the fetus are detectable by the 8th week of pregnancy and by day 100 peak at 20-30 ng/ml (Wallace, 1993).

In the sheep, fluctuations in pituitary, hypothalamic and adrenal hormone levels, either fetal or maternal, do not affect secretion of oPL. Evidence of this comes from experiments in which the fetus is hypophysectomized, adrenalectomized, or is anencephalic, where oPL concentrations in maternal or fetal serum are not altered (Taylor et al., 1983a; Taylor et al., 1983b; Wintour et al., 1982). Further support for this is found when oPL concentrations are not affected by infusion of TRH, dopamine agonists, or somatostatin (Taylor et al., 1983b). As seen with other species, there may be a fetal signal required for oPL production as a viable fetus is necessary for oPL synthesis and secretion (Taylor et al., 1983a). This signal may be necessary for placental maintenance in order to maintain the binucleate cell population. The increase in binucleate cell number observed during gestation is associated with the increase in maternal oPL concentrations (Kappes et al., 1992).

Transcriptional Regulation of Placental Lactogen

It is believed that the PL genes arose as a result of separation from either the GH or the PRL genes, as the GH/PRL gene family is thought to have emerged from one precursor gene perhaps 350-400 millions years ago (Barsh et al., 1983; Miller and

Eberhardt, 1983). There is a common structural characteristic among the PL genes, that being 5 exons and 4 introns, suggesting the separation may have occurred 80 million years ago, with the separation of the main order of mammals, because the non-primate and primate PL genes derive from different precursor genes, either PRL or GH respectively (Miller and Eberhardt, 1983; Walker et al., 1991; Wallis, 1993).

All eukaryotic genes contain a core promoter region located upstream of, or even overlapping the transcription initiation site, and comprised of initiator elements and/or TATA boxes (Eloranta, 1996; Struhl, 1999). This transcription initiation site is necessary in all eukaryotic genes and functions as a control point in gene expression, as it acts to nucleate the preinitiation complex, by recruiting RNA polymerase II, and to bind TATA binding protein (TBP), initiator proteins or TBP associated factors (TAF's) (Eloranta, 1996; Tansey and Herr, 1997). It is the assembly of the preinitiation complex centered at the core promoter that allows basal stimulation to occur, while additional *trans*-acting factors function to further regulate transcription (Eloranta, 1996). The *trans*-acting factors that interact with the basal machinery are modular in nature with separate activation and DNA-binding domains. Classification of these activator domains is based on the type of amino acid residues found within the site, which includes proline and glutamine-rich regions as well as acidic residues (Colgan and Manley, 1992; Klages and Strubin, 1995; Klein and Struhl, 1994; Sauer et al., 1995). The tissue-specific regulation of gene expression requires multiple or even single regulatory factors and trophoblast-specific expression of placentally expressed genes, such as PL, have been observed in ruminants, rodents and primates, suggesting the regulation of these genes may occur

through conserved *cis*-acting elements that modulate placental transactivation (Eloranta, 1996).

Human

The transcriptional regulation of the placental lactogen genes has been more thoroughly examined for the human and rodent than for ruminants. The locus for the human GH/PL gene is on chromosome 17, with the five genes encoding hPL and hGH centered within a 66 kilobase region (Chen et al., 1989; Owerbach et al., 1980). In contrast, the location for the hPRL gene is found on chromosome 6 (Barsh et al., 1983). There is a 90% homology within the proximal 500 bp of the promoter region for the hPL and the hGH genes. In addition, there is 93.5% nucleotide sequence identity between hPL and hGH, while hPL and hPRL share only 42% homology, and all five genes in the hGH/hPL cluster share greater than 90% sequence identity. The hGH/hPL gene locus 5' to 3' occurs in the order: hGH-N (pituitary GH), hPL-1, hPL-4, hGH-V (placental GH), and hPL-3 (Chen et al., 1989; Hirt et al., 1987; Kidd and Saunders, 1982). Human PL-3 and PL-4 both encode an identical mature protein, while hPL-1 appears to be a pseudogene (Hirt et al., 1987; Walker et al., 1991). Human GH-V is the main GH circulating during late gestation and is expressed only within the placenta (Handwerger, 1991; Walker et al., 1991).

When mRNA expression for the hPL-3 and hPL-4 genes was analyzed, hPL-4 was found to account for 60% of the placental PL mRNA (Selby et al., 1984). In the human, the PL transcriptional start site is located 30 bp downstream of a consensus TATA box initiator element (Selby et al., 1984; Selvanayagam et al., 1984). It was determined that all of the hPL genes were transcribed by RNA polymerase II, although hPL-1 does not

produce a mature protein (Selvanayagam et al., 1984). Cell-specific expression of the genes expressed in the placenta and pituitary necessarily occurs through unique transcriptional regulatory factors, based on the similarity within their promoter regions. These tissue-specific regulatory factors may be due to differences within the proximal 500 bp promoter regions or the location of these *cis*-elements in relation to the start site (Selby et al., 1984).

Within the first 500 bp of the proximal promoter region of the hPL-3 gene there are general enhancers functioning to confer basal promoter activity. The placental specific enhancers reside downstream of this gene and promote activated transcription. The general enhancers of the hPL and hGH genes include an SP-1 site, a TATA box and a trophoblast-specific initiator element (Fitzpatrick et al., 1990; Jiang et al., 1995). These enhancers aid in the formation of the RNA Polymerase II preinitiation complex. There is an enhancer region about 2.2 kb downstream of the hPL-3 gene, located within a 1.0 kb region. Although this region was not cell-type specific, activity was increased in placental cells in transfection assays (Jiang and Eberhardt, 1995; Rogers et al., 1986; Walker et al., 1990). This enhancer region was able to function independent of orientation and position and had the ability activate heterologous promoters (Rogers et al., 1986). The placental specific transcriptional enhancers are located within a 138 bp region located 2.2 kilobases downstream of the GH/PL gene locus. There are specific protein-DNA interactions which occur within this 138 bp region that act to regulate transcription (Rogers et al., 1986). Within this region there is a GT-IIC site and a *cis*-element that binds two proteins, TEF-1 and CSEF-1, with equal affinity (Jacquemin et

al., 1994; Lytras and Cattini, 1994). TEF-1 acts to reduce transactivation by inhibiting formation of the preinitiation complex (Jiang and Eberhardt, 1996).

Within the GT-IIC site there are additional elements required within the first 241 bp of the enhancer region to achieve maximal transactivation (Jacquemin et al., 1994; Jiang and Eberhardt, 1995; Lytras and Cattini, 1994). Three sites were identified, RF-1, DF-1 and GT-IIC. Mutations in the RF-1 repressed transcription, while a mutation in the DF-1 site activated transcription. Furthermore, placing the RF-1 site adjacent to the GT-IIC site resulted in decreased activation. There is a similarity between the DF-1 element and an Ets element, suggesting this site may bind a member of the Ets family (Lytras and Cattini, 1994).

Various experiments confirmed the importance of the 241 bp within the enhancer region. Four protected regions positioned downstream of the hPL-3 gene were identified through DNase I protection assays. Two of these, located upstream of the enhancer region, had no affect on transcription in transfected cells, however, DF-3 and DF-4 were within the enhancer region and deletion of these sites decreased transactivation (Jacquemin et al., 1994). Furthermore, multiple copies of either DF-3 or DF-4 functioned to stimulate transcription in JEG-3 cells (Jacquemin et al., 1994). In another set of experiments, five footprints were reported within the enhancer region that coincided with those previously reported. Through mutational analysis, similar results were achieved indicating that mutating the GT-IIC region caused the greatest decrease in activation (Jiang and Eberhardt, 1995). This enhancer region was labeled the chorionic somatomammotropin gene enhancer (CSEn).

Further *trans*-acting factors were postulated to bind the enhancer region of the hPL gene. One of these, TEF-1, was thought to bind at the GT-IIC element, while another novel protein, CSEF-1, discovered in BeWo and COS cells, also bound the GT-IIC site with high affinity (Azakie et al., 1996; Jiang and Eberhardt, 1994; Martin et al., 1988). CSEF-1 did not immunoreact with anti-TEF-1 indicating they are distinct proteins. Multiple GT-IIC sites had greater transcriptional activity in COS-1 cells than in BeWo cells, and no stimulation was found in HeLa cells (Jiang and Eberhardt, 1995) suggesting that CSEF-1 may function as an enhancer. In addition, either up-regulation or down-regulation of TEF-1 in BeWo cells, combined with multimers of CSEn or GT-IIC sites, resulted in stimulation or repression respectively (Jiang and Eberhardt, 1995). TEF-1 interacts with TBP and inhibits TBP binding to the TATA box, thus functioning as a repressor by preventing preinitiation complex formation, supporting its role as a repressor in the hPL promoter (Jiang and Eberhardt, 1996). However, CSEF-1 may interact with the GT-IIC element in order to stimulate transcription.

Although there are similarities within the 5' proximal promoter regions of the five hGH/hPL genes, unique *cis*-elements differentially stimulate transactivation of these genes (Walker et al., 1991). For instance, in placental cells, the hPL promoter functionally activates transcription to a greater degree (3- to 5- fold more) than the hGH promoter. When combined with the CSEn region, the hPL promoter construct revealed a significant decrease in activation when the -142 to -129 region was deleted, suggesting the necessity of this region for stimulation of transcription (Fitzpatrick et al., 1990). This region represents a potential binding site for the Sp-1 transcription factor and further evidence was provided by gel mobility shifts assays which indicated that Sp-1 could

specifically compete for binding, although the element was not tissue-specific. Thus, Sp-1 could function to enhance activation of the hPL-3 enhancer. Additional reports with another promoter from this gene family, the rhesus monkey GH-v promoter, revealed a similar element at -140/-131 that interacted with both Sp-1 and Sp-3 (Jiang et al., 1995; Jiang and Eberhardt, 1995; Schanke et al., 1998). Mutational analysis revealed that the Sp-1 site is important for stimulation of basal and enhancer mediated transcription, although mutating this site did not bring transactivation down to basal levels, indicating other elements are involved in transactivation. Therefore, deletion analysis was performed and additional *cis*-elements regulating transcription were identified within the promoter region, including a TATA box at -30 bp, and an initiator element (InrE) at -15/+1 bp (Jiang et al., 1995). The CSEn region, although not able to stimulate transcription alone, functions to activate transcription in conjunction with a promoter region, suggesting that it cooperates with additional transcription factors or promoter elements, including the InrE, to activate transcription (Jiang et al., 1995).

Within the hGH/hPL gene 5' flanking regions another element involved in transcription was a functional GHF-1/Pit-1 site (Bodner and Karin, 1987; Castrillo et al., 1989; Lemaigre et al., 1989; Mangalam et al., 1989; Nelson et al., 1986; Nelson et al., 1988). It was found that GHF-1/Pit-1 bound to 150 bp of the promoter and also stimulated transcription in pituitary cell lines with -469 bp of the hGH-N or the hPL-4 promoters (Lemaigre et al., 1989; Nachtigal et al., 1993). In the pituitary, repression of the placental-specific genes occurs through two orientation dependent PSF sequences located upstream (1.7 to 2.1 kb) of the placentally expressed genes (Nachtigal et al., 1993). Two sites, PSF-A and PSF-B were protected in DNase I analysis in pituitary cell

nuclear extracts, but not in placental cell nuclear extracts, indicating that the placentally expressed genes are inhibited in the pituitary by repressors (Nachtigal et al., 1993). Therefore, the hPL gene is regulated by positive and negative factors, including the CSEn, InrE and PSF elements. Thus, the transcriptional regulation of the hPL gene involves various transcription factors that act to stimulate and repress expression of this gene, depending on the tissue.

Rodent

In rats PRL, PL-I and PL-II are located on chromosome 17 while the rat GH gene is located on chromosome 10 (Cooke et al., 1986). In comparison, mouse PL-I and PL-II are clustered with mPRL on chromosome 13 and mouse GH is found on chromosome 11 (Jackson-Grusby et al., 1988). The two rodent PL genes are expressed at unique times during gestation and they are expressed by the trophoblastic giant cells of the placenta (Talamantes F and Ogren L, 1988).

The gene locus for rodent PL/PRL is similar to that found in the human PL-GH family. The expression of the PL-I gene in mice is regulated by a 274 bp region 5' of the transcriptional start site (Shida et al., 1993). Based on deletion analysis of the mPL-I promoter, this 274 bp region appears to be necessary for placental-specific regulation of the mPL-I gene (Shida et al., 1993). There are two binding sites for activating protein 1 (AP-1) located within this 274 bp region, at -246 and -80 bp, that appear to act as general enhancers of the mPL-1 gene (Shida et al., 1993). DNase I protection analysis revealed three GATA sites within this region, at -224, -215 and -93 that regulate trophoblast-specific transcription upon binding of GATA-2 and GATA-3 proteins (Ng et al., 1994). These sites have all been shown to bind GATA-2 and GATA-3 proteins and

differentiated Rcho-1 cells were found to contain mRNA transcripts for these transcription factors (Ng et al., 1994). Additionally, when GATA-2 or GATA-3 expression vectors and the mPL-I promoter construct were co-transfected into mouse L cells lacking endogenous expression of the GATA proteins, transcription was stimulated (Ng et al., 1994). Furthermore, experiments in which the mice had disruptions in the GATA-2 or GATA-3 genes resulted in a 50% reduction in mPL-I production (Ma et al., 1997). Thus, it appears that AP-1 and GATA proteins act to regulate the trophoblast-specific transcription of the mPL-I gene. However, additional *cis*-elements may be involved in the regulation of the mPL-I gene.

A basic helix-loop-helix protein (bHLH), Hand 1, also called *hxt*, Thing-1 or e-Hand, was found to stimulate mPL-1 transcription in Rcho-1 cells, but no direct binding to a *cis*-element in the mPL promoter has been observed (Cross et al., 1995). However, Hand 1 did function to influence differentiation of trophoblast cells and to increase the number of differentiated cells (Cross et al., 1995; Scott et al., 2000). In addition, Hand-1 and E47 heterodimers have been shown to stimulate transactivation in fibroblast cells via interaction with a Th1 box. Hand-1 has also been shown to interact with other bHLH proteins, including ITF2, Mash2 and itself to stimulate transcription. Thus, Hand-1 may function either directly or indirectly to transactivate the mPL-I promoter (Scott et al., 2000).

Although no specific *trans*-acting factors have been identified in the regulation of the mPL-II gene, the region between -1778 and -1471 bp provides trophoblast giant cell-specific activation of transcription (Lin and Linzer, 1998). In transgenic mice containing 2.7 kb of the 5'-flanking sequence this region was able to confer trophoblast giant cell-

specific regulation (Shida et al., 1992). Additional experiments utilizing promoter constructs with 2.0 kb of the 5'-flanking region also exhibited significant placental activity compared to the fetus (Lin and Linzer, 1998). Transient transfection assays in Rcho-1 cells indicated that elements within the -1.3/-0.6 kb region of the mPL-II promoter were required for trophoblast-specific activation. Further experiments revealed an enhancer element residing within a 132 bp region located between -1471 and -1340 bp. There is also a specific interaction with a protein present in Rcho-1 cells that occurs with the -1457/-1471 region (Lin and Linzer, 1998).

There is less known about the rat PL-I and PL-II genes compared to the mouse. Trophoblast-specific transactivation of the rPL-II gene appears to reside within -3000 to -765 bp of the 5'-flanking sequence (Shah et al., 1998). The region from -1435 to -765 bp provides minimal support while an enhancer element exists between -2838 and -1729 bp (Sun and Duckworth, 1999). The regulation of the rat PL-II gene appears to reside within a 65 bp enhancer region adjacent to two *cis*-acting elements, an Ets element at -1743 and an AP-1 site at -1759 (Sun and Duckworth, 1999). Transactivation was significantly reduced when these sites were mutated and transfected into Rcho-1 cells, indicating they are important for transcriptional regulation (Sun and Duckworth, 1999). Specific binding between the AP-1 element and a Fos/Jun heterodimer occurred, but no binding between Ets1 or Ets2 and the 65 bp region was observed. However, co-transfection studies with the 65 bp element revealed that both Ets2 and Fos/Jun expression vectors were able to stimulate transcription (Sun and Duckworth, 1999). Therefore, in the rPL-II promoter, the AP-1 element is functional and transcription can be augmented via interaction with Fos/Jun heterodimers.

Ruminant

In ruminants bovine placental lactogen (bPL) and bovine prolactin (bPRL) are located on chromosome 23 (Dietz et al., 1992). The bovine PL gene consists of 5 exons and 4 introns and spans 12 kb (Kessler and Schuler, 1991). The oPL gene, like bPL, contains 5 exons and 4 introns which span a genomic sequence of 12 kb, and 4.5 kb of 5'-flanking sequence has been characterized (Liang et al., 1999). Although, the bPL gene most likely exists as a single copy gene, there is evidence of allelic variants and differential splicing (Kessler and Schuler, 1991; Yamakawa et al., 1990). Ovine PL, like bPL, also exists as a single copy gene (Liang et al., 1999). In both the bovine and ovine PL genes, the transcriptional start site is located at an identical nucleotide ahead of the start codon for translation (Kessler and Schuler, 1991; Liang et al., 1999). Although, putative regulatory elements have been identified in the bPL promoter, AP-1, AP-2, and thyroid hormone responsive elements, their functionality has yet to be tested (Kessler and Schuler, 1991). There is an 86% nucleotide sequence identity between oPL and bPL in the first 369 bp of the 5' flanking region.

Previous analysis of the ovine placental lactogen (oPL) promoter has identified specific regions responsible for activated stimulation of transcription. Various footprints were identified in the oPL proximal promoter using DNase I protection analysis (Liang et al., 1999). Using various deletion constructs of the proximal promoter region, transcriptional regulation of the oPL gene has been examined in transient transfection assays using trophoblast derived BeWo and Rcho-1 and non-trophoblast derived HeLa and C127 cell lines. Maximal stimulation of trophoblast-specific activity was localized

to the sequence between -383 to +16 relative to the transcriptional start site and six of the footprints lie within this region (Liang et al., 1999). The minimal promoter region from -124/+16 provides trophoblast-specific transactivation and three footprints lie within this region, FP1 (-12/+7), FP2 (-74/-48) and FP3 (-123/-95). Using a sequence from -217/+16 resulted in loss of trophoblast-specific activity however, the sequence from -217 to +16 was able to confer basal stimulation of the oPL gene in trophoblast cells and this region contains FP4 (-173/-137). The region encompassing -383 to -217 appears to enhance activation of the minimal promoter and two footprints, FP5 (-284/-246) and FP6 (-319/

-349), lie within this region (Liang et al., 1999). The construct containing -383 bp of the oPL 5'-flanking sequence was able to confer activity equivalent to the maximal activity observed with constructs containing additional promoter sequence, indicating that the trophoblast-specific elements necessary for full transactivation reside within this -383/+16 sequence (Liang et al., 1999; Limesand, 2000). The construct from -124/+16 bp of the oPL promoter was able to activate trophoblast-specific activity although, the transactivation was reduced compared to that observed with the -383/+16 construct, indicating it functions as a minimal promoter (Liang et al., 1999; Limesand, 2000).

As there were variations in the promoter activity for the Rcho-1 and BeWo trophoblast cell lines, alternative *cis*-elements may function in transactivation of the oPL gene. Analysis of protein-DNA interactions within the proximal 1.1 kb promoter region of the oPL gene using DNase I protection assays and ovine binucleate cell (BNC) nuclear extracts identified 19 protected regions. These included 10 GATA elements, two within the minimal promoter region at FP2 and FP3 (Liang et al., 1999; Limesand, 2000).

Additional elements within the minimal promoter included an AP-2 site and an E-box element (Liang et al., 1999). A potential initiator element was found which encompassed the transcriptional start site. As the non-canonical TATA box was not protected in the DNase I assays, this initiator element may be required for transcription. There was also a direct repeat of a GAGGAG sequence in FP5 and FP6, both within the region required for maximal trophoblast-specific stimulation of transcription (Liang et al., 1999).

Three footprints, identified through DNase I protection analysis, reside within the minimal promoter region (-124/+16) of the oPL gene (Liang et al., 1999) An AP-2 *cis*-acting element was identified at -58 bp relative to the transcriptional start site, indicating it may be involved in transactivation. Through EMSA and supershift assays it was determined that this protein interacted with the minimal promoter region of the oPL gene (Liang et al., 1999). Further experiments were conducted to functionally test AP-2 in the transcriptional activation of the oPL gene. Mutational analysis of this *cis*-element in BeWo cells confirmed its ability to stimulate transcription with a -124/+16 oPL promoter construct. AP-2 elements have been identified in other placental genes and AP-2α is involved in transactivation of hCG α- and β- subunit genes (Johnson et al., 1997). The presence of AP-2α in the ovine placenta was confirmed via Northern hybridization and immunocytochemistry and an ovine cDNA library was subsequently screened. Four AP-2 cDNA's were isolated from this ovine placenta cDNA library and, after nucleotide sequencing, the AP-2 mRNA from ovine placenta was found to share identity with human AP-2α. Although, the AP-2α cDNA's were similar to both mouse and human AP-2α sequences, there were variations in the predicted N-terminus revealing the identification of three unique AP-2α splice variants (Limesand and Anthony, 2001).

Two of the clones were 100% identical throughout their coding regions (AP-2α v4). However, they were not able to transactivate the oPL promoter in functional transfection assays. Two of the clones, AP-2-α v6 and AP-2-α v7 stimulated transactivation of the oPL minimal promoter suggesting they act through the *cis*-element within the oPL promoter and are functionally involved in tranasctivation of the oPL gene (Limesand and Anthony, 2001).

Although there was a functional GATA element at -102 bp located within the protected region from -123/-95 bp within the oPL promoter (FP3), mutational analysis of this element from -99/-92 did not significantly reduce transactivation in choriocarcinoma cells. However, an additional mutation within the FP3 region from -109 to -102 resulted in significant decreases in transactivation in BeWo, but not in Rcho-1 cells, indicating that this element is not critical in all placental cell types. These results suggested that another *cis*-element within the FP3 region was functioning to stimulate transcription (Limesand et al., 2004). Further investigations of this FP3 region were performed to determine whether another nuclear protein was interacting with this sequence. EMSA analysis utilizing a labeled oligonucleotide from -116 to -102 bp identified a specific protein-DNA complex. Specific competitors for oGATA and oAP2 were not able to reduce complex formation, indicating the protein is distinct from the GATA protein previously found to interact with the FP3 region. However, the nucleotides identified within the oPL promoter as binding this nuclear protein were identical to a binding site found within the hPL promoter (Limesand et al., 2004). Using this sequence, an ovine cDNA library was screened and positive cDNAs were identified and found to be similar to both human and mouse Purα, a single-stranded DNA binding protein. Functional co-

transfection studies using a Purα expression vector were performed in choriocarcinoma cells and the results showed that Purα was able to stimulate transactivation of the oPL minimal promoter (Limesand et al., 2004).

In summary there have been various transacting factors demonstrated to interact with the minimal promoter region of the oPL gene (Liang et al., 1999; Limesand et al., 2004). These include an initiator element at FP1, GATA elements within FP2 and FP3, a Pur-α element within the -124 FP3 region and an E-box at FP4. Further interactions have been tentatively identified at FP5 and FP6, including direct repeats of a GAGGAG sequence within FP5 and FP6 (Limesand et al., 2004). Both FP5 and FP6 contain specific sequences shown to be functional through mutation analysis. Thus, further examination of these regions, specifically footprint 6, is necessary to identify the factors activating transcription in a trophoblast-specific manner.

Previous results from transfection and EMSA analysis with the footprint 6 region show decreased activity when the GAGGAG region within footprint 6 was mutated (Liang et al., 1999; Limesand, 2000). EMSA analysis using ten 2 base pair mutations within the footprint 6 region (encompassing this GAGGAG sequence) showed a decrease in binding of the mutated bases centering around this region (Limesand, 2000). Excess addition of these unlabeled mutated bases was unable to compete with binding of the labeled oligonucleotide (Limesand, 2000). However, this assay failed to define a single contiguous *cis*-element interacting with the BNC nuclear proteins. In addition, computer analysis of the oPL proximal promoter region from -383/+16 identified potential binding sites for Sp and CEBP-α proteins within the FP6 region, indicating these transcription factors may interact with the FP6 region. Therefore continued exploration of this region,

through transient transfection and mutation analysis is necessary to identify the specific nucleotides involved in transactivation of the oPL gene.

Sp1 and Sp3

Genes with proximal promoters (located within the first few hundred base pairs relative to the transcriptional start site), such as the placental lactogens, usually contain a TATA sequence, necessary to bind TFIID. However, many promoters, including the oPL promoter, do not have a functional TATA box (Liang et al., 1999). These promoters require binding sites for other factors, such as the specificity proteins (Sp) to facilitate activation of transcription. The Sp family of transcription factors interacts with the promoters of genes through binding of GC-rich regions (Tjian and Maniatis, 1994). This family includes Sp1, Sp2, Sp3 and Sp4. Sp4 is found predominantly in neuronal tissues, while Sp1, Sp2 and Sp3 exist in many different tissues. These factors bind to GT/A rich motifs as well as to GC boxes (Suske, 1999). The Sp1 and Sp3 proteins are involved in the transcriptional activation of various genes and bind to the same *cis*-elements. Sp3 has been shown to be involved in transcriptional activation in a number of developmental genes (Kishikawa et al., 2002; Schanke et al., 1998; Wong and Lee, 2002). In the monkey, Sp1 and Sp3 are necessary for transcriptional activation in syncytiotrophoblast cells (Schanke et al., 1998). Sp3 stimulates transcription from the rat connexin 40 promoter, and regulates transcription of the mouse *mer* gene (Teunissen et al., 2002; Wong and Lee, 2002). Sp3 also plays a role in the transcriptional enhancement of human endometrial stromal cells (Gao and Tseng, 1996).

The transcription factors Sp1 and Sp3 share more than 90% sequence homology in their DNA-binding domains. They both bind to the same GC-rich promoter elements and have been found to exert their regulatory effects in various cellular and viral genes. In addition, it has been found that all mammalian cells that express Sp1 also express Sp3 mRNA (Hagen et al., 1994; Kingsley and Winoto, 1992). However, transcriptional activity between the Sp family members is not functionally equal. While Sp1 acts mainly to stimulate transcription, Sp3 functions as both a repressor and activator of transcription (Birnbaum et al., 1995; Kumar and Butler, 1997; Liang et al., 1996). Sp3 represses Sp1 activation of the ornithine decarboxylase promoter (Kumar and Butler, 1997), the dhfr promoter (Birnbaum et al., 1995) and the SV40 early promoter (Hagen et al., 1994; Majello et al., 1997). Sp3 has been found to activate transcription of various promoters including human thymidine kinase (Birnbaum et al., 1995), PDGF-B (Liang et al., 1996) and human α2 (I) collagen (Ihn and Trojanowska, 1997). Although Sp1 and Sp3 are ubiquitously expressed, and may in fact replace each other in some manner, knockouts reveal their *in vivo* actions to be distinct (Bouwman et al., 2000). In mice, Sp1 *null* embryos die at day 10 and are severely growth retarded (Marin et al., 1997). In comparison, Sp3 *null* mice develop until birth with no obvious abnormalities except reduced body weight. However, these Sp3 knockout mice die within 10 minutes after birth due to respiratory failure (Bouwman et al., 2000). Therefore, while Sp1 and Sp3 may have similar functions during early development, these functions become more distinct as development progresses.

Although Sp1 acts specifically as an activator of transcription, Sp3 is bi-functional, and may stimulate or repress transcriptional activity. Sp3 has been shown to be inactive

for promoters that contain multiple Sp binding sites, effectively competing with Sp1 for DNA binding (Yu et al., 2003). Sp1 acts synergistically to activate transcription of promoters containing adjacent Sp binding sites. In contrast, Sp3 competes with Sp1 for promoter occupancy, reducing Sp1 mediated activation of promoters with multiple Sp binding sites (Yu et al., 2003).

While most studies reveal that Sp1 acts to activate transcription and Sp3 either activates or repress transcription, depending on the promoter, Sp1 and Sp3 can also act together to negatively influence transcription. In the expression of growth hormone by monocyte cells, over-expression of Sp3 causes an inhibition of transcription while over-expression of Sp1 does not significantly affect transcriptional activation. Furthermore, co-transfection of both Sp1 and Sp3 also inhibits transactivation, indicating that Sp3 alone or in conjunction with Sp1 may inhibit transcription (Vines and Weigent, 2000). Sp1 and Sp3 have been shown to interact with the promoters of various genes, including those expressed in the placenta, such as the hPL3 gene and the rhesus monkey GH-v gene (Fitzpatrick et al., 1990; Jiang and Eberhardt, 1995; Schanke et al., 1998). Thus, these *trans*-acting factors may function to enhance activation of the genes expressed during gestation.

CEBP-α

C/EBP-α is a CCAAT enhancer binding protein belonging to the basic region leucine zipper (bzip) family of transcription factors that includes CEBP-α, -β, -δ, -γ, -ε and -ζ (Hanson, 1998; Yamanaka et al., 1998). The CCAAT enhancer binding protein is a sequence-specific, heat stable, DNA-binding protein that is involved in the transcriptional

regulation of a number of developmental genes (Johnson et al., 1987). The CEBP proteins bind to the CCAAT sequence found in the promoters of various genes and have been implicated in the development of fetal tissues through transactivation of various cell specific genes (Birkenmeier et al., 1989; Christy et al., 1989). The CEBP proteins interact with various *trans*-acting factors to exert their regulatory effects on transcription. There is evidence that CEBP-α interacts with Ets family members to stimulate transcription in eosinophils (McNagny et al., 1998). CEBP-α has also been shown to stimulate transcription in rat liver (Descombes et al., 1990). In myeloid cells CEBP-α acts to regulate transcription through interactions with members of the Sp family (Lopez-Rodriguez et al., 1997).

There is evidence that CEBP proteins interact with other *trans*-acting factors, such as the Sp proteins, to stimulate transcription in genes involved in growth and development (McKnight S, 1992). CEBP proteins have been shown to cooperatively activate transcription with members of the Sp family. In order to stimulate the promoter for the chum salmon insulin-like growth factor II gene CEBP-β requires the transcription factor Sp1 (Palamarchuk et al., 2001). The ability of CEBP-α to stimulate activation of the CD11c integrin gene promoter is also dependent on cooperation by adjacent Sp1 elements, indicating a functional interplay between CEBP and Sp proteins (Lopez-Rodriguez et al., 1997). The CEBP-α protein has also been implicated in promotion of granulocyte differentiation (Radomska et al., 1998). While the function of CEBP proteins is varied they do appear to play important roles in growth and differentiation. These transcription factors act in conjunction with other *trans*-acting factors, specifically the Sp proteins, to regulate developmental genes. This cooperation may be important in

the regulation of other placentally expressed genes such as the placental lactogens. Thus, further investigation of the CEBP proteins as potential regulators of the genes expressed during gestation is necessary.

Summary

The regulation of transcription is the basis by which specific genes are turned on in some cells and remain inactive in others. During gestation this specific activation is even more apparent. The control of gene expression is complex and involves the coordination of various transcription factors. It is important to understand the interactions between *trans*-acting factors and their target genes in order to begin to define the control of gene expression. Studying the transcriptional regulation of specific genes involved in fetal growth and development will provide insight into those factors governing fetal growth. The identification of the genes responsible for fetal development and the analysis of their transcriptional regulation will provide avenues through which genetic therapies can be explored.

Placental lactogen, a gene expressed only during pregnancy, is believed to impact fetal growth. The regulation of this gene is essential in understanding the factors involved in promoting and maintaining fetal development. Various *cis*-elements and *trans*-acting factors involved in the regulation of the oPL gene have been identified. Through deletion analysis, the region within the proximal promoter (-383/+16) necessary for maximal trophoblast-specific activation has been confirmed. Through DNase I footprint analysis specific sequences within this -383/+16 bp region have been shown to interact with

nuclear proteins. Mutational analysis of a GAGGAG sequence found in both FP5 and FP6 confirmed the necessity of this element in activation of transcription in choriocarcinoma cell lines. Although EMSA analysis confirmed the binding of nuclear proteins to the FP6 region, the specific *cis*-elements involved in transactivation have not been defined. Therefore, further analysis of the FP6 region within the oPL proximal promoter is necessary to identify the factors involved in transactivation of the oPL gene.

Chapter III

IDENTIFICATION OF A *TRANS*-ACTING FACTOR INTERACTING WITH THE FOOTPRINT 6 REGION OF THE OVINE PLACENTAL LACTOGEN GENE PROMOTER

Introduction

The placenta is a transitory organ, with the sole purpose of providing an optimal environment for the growth and development of the fetus. The endocrine functions of the placenta influence fetal and maternal tissues in order to re-direct the flow of nutrients towards the fetus. This is accomplished through the production of a plethora of hormones and growth factors, including the placental lactogens. This hormone, a member of the growth hormone/prolactin gene family is found across a wide range of species, including ruminants, primates, and rodents, and it is believed to alter maternal metabolism such that the fetal nutrient pool increases, providing sufficient reserves for fetal growth (Anthony et al., 1995a).

Although, the human placenta is quite different from that of the sheep from a strict anatomical perspective, from a functional standpoint, they are quite similar. The multi-villous nature of the maternal-fetal vasculature in the sheep and human is more similar

than either the sheep and rodent or the human and rodent (Steven, 1975). Thus, the sheep provides a useful model for investigating the hormones involved in the regulation and maintenance of fetal development (Steven, 1975). During intrauterine growth restricted pregnancies, the fetus is compromised and the risk of death increases with gestational age. This disease affects 8% of all pregnancies and in severe cases requires early delivery of the fetus in order to avoid fetal mortality (Brar and Rutherford, 1988; Pollack and Divon, 1992). This condition is caused by a variety of factors, including aberrations in hormones and growth factors, decreased maternal nutrient intake and restricted nutrient flow to the fetus (Anthony et al., 2003). Placental lactogen is one hormone found to be decreased in growth restricted pregnancies, providing support for its role in maintaining the developing fetus throughout gestation (Regnault et al., 1999).

The transcriptional regulation of the ovine placental lactogen gene has been studied through investigation of 4.5 kb of the 5'-flanking sequence, relative to the transcriptional start site (Liang et al., 1999). Trophoblast-specific activation of the reporter gene occurred with 1.1 kb of the 5' flanking sequence and within this region 19 footprints were identified through DNase I digestion analysis (Liang et al., 1999). Maximal transcriptional activation in trophoblast-derived cell lines was found to lie within the proximal -383 bp of the oPL gene and six of the footprints are found within this region (Liang et al., 1999). The minimal promoter region from -124/+16 bp provides trophoblast-specific transactivation and includes an AP-2 element, two GATA elements and a Purα element (Liang et al., 1999; Limesand et al., 2004; Limesand and Anthony, 2001).

The region encompassing -383/-217 bp of the oPL promoter appears to enhance activation of the minimal promoter. Within this region two footprints were identified,

footprint 5 (-286/-246) and footprint 6 (-349/-319). Although no previously defined *cis* elements were identified within these regions, a direct repeat of the GAGGAG sequence, located within these footprints, was found to be functional through mutation analysis (Liang et al., 1999). However, the *trans*-acting factors binding to FP6 have yet to be identified. Therefore, the focus of this project was to identify and analyze the transcription factors interacting with the FP6 region of the oPL promoter

Materials And Methods

Binucleate Cell Isolation and Separation

Mature ewes were bred at behavioral estrus (day 0) and at 100 day post coitus (dpc), they were hysterectomized by midventral laparotomy after pentobarbital induced euthanasia and exsanguination. Fetal cotyledonary tissue was manually removed from the placenta, rinsed three times in physiological saline and digested. Digestion was performed in a 10% Trypsin, PBS solution (405 ml H_2O, 45 ml 1X PBS, 50 ml 10X Trypsin) as described by Reimers, *et al.* for 15 minutes at 37 °C (Reimers et al., 1985). The partially digested tissue was then filtered through 4 layers of cheesecloth and digested for an additional 60 minutes. The tissue was again filtered through 4 layers of cheesecloth, the flow-through collected, and the undigested tissue subjected to another 60 minute digest. The flow-through was collected, added to the first, and centrifuged at 1200 x g for 10 minutes. The supernatant was discarded and the pellet resuspended in a 4% glycerol/PBS solution, to lyse the red blood cells, and incubated at 37 °C for 30 minutes. The cells were subsequently washed and centrifuged 3 times in a 1X PBS solution for 5 minutes at 600 x g. Prior to the final wash, the cells were filtered through 8 layers of cheesecloth and

resuspended at a concentration of 1 x 10^7 cells per ml. In order to isolate the binucleate cells, 4 ml of the cell solution was layered in a Percoll gradient. The gradient was generated by mixing 9.5 ml Percoll (Sigma) solution at a density of 1.13 ± 0.005 g/ml with 2.5 ml 10 X PBS and 13 ml H_2O then, centrifuging for 30 minutes at 25,000 x g. This created a gradient through which the larger binucleate cells migrated, forming a band distinct from the smaller uninucleate cells. In the gradient the lower band represented the binucleate cells while the upper band represented the uninucleate cells. Once the cell mixture was layered onto the gradient, they were centrifuged at 1600 x g for 15 minutes. Following separation, the lower band of cells was removed, rinsed twice in PBS and counted. The nuclear protein from these cells was subsequently extracted using the procedure of Dignam *et al.* (Dignam et al., 1983).

Electrophoretic Mobility Shift Assays and Nuclear Extractions

Nuclear extractions were performed on ovine binucleate cells and BeWo cells expanded in culture according to the procedure of Dignam *et al.* (Dignam et al., 1983). Binucleate cells from gestation day 100 ovine cotyledonary tissue were isolated and purified (Liang et al., 1999; Morgan et al., 1990; Reimers et al., 1985). After collection the cells were rinsed in 1X PBS, centrifuged at 3,000 x g and resuspended in hypotonic buffer (10mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT) and allowed to swell on ice for 10 minutes. The cells were homogenized using a Dounce homogenizer and centrifuged for 20 minutes at 15,000 x g. The pellet was resuspended in equal amounts of low salt NET buffer (0.15 M NaCl, 0.1 mM EDTA, 20 mM Tris, 0.2 mM PMSF, 0.5 mM DTT) and high salt NET buffer (1 M NaCl, 0.1 mM EDTA, 20 mM Tris,

0.2 mM PMSF, 0.5 mM DTT) and placed on ice for 30 minutes to allow the nuclei to extract. Following extraction and centrifugation at 20,000 x g for 30 minutes the nuclear proteins were dialyzed against Dignam D buffer (20 mM HEPES pH 7.9, 0.2 mM phenylmethylsuphonyl fluoxide (PMSF), 0.5 mM dithiothreitol (DTT), 0.2 mM EDTA and 20% glycerol) in dialysis tubing with a molecular weight cutoff of 25,000 (Pharmacia). Following dialysis the nuclear protein was centrifuged for 20 minutes at 20,000 x g to remove any remaining cell debris. The protein concentration was determined using a Bradford assay after which they were snap frozen and stored at -80 °C.

Electrophoretic mobility shift assays were performed using T4 polynucleotide kinase (MBI Fermentas, Hanover, MD) to radiolabel 10 picomoles of the antisense strand of a synthetic FP6 (-325/-345) oligonucleotide by phosphorylation of the 5' end with [γ-^{32}P] ATP (MP Biomedicals, Irvine, CA). After labeling of the anti-sense strand, 10 picomoles the sense strand was added to the reaction, heated to 95 °C for 5 minutes to completely separate the strands then, the DNA was cooled to room temperature and allowed to anneal overnight at 4 °C. The binding assays were performed in Dignam D buffer: 20% glycerol (v/v), 100 mM KCl, 0,2 mM EDTA, 0.25 mM DTT, 20 mM HEPES (pH 7.9 at 4 °C), and 0.2 mM PMSF. Spermidine (1 mM) and poly (dI-dC)-poly (dI-dC) were also added to the binding assays. The nuclear proteins (20 μg), the reaction buffer, and the unlabeled competitor oligonucleotides (100-fold molar excess) were gently mixed and placed on ice for 5 minutes, incubated at 30 °C for 5 minutes then, placed on ice for an additional 5 minutes. Five fmoles of the labeled oligonucleotide (50,000 cpms per reaction) was then added to the reaction mixture and incubated for 20 minutes at 30 °C. The mixture was subsequently cooled on ice for 5 minutes, and electrophoresed through a pre-run non-

denaturing polyacrylamide 5% TBE gel for 3 hours at 4 °C. The gel was then dried onto Whatman paper for 60 minutes, and exposed to x-ray film overnight at -80 °C. Four labeled oligonucleotides were used in the EMSA and supershift assays, FP6 -339/-359, FP6 -325/-345, FP6 -321/-341 and FP6 -318/-338 **(Table 1)** to determine whether one sequence provided a stronger interaction with the nuclear extracts and anti-serum.

Southwestern Analysis

Southwestern analysis was performed on BeWo, HeLa, Jurkat, JAR, Rcho-1 and binuleate cell nuclear extracts. A 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate the nuclear proteins. Electrophoresis was carried out at 4 °C. After separation, the size-fractionated proteins were transferred to a 0.22 μm nitrocellulose membrane (Micron Separation, Inc., Westborough, MA) and renatured in 10 mM HEPES pH 7.9, 10% glycerol, 0.1% Igepal CA-630 (Sigma Chemical Co.), 50 mM KCl, 0.5 mM DTT, 5 μg/ml sonicated Herring Sperm DNA, 5% non-fat dry milk, 100 μg/ml BSA, 25 μg/ml Yeast tRNA and 1 mM spermidine. The binding reaction contained 20 mM HEPES pH 7.9, 0.1 % Igepal CA-630, 10% glycerol, 100 mM KCl, 1 mM spermidine, 0.5 mM DTT, 5 μg/ml sonicated Herring Sperm DNA, 10 μg/ml BSA, 2.5 μg/ml Yeast tRNA, 0.5% non-fat dry milk and 2 x 10^6 cpm/ml radiolabeled double stranded oligonucleotide, end labeled with T4 polynucleotide kinase [γ-^{32}P] ATP. The oligonucleotide used was a concatamer of footprint 6: FP6F, 5'-AAG ACC CCT GGA GGA GGG CAT GGC AAC CAG ACC CCT GGA GGA GGG CAT GGG AAC CAG ACC-3'. The binding reactions were performed at 4 °C for four hours. The membranes were incubated with either radiolabeled FP6 oligonucleotide alone or in conjunction with

Table 1: Footprint 6 double stranded EMSA and supershift oligonucleotides.

FP6	Double Stranded Oligonucleotides
-359 / -339	**5'** CCTGAGTAGGGAAGACCCCTG GGACTCATCCCTTCTGGGGAC **5'**
-345 / -325	**5'** ACCCCTGGAGGAGGGCATGGC TGGGGACCTCCTCCCGTACCG **5'**
-341 / -321	**5'** CTGGAGGAGGGCATGGCAACC GACCTCCTCCCGTACCGTTGG **5'**
-338 / -318	**5'** GAGGAGGGCATGGCAACCCAT CTCCTCCCGTACCGTTGGGTA **5'**

100- or 200- fold molar excess unlabeled FP6 oligonucleotide. Following the binding reactions, the membranes were washed in ice cold 20 mM HEPES (pH 7.9), 0.5 mM DTT, 1 mM spermidine, 10% glycerol and 100 mM KCl three times for 10 minutes and subsequently exposed to x-ray film for 24-72 hours.

FP6 Block Mutation Constructs

Three block mutations were generated within the DNAse I protected region of FP6 using dual PCR (Perkin Elmer, Branchburg, NJ) amplification, in the -380 pGL3 vector to encompass the GAGGAG sequence. For each mutation over-lapping oligonucleotides were designed containing a *Not* I restriction endonuclease recognition sequence at the 5' end. The following primers were used in conjunction with the -380 pGL3 vector as the template and either the forward (RV3) or reverse (GL2) pGL3 primers (Promega, Madison, WI): 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 were used to amplify the 5' or 3' portion of the proximal -383 base pairs of the oPL gene. In the first PCR reaction, the solution contained 6.6 fmol of linearized -383 pGL3, 0.2 μM of the forward and reverse oligonucleotides, 0.1 μM of each dNTP, 1 U of *Taq* DNA Polymerase (Gibco BRL, Gaitherburg, MD), in a 1X reaction buffer containing 1.5 mM $MgCl_2$. The parameters for the PCR procedure consisted of a 5 minute incubation at 95 °C, a denaturation step for 1 minute at 94 °C, a 2 minute annealing step at 48 °C, followed by a 1 minute extension step

at 72 °C. After 10 cycles the annealing temperature was increased to 60 °C for an additional 30 cycles. The DNA fragments generated from the PCR were then purified on an agarose gel using DEAE membranes (Ausubel *et al.* 1995) and added in equal molar ratios to a second PCR for use as the template DNA with the pGL3 forward and reverse oligonucleotides. The resulting PCR products were digested with *Kpn* I and *Hind* III restriction endonucleases, purified using agarose gel electrophoresis, and subsequently ligated into the pGL3 Basic vector. The FP6 block mutations Δ1, Δ2 and Δ3, **(GAAGACCCCTGGAGGAGGGCATGGCAACCCAT),** were confirmed using

Δ3 Δ2 Δ1

Southern analysis and nucleotide sequencing. An alkaline-lysis procedure (Ausubel *et al.*, 1995) followed by CsCl equilibrium centrifugation was used to obtain covalently closed circular DNA.

Construction of Two-base Pair Mutation Constructs

Two base pair transversion mutations, encompassing FP6 (-349 to -318) were generated using dual PCR amplification. Each primer contained a two base pair transversion mutation flanked by 10 base pairs on each side of the wild type (WT) -380 oPL promoter sequence. Sixteen forward and sixteen reverse primers containing the mutations were generated starting at -349 and ending at -318 (**Table 2**). Polymerase chain reaction (PCR), using *Taq* DNA Polymerase, was employed using the linearized -380 pGL3 WT plasmid as the template with a separate reaction for the forward and the reverse primers. The PCR products were then agarose gel purified using DEAE membranes and annealed together to provide the template for the second round of PCR. The second PCR was performed using

Table 2: Primers used in generating two-base pair transversion mutations in footprint 6.

Mutation #	Forward Primers	Reverse Primers
MUT 1	cctgagtagg **CT** agacccctgg	ccaggggtct **AG** cctactcagg
MUT 2	tgagtaggga **TC** acccctggag	ctccaggggt **GA** tccctactca
MUT 3	agtagggaag **TG** ccctggagga	tcctccaggg **CA** cttccctact
MUT 4	tagggaagac **GG** ctggaggagg	cctcctccag **CC** gtcttcccta
MUT 5	gggaagaccc **GA** ggaggagggc	gccctcctcc **TC** gggtcttccc
MUT 6	gaagacccct **CC** aggagggcat	atgccctcct **GG** aggggtcttc
MUT 7	agacccctgg **TC** gagggcatgg	ccatgccctc **GA** ccaggggtct
MUT 8	acccctggag **CT** gggcatggca	tgccatgccc **AG** ctccaggggt
MUT 9	ccctggagga **CC** gcatggcaac	gttgccatgc **GG** tcctccaggg
MUT 10	ctggaggagg **CG** atggcaaccc	gggttgccat **CG** cctcctccag
MUT 11	ggaggagggc **TA** ggcaacccat	atgggttgcc **TA** gccctcctcc
MUT 12	aggagggcat **CC** caacccattc	gaatgggttg **GG** atgccctcct
MUT 13	gagggcatgg **GT** acccattcca	tggaatgggt **AC** ccatgccctc
MUT 14	gggcatggca **TG** ccattccagc	gctggaatgg **CA** tgccatgccc
MUT 15	gcatggcaac **GG** attccagcat	atgctggaat **CC** gttgccatgc
MUT 16	atggcaaccc **TA** tccagcattc	gaatgctgga **TA** gggttgccat

this template with the pGL3 forward and reverse primers, RV3 and GL2. The second PCR product was agarose gel purified and digested, along with the pGL3 Basic plasmid, for one hour at 37 °C with *Kpn* I and *Hind* III. Both digestion mixtures were gel purified and a ligation reaction was performed overnight at room temperature using a 1:3 molar ratio of plasmid to PCR insert. Competent DH5α cells were transformed with the ligation mixture and grown with antibiotic selection at 37 °C for 15 hours. Plasmid DNA was isolated from resulting colonies and analyzed by restriction digest with *Kpn* I and *Hind* III. Digested DNA was separated on a 1% agarose gel to confirm the size of the insert. DNA with the correct insert size was analyzed via nucleotide sequencing. The sequences were subsequently compared to the WT -380 pGL3 sequence using BLAST analysis to confirm that the mutation was correct and there were no polymerase errors. Once the mutations were correctly obtained the plasmids were amplified and isolated using a CsCl centrifugation gradient.

DNA Amplification and Purification

All DNA utilized in the transient transfections and co-transfections was amplified using *E. coli*, isolated and purified using an alkaline-lysis procedure and CsCl equilibrium gradient centrifugation (Sambrook, 1989). The bacterial cultures containing Luria Broth and ampicillin (50 μg/ml), were spiked with one colony from the DNA plasmid stock and allowed to grow overnight with agitation at 37 °C. Following this overnight growth, the resulting culture was centrifuged to pellet the cells, the cells were lysed and neutralized and the plasmid collected and precipitated overnight at -20 °C. The precipitation mixture was then centrifuged to pellet the DNA, incubated with RNase at 37 °C then, extracted with a

1:1 chloroform/phenol solution. The DNA was allowed to precipitate again overnight then, mixed with CsCl (1.0 gm of CsCl for every 1.0 ml of solution, for a continuous gradient resulting in a density of 1.5-1.6 gm/ml) and ethidium bromide (EtBr) and subjected to ultcentrifugation in a Beckman (Beckman, Palo Alto, CA) NVT 90 nearly vertical rotor at 484,481 x g for 4 hours at 20 °C to separate the plasmid from the linear DNA. The DNA was subsequently extracted with isoamyl alcohol to remove all CsCl and EtBr and precipitated overnight at -20°C. The final DNA solution was centrifuged a final time to remove all debris, precipitated with ethanol then, allowed to air dry before being resuspended in a TE solution. All DNA was quantified using a 260/280 UV spectrophotometer ratio, and stored at -20 °C.

Cell Culture and Transient Transfections

BeWo cells (a human choriocarcinoma cell line) were obtained from American Type Culture Collection (Rockville, MD). BeWo cells were maintained in F12K medium (Mediatech, Inc., Herndon, VA), supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-Products, Inc., Calabasas, CA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO). Cells were maintained in monolayer cultures at 37 °C in 95% air, 5% CO_2 and 100% humidity. Passage numbers for the transient transfection analysis were kept at less than 10 to ensure optimal results. Cells were additionally treated for 48 hours with 80 μM forskolin (Sigma Chemical Co., St. Louis, MO) to cause them to differentiate into syncytiotrophoblasts, producing a more homogenous population (Kudo and Boyd, 2002; Wice et al., 1990).

Transient transfections were performed in 6-well dishes at a density of 0.2 x 10^6 cells/well. The formation of the polycationic lipid/DNA complex reaction was performed at room temperature for 15 minutes using Polyfect (Qiagen, Valencia, CA) transfection reagent. For each reaction, 5 μg of pGL3 plasmid DNA (either block mutations or -380 wild-type), 12μl Polyfect, 0.25 μg of control plasmid pβ-galactosidase (RSV promoter and enhancer, ClonTech Laboratories, Inc., Palo Alto, CA) and F12K culture medium without serum or antibiotics in a total volume of 100 μl. The cells were washed twice with 3 mls serum- and anitibiotic-free medium, then 3 mls of complete (10% heat-inactivated fetal bovine serum (Gemini Bio-Products, Inc., Calabasas, CA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO)) medium was added to each well. After the 15 minute incubation, 900 μls of complete F12K medium was added to the Polyfect-DNA mixture, mixed well then, added to the cells for 48 hours.

After the 48 hour transfection reaction, the cells were harvested, lysed and analyzed for luciferase and β-galactosidase activities using a Luciferase Assay System (Promega, Madison, WI) and a Galacto-Light Plus Assay kit (Applied Biosystems, Bedford, Massachusetts). Light emission generated by both luciferase and β-galactosidase activity was measured using a Turner TD-20/20 luminometer with an integration time of 10 seconds. To control for intra-assay variation the number of light units generated by luciferase activity was normalized to β-galactosidase activity for each transfection. The relative luciferase activity for each construct is expressed as percent activity of the wild-type -380 pGL3 construct. Data are presented as the mean percent activity ± sem and analyzed by Student's paired t-test.

Transcription Factor Binding Site Analysis

In order to analyse the footprint 6 region of the oPL proximal -380/+16 promoter, this sequence was subjected to computer analysis utilizing the TESS program. This program identified binding sites within the FP6 region for potential *trans*-acting factors. Based on the results of this analysis, various transcription factors were identified as possibly interacting with FP6, and further investigation was pursued. These binding sites tended to be grouped around the GAGGAG sequence within FP6 and included those for the Specificity proteins (Sp) and CCAAT-enhancer binding proteins (CEBP).

Transient Co-transfections

For transient co-transfections the BeWo cells were grown as described and plated at a density of 200,000 cells per well. The cells were gown an additional 24 hours in complete medium after being plated for co-transfection. Cells were then treated for 48 hours with 80 μM forskolin (Sigma Chemical Co., St. Louis, MO) in complete F12K medium (10% heat-inactivated fetal bovine serum (Gemini Bio-Products, Inc., Calabasas, CA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO)), 3 mls per well. The forskolin was changed after 24 hours and replaced with fresh medium that remained for an additional 24 hours until transfection. For the CEBP-α, -β, and -δ over-expression vectors, (kindly provided by Dr. Norman Curthoys, Colorado State University) (Liu et al., 2001), 5 μg of the wild-type -380 oPL promoter construct was added to the cells with either 5 μg of control plasmid (pGL2), CEBP-α, -β, or -δ plasmids. The dominant negative (DN) co-transfections were performed in the same manner, with the wild-type construct being added to the cells in addition to either control plasmid, DN A-CEBP,

(generously donated by Dr. Charles Vinson, National Cancer Institute, NIH) (Moitra et al., 1998), or DN CEBP-α, (a kind gift of Dr. Daniel Tenen, Harvard Medical School) (Pabst et al., 2001). All co-transfections were performed in triplicate, with at least three separate DNA isolations. Results are expressed as percent of wild-type, with results reported as means ± sem.

The transient co-transfections using the Sp1 and Sp3 over-expression constructs were also performed in triplicate, with at least three DNA isolations for each construct. The Sp1 construct was provided by Dr. Robert Tjian, (University of California, Berkeley) (Naar et al., 1998), and the Sp3 construct was obtained from Dr. Jon Horowitz, (North Carolina State University) (Kennett et al., 1997). Over-expression co-transfections were performed using the wild-type -380 promoter construct with either the control plasmid or the Sp1 or Sp3 expression constructs. The co-transfections were also performed with a construct containing the FP6 (-319/-349) sequence in front of the minimal ovine prolactin promoter (FP6/PRL) construct and the Sp1 or Sp3 expression plasmids or the -124 pGL3 construct and the Sp1 or Sp3 expression plasmids. All DNA concentrations remained constant at 5 μg/reaction.

For small interfering (si) RNA co-transfections, the Sp1 and Sp3 siRNA constructs were purchased from Panomics (Redwood City, CA). The siRNA vectors are comprised of templates for Sp1 or Sp3 cloned into RNA Polymerase III expression vectors containing the first 27 nucleotides of human U6 RNA promoter (U6 + 27). Transfections were carried out in the manner described above, with 48 hour forskolin treatment followed by 48 hours of transfection. However, the concentration of DNA used in the interfering RNA experiments was 2.5 μg/reaction. The wild-type -380 promoter construct was co-

transfected with either the pU6 + 27 control plasmid (the backbone vector for the siRNA plasmids), the Sp1 or the Sp3 siRNA plasmids. The transfections were also performed with the FP6 sequence in front of the minimal prolactin promoter construct (FP6PRL) to assess the ability of Sp1 and Sp3 to interact with the FP6 sequence. All experiments were carried out in triplicate with at least three separate DNA isolations. All results are expressed as a percent of the wild-type construct with data shown as means ± sem.

Supershift Analysis

For supershift analysis, the antibodies (2 μl of rabbit polyclonal crude serum) to either Sp1, Sp3 or CEBP-α (Active Motif, Carlsbad, CA) were added to the reaction mixture containing the nuclear protein and the buffer. This was mixed gently and allowed to incubate overnight at 4 °C. Following pre-incubation with the antiserum, the unlabeled competitors were then added to the reaction mixture and incubated at room temperature for 5 minutes, after which the labeled oligonucleotides were added and incubated at room temperature for an additional 20 minutes before electrophoresis. The nuclear protein mixture was electrophoresed through a 5% TBE polyacrylamide non-denaturing gel for 3 hours. The gel was subsequently dried onto Whatman paper and exposed to x-ray film for 24 hours at -80 °C.

Western Immunoblot Analysis

Western analysis was performed using sheep nuclear extracts from day 100 of gestation or nuclear extracts from HeLa and Jurkat cell cultures. The nuclear proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After

electrophoresis, the nuclear proteins were transferred to nitrocellulose membranes (Micron Separations, Inc.) in 2 mM Tris Ph 8.3, 0.1% SDS buffer, 20% methanol and 150 mM glycine. The membranes were subsequently blocked overnight in a 5% nonfat dry milk/ 1X TBS (10 mM Tris pH 8.0, 0.05% Tween 20, 150 mM NaCl) solution. Immunoblot detection of Sp1 and Sp3 was performed by incubating antibodies (0.2 μg/ml) provided by Santa Cruz Biotechnologies (Santa Cruz, CA) at a concentration of 1:200 (250 μl rabbit polyclonal anti-sera, 50 μg, in 50 ml 1X TBST solution) for 2 hours at room temperature with gentle agitation. The membrane was washed 3 x 10 minutes in 1X TBST, followed by incubation with anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnologies) at a dilution of 1:1000 for 1 hour at room temperature. The membrane was washed an additional 5 times in 1X TBST and incubated for 5 minutes at room temperature in Super Signal West Femto chemiluminescent reagent (Pierce, Rockford, IL). Following incubation the membranes were exposed to x-ray film for visualization.

For Southwestern and Western immunoblots performed on the same membrane, the membranes were first subjected to Western analysis as described above. The membranes were then washed in TBS for 5 minutes at room temperature, stripped using Western stripping buffer (Pierce, Rockford, IL) for 20 minutes at room temperature, then washed in TBST for 10 minutes and placed in blocking buffer (5% NFDM in 1X TBST) overnight at 4 °C. These membranes were then subjected to Southwestern analysis as described above.

Statistical Analysis

All transient transefction and co-transfection analyses were performed using n = 3 wells for each construct, with at least three separate experiments and three different DNA isolations for each construct. Luciferase values were normalized against β-galactosidase values and the means were reported as a percentage of the wild type -380 (for transfections) or -380 + control (for co-transfections). Results were shown as means ± sem. Statistical analysis for the transient transfections was done using the Student's t-test with means compared to the wild type, or wild type + control, and significance accepted as $p \leq 0.05$.

Results

Characterization of FP6

Previous footprint analysis of the oPL proximal promoter identified a protected region between -319/-349 (FP6), suggesting a nuclear protein was binding to this sequence (Liang et al., 1999). Further analysis was needed to define this protein and test its functionality. EMSA analysis was used to determine binding interactions between this FP6 region and the specificity of these interactions. Nuclear proteins from BeWo and binucleate cell extracts were found to interact with the FP6 sequence and increasing amounts of unlabeled homologous FP6, but not heterologous oligonucleotide, Purα (Limesand et al., 2004), specifically inhibited binding of the labeled FP6 oligonucleotide to the nuclear proteins **(Figure 1).** To further define this FP6 region Southwestern analysis was employed to delineate the specificity of the nuclear protein in binding to the footprint 6 region. Southwestern results suggest a nuclear protein with an apparent M_r of 105,000 - 120,000

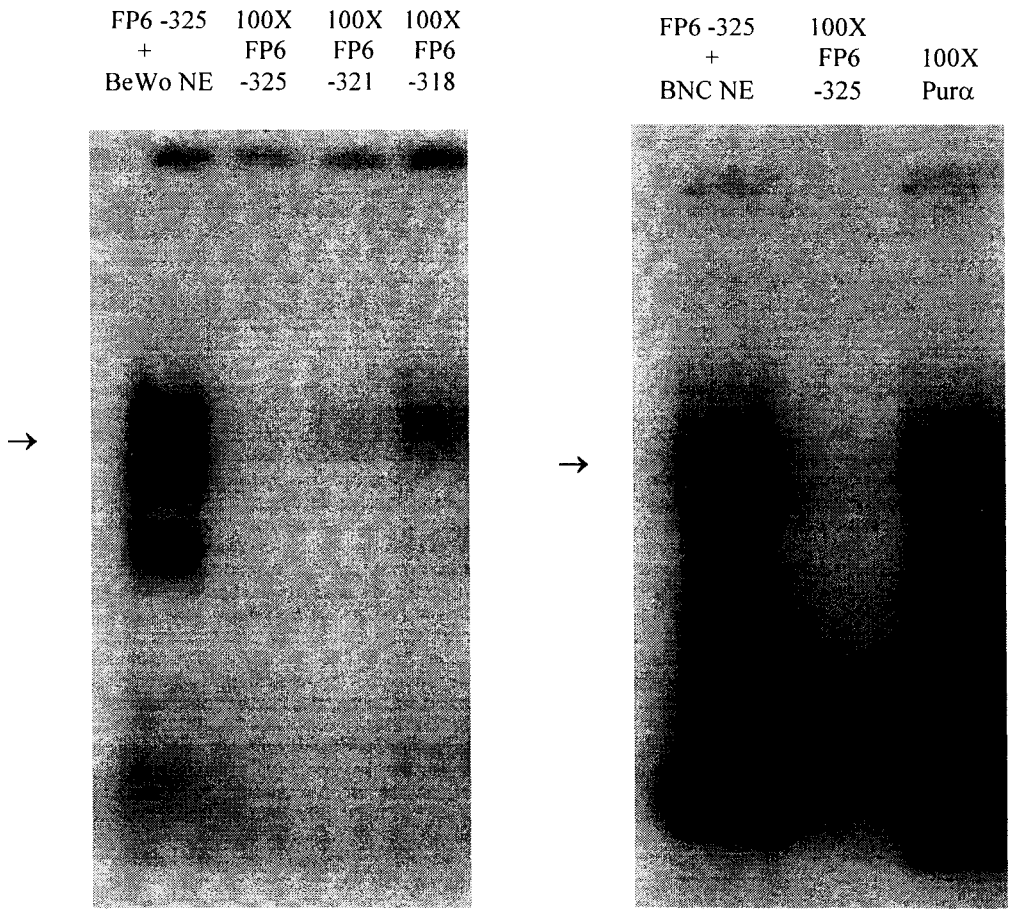


Figure 1: EMSA analysis with a labeled FP6 oligonucleotide and nuclear extracts from BeWo and binucleate cells (BNC). The membrane on the left shows the FP6 labeled oligonucleotide -325 (-325/-345) and nuclear extracts from BeWo cells. The first lane shows the shift observed with the BeWo cells, indicating there is a nuclear protein interacting with the FP6 region. Lanes 2, 3 and 4 show 100X excess unlabeled FP6 oligonucleotides -325, -321 (-321/-341) and -318 (-318/-338) are able to compete for binding with the FP6 labeled oligonucleotide. The -325 oligonucleotide competes more efficiently than the -321 or the -318 oligonucleotides. In the membrane on the right a shift is observed with nuclear extracts from BNC and the -325 labeled FP6 oligonucleotide. In lane 2, excess homologous unlabeled FP6 oligonucleotide efficiently competes for binding, but a heterologous oligonucleotide (Purα) in lane 3 does not, indicating the binding is specific to the FP6 sequence.

binds to FP6 and homologous competitor oligonucleotides inhibit binding (**Figure 2).** This suggests the interaction is sequence specific to the FP6 region. Together with the SW data, these results suggest there is a nuclear protein specifically interacting with the FP6 region of the oPL proximal promoter in both binucleate and BeWo cells.

Functional Analysis of FP6

In order to test the functionality of the footprint 6 sequence in the transcriptional regulation of the oPL gene, transient transfection analysis using the FP6 block mutation constructs was conducted with forskolin treated BeWo cells to reduce intra-assay variability and to utilize a homogenous population of syncytiotrophoblast cells. The results **(Figure 3)** indicate that while all three mutations significantly reduce ($p \leq 0.01$) activity, mutations 1 and 2 were most effective. To determine if a single, contiguous *cis*-element within the FP6 region is responsible for transcriptional stimulation, 16 two-base pair mutations were created encompassing the FP6 sequence, and the functionality of these mutations was analyzed in transient transfection analysis of forskolin (80 μM) treated BeWo cells. The results of these transient transfections identified specific base pairs that, when mutated, significantly decreased transcription compared to the wild-type -380 promoter construct (**Figure 4)**. Specifically, mutation constructs 2, 4, 5, 6, 8, 9, 10 and 12 caused significant (2, 4, 5, 6, 10 $p \leq 0.01$; 8, 9, 12 $p \leq 0.05$) decreases in transactivation when compared to the wildtype -380 oPL promoter construct, suggesting these bases are necessary for transactivation. The base pairs (-349 GA**AG**AC**CCCTGG**AG**GAGGGC**AT**GG**CCACCCAT -318) were identified from these

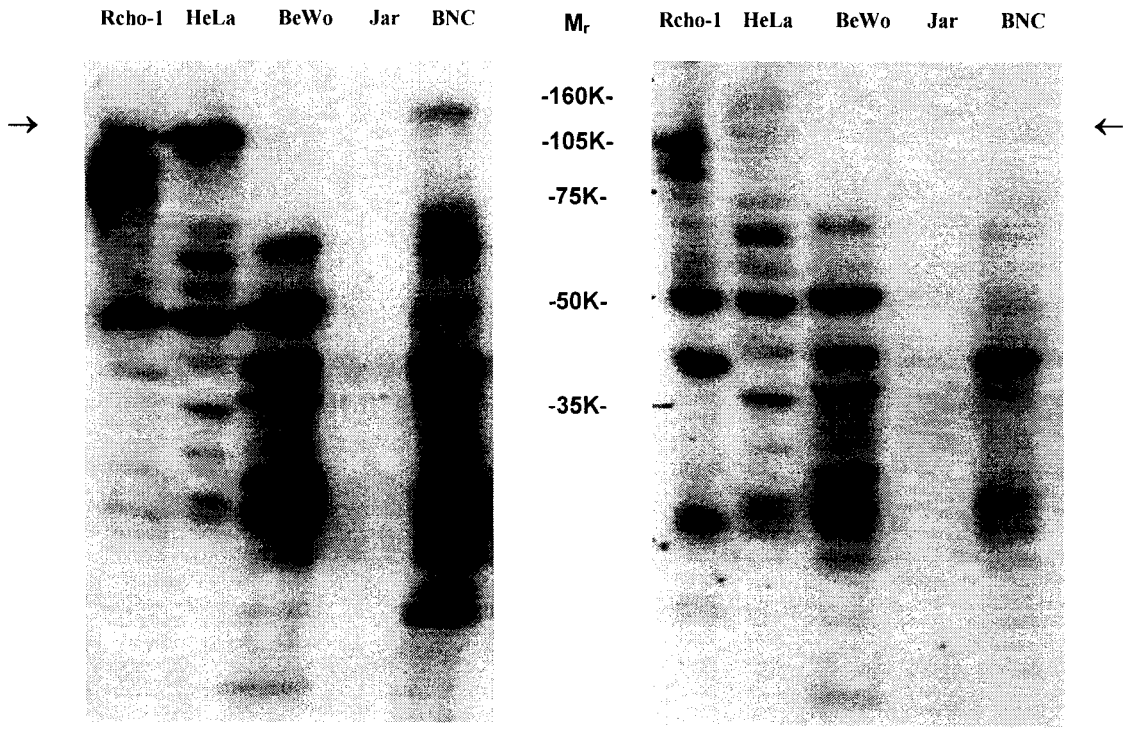
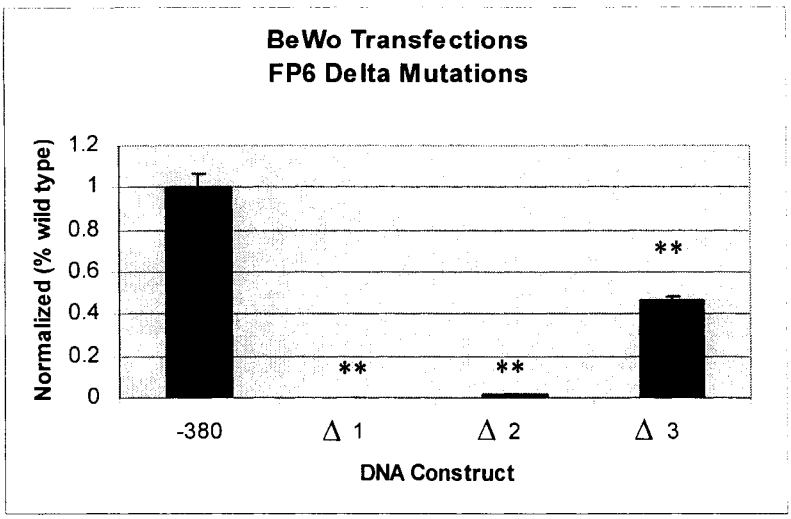


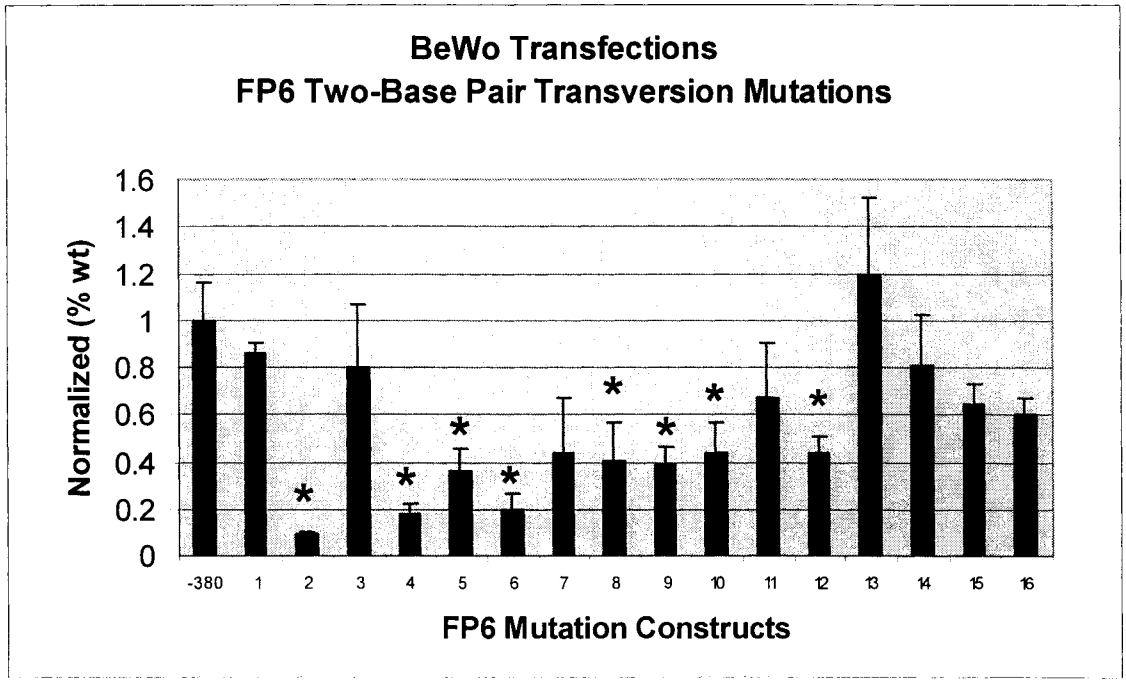
Figure 2

Southwestern analysis with a FP6 FC labeled oligonucleotide and nuclear extracts from Rcho-, HeLa, BeWo, Jar and binucleate cells (BNC). The membrane on the left was incubated with 2 x 10^6 cpms of FP6 FC per ml of binding buffer for 4 hours at 4 °C. The membrane on the right was incubated with 2 x 10^6 cpms of radiolabeled FP6 FC oligonucleotide plus 200X excess unlabeled FP6 FC. The unlabeled oligonucleotide competed for binding with the labeled oligonucleotide at an apparent M_r of 105,000 - 120,000 in Rcho-1, HeLa and BNC cells, shown with the arrows, indicating a nuclear protein specifically bound to the FP6 sequence.



FP6 GA<u>AGACCCCTG</u><u>GAGGAGGG</u>CA<u>TGGCAACC</u>CAT
Δ3 **Δ2** **Δ1**

Figure 3: Three block mutations were created along the length of FP6 and the DNA constructs were transfected into forskolin treated BeWo cells. The DNA mutation constructs, Δ1, Δ2 and Δ3 shown below the graph, and the wild type -380 construct were transfected at a concentration of 5 μg/reaction. β-galactosidase was also transfected at a concentration of 0.25 μg/reaction to control for variation within assays. Significant reductions ($p \leq 0.001$) were observed with all mutation constructs compared to the wild type -380 plasmid. This indicates that the FP6 sequence is necessary for transactivation of the oPL promoter specifically, the region encompassed by mutations 1 and 2.



FP6 -349 GA AG AC CC CT GG AG GA GG GC AT GG CC AC CC AT -318
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 4: BeWo Cells were transiently transfected with two-base pair transversion mutations of the FP6 sequence. The FP6 sequence is shown below the graph with the corresponding mutation number under each base-pair. All cells were treated with forskolin for 48 hours before transfection. The concentration of DNA used was 5 μg/reaction for all constructs. β-galactosidase was used as an intra-assay control at a concentration of 0.25 μg/reaction. Results are calculated as luciferase/β-galactosidase and expressed as a percent of the wild type -383 construct. Mutations 2, 4, 5, 6, 8, 9, 10 and 12 resulted in significant decreases ($p \leq 0.01$ for mutations 2, 4, 5, 6 and 10 and $p \leq 0.05$ for mutations 8, 9, 10 and 12) in transactivation compared to the wild type promoter construct, indicating they are necessary for transcriptional activation of the oPL promoter.

results as necessary for activated transcription in BeWo cells, suggesting they play an important role in regulation of the oPL gene.

Interaction of CEBP with FP6

Based on transcription factor analysis of the FP6 region the sequence from -339/-332 (GGAGGAGG) represents a potential binding site for the CEBP proteins while the sequence from -340/-325 (TGGAGGAGGGCATGGC) represents a potential binding site for the Sp proteins. Functional analysis of CEBP proteins was carried out in co-transfection experiments using over-expression constructs for CEBP-α, -β and -δ (in pGL2 plasmids) and dominant negative constructs for A-CEBP (in CMV500 plasmid) and CEBP-α (in the pcDNA 3.1 plasmid). The results from the co-transfections with CEBP -α, -β, -δ over-expression constructs showed no significant increases compared to the control constructs, indicating that perhaps CEBP proteins were not involved in activation of transcription of the oPL promoter. However, there was a significant decrease in transactivation with over-expresssion of the CEBP-δ construct, implying that this protein may interact with the oPL promoter and inhibit complex formation of other transacting factors necessary for activation (**Figure 5**). Results from the A-CEBP and CEBP-α dominant negative co-transfections showed no significant decreases in transactivation (**Figure 6**), providing further evidence that the CEBP proteins alone may not function to activate transcription of the oPL gene.

To further investigate a possible interaction between FP6 and CEBP-α, supershift analysis was performed using both BeWo cell and binucleate cell nuclear extracts, a FP6 labeled oligonucleotide and anti-serum raised against CEBP-α. There was no binding of

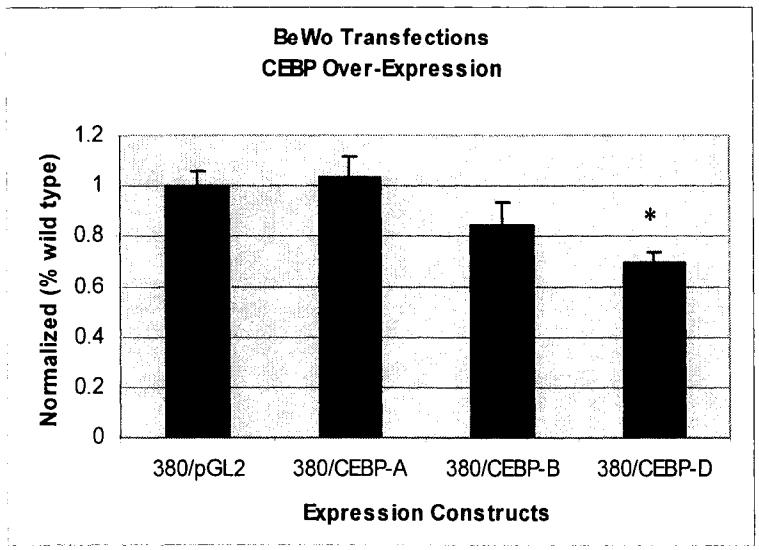


Figure 5: Co-transfections with the -380 oPL promoter construct and over-expression constructs for CEBP-α (CEBP-A), CEBP-β (CEBP-B) and CEBP-δ (CEBP-D) were performed in forskolin treated BeWo cells. The amount of DNA was kept constant at 5 μg/reaction for the wild type -380 construct, the control plasmid (pGL2) and the CEBP expression constructs. β-galactosidase was added to each reaction to control for intra-assay variation (0.25 μg/reaction). Luciferase reporter activity was normalized against β-galactosidase activity and values are expressed as a percent of the wild type (380/pGL2) activity. The data is presented as means ± sem. There were no significant increases in transactivation with the CEBP-α, -β, or -δ over-expression constructs compared to wild type. However, over-expression of the CEBP-δ construct did significantly decrease transcription, indicating it may bind to the FP6 region thus, inhibit binding of other transacting factors.

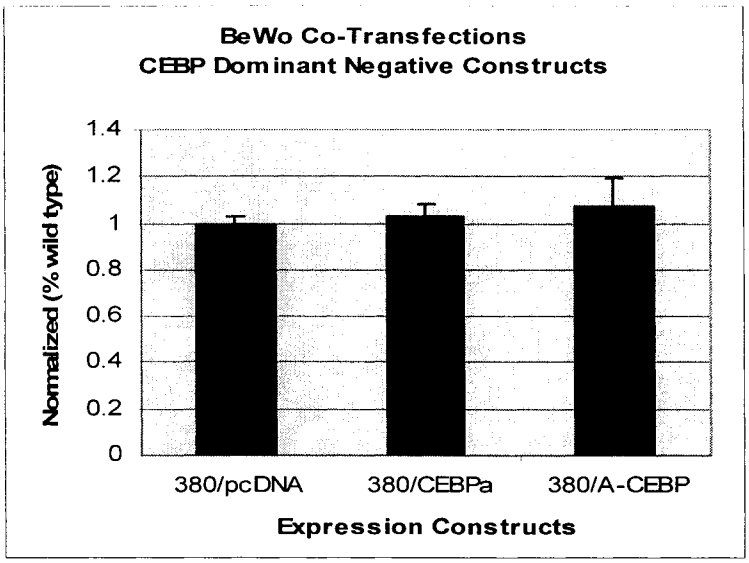


Figure 6: The -380 promoter construct was co-transfected with either a CEBP-α specific (CEBPa) or a CEBP (A-CEBP) dominant negative construct into 48 hour forskolin treated BeWo cells. All DNA plasmids were transfected at a concentration of 5 μg/reaction for the -380 wild type and 5 μg/reaction for the dominant negative constructs. A β-galactosidase control plasmid was also transfected at a concentration of 0.25 μg/reaction to control for variation within assays. Results are expressed as a percent of the wild type and data shown as means ± sem. The CEBPa and A-CEBP dominant negative constructs were not significantly different from the wild type -380 construct. These results indicate that the CEBP proteins may not be specifically interacting with the FP6 region to activate transcription of the oPL promoter.

the CEBP-α antibody to the nuclear protein-DNA complex (**Figure 7 and 8**). Together, these results suggest that the CEBP proteins, and CEBP-α specifically, do not interact with the FP6 region of the oPL promoter in a manner that activates transcription.

Sp1 and Sp3 Interaction with FP6

Based on transcription factor anaylsis, the Sp proteins were also implicated as potentially binding to a core sequence of the FP6 region. To determine whether Sp1 and/or Sp3 interact with FP6, supershift analysis utilizing both BeWo and BNC nuclear extracts was performed. Antibodies to either Sp1 or Sp3 were incubated with the nuclear extracts and the FP6 radiolabeled oligonucleotide (-325/-345). Results from the supershift assays indicated that both Sp1 and Sp3 proteins interact with the FP6 sequence, as antiserum to both proteins caused a shift in the radiolabeled FP6 oligonucleotide-nuclear protein complex, indicating the proteins were binding to FP6 **(Figure 7 and 8).** These results provide evidence that both transcription factors (Sp1 and Sp3) can interact with the FP6 region in a sequence-specific manner.

Co-transfections were performed in forskolin treated BeWo cells with the -383 pGL3 construct as the wildtype and either Sp1 (CMV-Sp1 plasmid) or Sp3 (pCMV4-Sp3/flu) expression constructs. The co-transfections showed that both Sp1 and Sp3 resulted in significantly ($p \leq 0.01$) increased expression of the luciferase reporter gene when compared to the wildtype -383 construct (**Figure 9**). Co-transfections were also performed in BeWo cells with a FP6/PRL promoter construct (in the pGL3 plasmid) to confirm the interaction was specific to FP6. Results from these Sp1/Sp3 co-transfections indicate that the interaction is indeed FP6 specific as the Sp1 and Sp3 over-expression constructs also

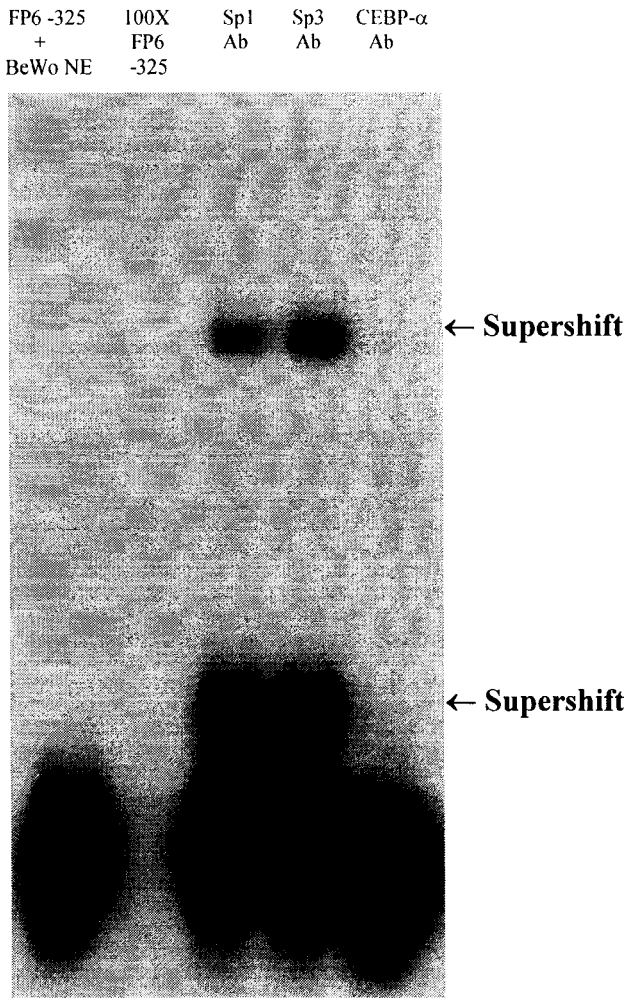


Figure 7: Supershift analysis was performed using nuclear extracts from BeWo cells and a labeled FP6 oligonucleotide. This analysis was used to determine whether Sp1, Sp3, or CEBP-α proteins were binding to the FP6 region of the oPL promoter. The labeled oligonucleotide encompassing the FP6 region was FP6 -325/ -345 (-325). A shift was observed with BeWo nuclear extracts and addition of excess unlabeled -325 FP6 was able to compete for binding. Antiserum to Sp1 and Sp3, but not CEBP-α, was able to supershift the nuclear complex. This indicates that Sp1 and Sp3 interact with the FP6 region of the oPL promoter while CEBP-α does not.

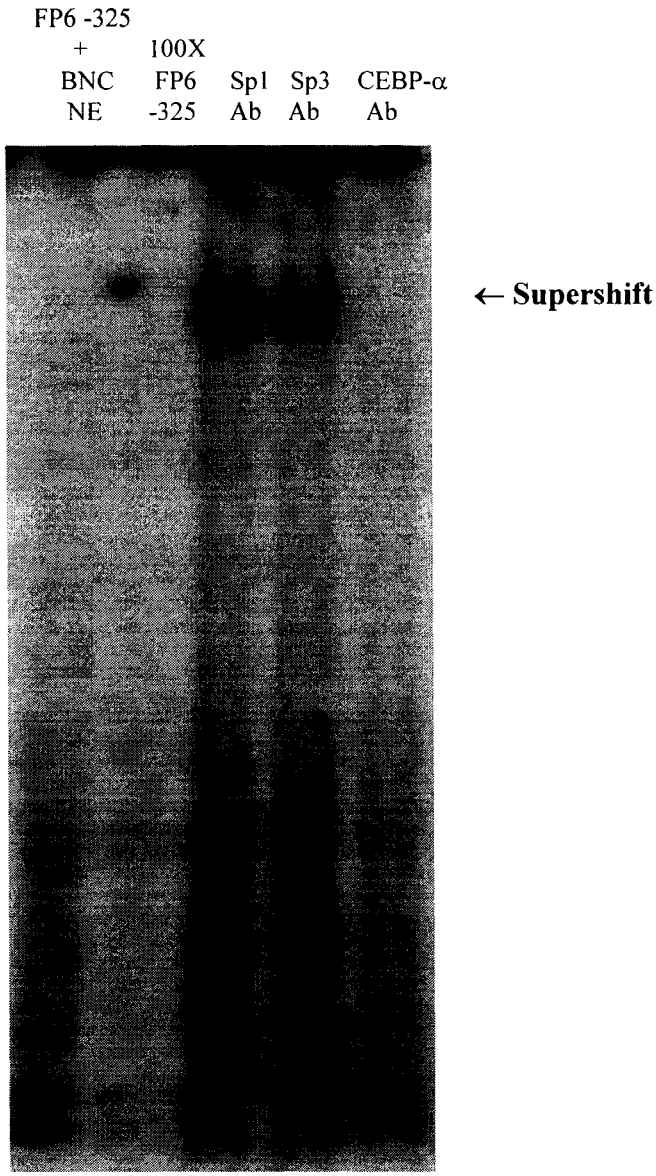


Figure 8: Supershift with binucleate cell (BNC) nuclear extract, FP6 -325/-345 labeled oligonucleotide and Sp1, Sp3 and CEBP-α antiserum. Nuclear extracts were incubated with 50,000 cpms of labeled FP6 oligonucleotide per reaction. BNC nuclear extracts produced a shift shown in lane 1. Excess unlabeled homologous competitor oligonucleotide (FP6 -325/-345) competed for binding with FP6 labeled oligonucleotide, shown in lane 2. Antiserum to Sp1 and Sp3 resulted in supershifts, shown in lanes 3 and 4, indicating these proteins were present in BNC nuclear extracts and bind to FP6. Antiserum to CEBP-α was not able to produce a supershift, shown in lane 5.

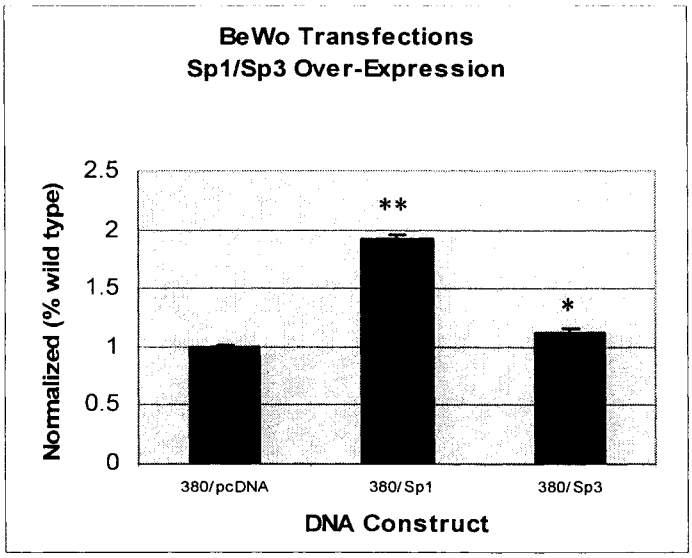


Figure 9: BeWo co-transfections with over-expression constructs for Sp1 and Sp3 and the -380 oPL promoter construct. These transfections were performed to determine whether over-expression of Sp1 and Sp3 increased transcription. The empty pcDNA 3.1 vector was used as a control. All plasmid DNA, -380 + pcDNA (380/pcDNA), -380 + Sp1 (380/Sp1) and -380 + Sp3 (380/Sp3) was transfected at a concentration of 5 μg/reaction. The β-galactosidase plasmid was transfected at 0.25 μg/reaction as a control. Luciferase values were normalized against β-galactosidase and expressed as a percent of wild type. Results are reported as means ± sem. Over-expression of Sp1 and Sp3 resulted in significant ($p \leq 0.001$ and $p \leq 0.05$ respectively) increases in transactivation compared to the wild type -380 construct. These results indicate that Sp1 and Sp3 functionally interact with the FP6 region of the oPl promoter to activate transcription.

significantly ($p \leq 0.01$) increased transactivation with the FP6/PRL promoter construct (**Figure 10**). These results provide functional evidence that Sp1 and Sp3 may interact with the FP6 region in a manner that activates transcription.

To further confirm that Sp1 and Sp3 were indeed interacting with the FP6 region to stimulate transcription, siRNA constructs were co-transfected with the -380 wild type plasmid to assess the effect on transcription of inhibiting these factors. Both Sp1 and Sp3 siRNA constructs (in a plasmid with the U6 +27 promoter) and the control plasmid (also driven by the U6 +27 promoter) were co-transfected into forskolin treated BeWo cells with the -380 wild type construct. Both Sp1 and Sp3 siRNA constructs significantly ($p \leq 0.001$) inhibited transactivation compared to the wild type construct, as measured by luciferase activity (**Figure 11**). Together these co-transfection experiments indicate that both Sp1 and Sp3 interact with the FP6 region of the oPL promoter in a sequence-specific manner and function to stimulate transcription. Over-expression of these transcription factors functions to activate transcription of the -380 oPL promoter construct while inhibition of these *trans*-acting factors decreases transcription, further implicating them as necessary for activation of the oPL promoter.

Western analysis was carried out using nuclear extracts from BNC, HeLa and Jurkat cells to assess whether Sp3 was present. Results from all cell types provided evidence that this transcription factor is indeed detectable in the nuclear portion where it can potentially play a role in transactivation (**Figure 12**). Further evidence that Sp3 interacts with the FP6 region was provided by Southwestern analysis utilizing the same membrane. After Western analysis, the membrane was stripped and subsequently subjected to Southwestern analysis. The results (**Figure 12**) showed that a labeled FP6 oligonucleotide was able to

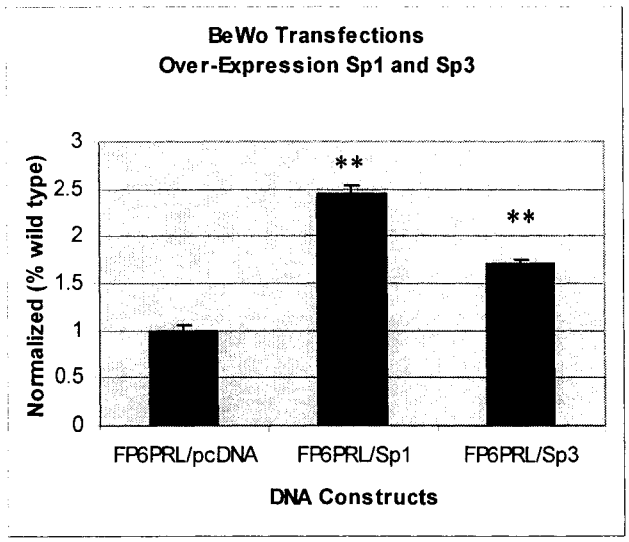


Figure 10: Co-transfections with Sp1 and Sp3 expression constructs in 48 hour forskolin treated BeWo cells. All plasmids were isolated and purified using a CsCl centrifugation gradient and concentrations were constant for each construct at 0.5 μg/reaction. β-galactosidase was transfected at a concentration of 0.25 μg/reaction to correct for intra-assay variation. A FP6 minimal prolactin promoter construct (FP6PRL) was used as the wild type to assess the ability of Sp1 and Sp3 to interact with FP6. The empty pcDNA 3.1 vector (pcDNA) was used as the control. Results were normalized as luciferase/β-galactosidase and expressed as a percent of wild type with results shown as means ± sem. FP6PRL/Sp1 and FP6PRL/Sp3 values are significantly different ($p \leq 0.0002$ and $p \leq 0.001$ respectively) than the wild type FP6PRL/pc construct. Over-expressing Sp1 and Sp3 with a FP6 sequence in front of the minimal prolactin promoter increases transcription indicating that these *trans*-acting factors functionally interact with the FP6 region.

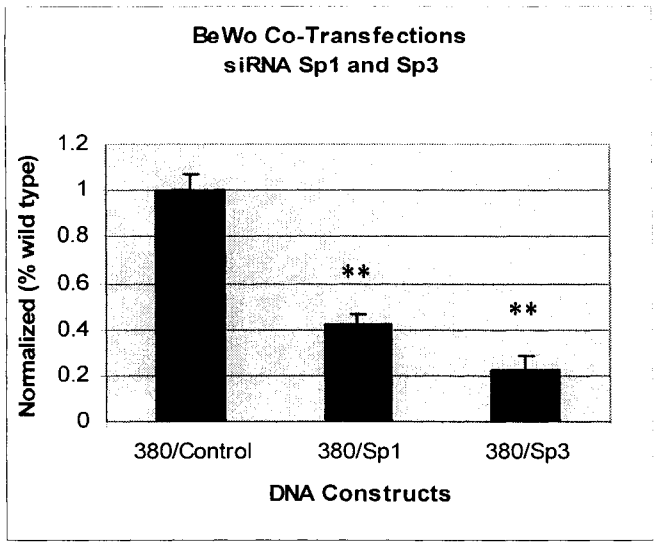


Figure 11: Co-transfections were performed in BeWo cells with the wild type -380 construct and siRNA constructs for Sp1 and Sp3. The -380 promoter construct was co-transfected with a control plasmid (the empty siRNA vector pU6 + 27) or the siRNA plasmids for Sp1 or Sp3. β-galactosidase was used as a control plasmid (0.25 μg/reaction) to reduce intra-assay variation. All DNA was transfected at a concentration of 2.5 μg/reaction. The BeWo cells were treated for 48 hours with forskolin before being transfected with the inhibitory RNA constructs. Results were normalized (luciferase against β-galactosidase) and expressed as a percent of wild type. The results are shown as means ± sem. When co-transfected with the wild type promoter construct, both siRNA constructs Sp1 (380/Sp1) and Sp3 (380/Sp3) significantly ($p \leq 0.002$ and $p \leq 0.001$ respectively) reduced activation of the -380 oPL promoter construct compared to wild type (380/Control). These results indicate that Sp1 and Sp3 interact with the FP6 region and function to activate transcription as inhibition of either *trans*-acting factor significantly decreases transactivation of the oPL promoter.

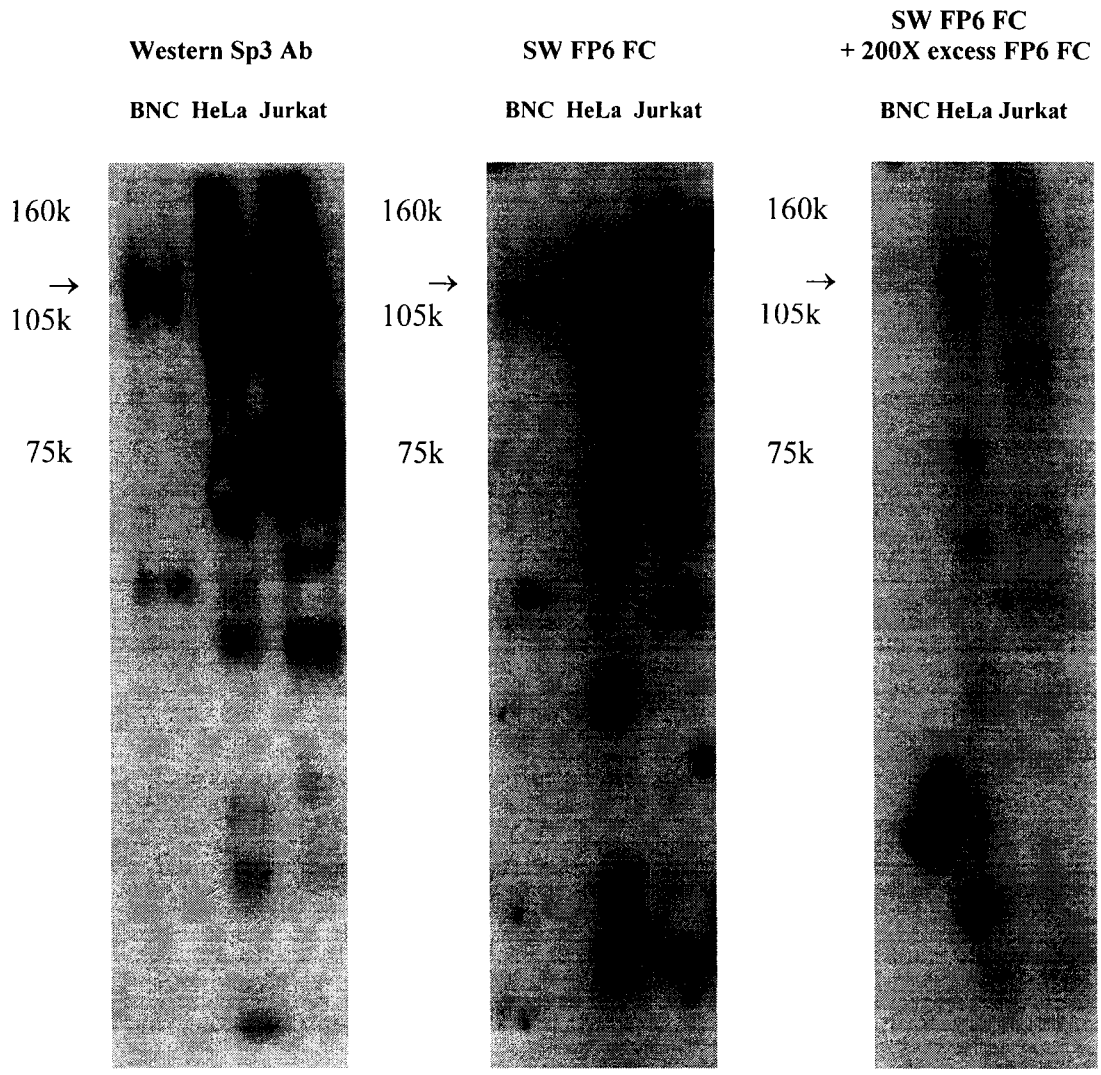


Figure 12: Western and Southwestern analysis with gestation day 100 binucleate cell, Hela cell and Jurkat cell nuclear extracts. The membrane on the left shows a Western assay using Sp3 antiserum. The arrow marks the nuclear protein, with an apparent M_r of 105k -120k identified by the Sp3 antibody. The same membrane was then subjected to Southwestern analysis, shown in the center, with a labeled FP6 oligonucleotide (FP6 FC) binding to specific nuclear proteins, specifically the one identified by the Sp3 antiserum, labeled with the arrow. The membrane on the right was also subjected to Southwestern analysis with a labeled FP6 oligonucleotide and 200X excess unlabeled FP6 oligonucleotide. The unlabeled FP6 efficiently competes with the FP6 radio-labeled oligonucleotide protein for binding with the nuclear protein identified in both Western and Southwestern analysis, as shown by the arrow. This suggests the Sp3 protein is present in BNC, HeLa and Jurkat cells and specifically binds to the FP6 sequence in these same nuclear extracts, indicating that Sp3 interacts with the FP6 sequence.

specifically bind to nuclear proteins in BNC, HeLa and Jurkat cells. Molar excess of unlabeled FP6 oligonucleotide specifically inhibited binding to these nuclear proteins corresponding to those previously identified with the FP6 oligonucleotide. Similar Western analysis of Sp1 did not coincide with the migration observed by Southwestern analysis (data not shown). However, this may have been the result of the antiserum used.

Discussion

DNase I protection analysis identified a protein-DNA interaction within the FP6 (-319/ -349) region of the oPL proximal promoter (Liang et al., 1999). Through transient transfection analysis utilizing block mutations, this FP6 region was shown to be functional and necessary for maximal trophoblast-specific transactivation of the oPL promoter. Within this FP6 region there was a GAGGAG sequence that was found to be necessary for binding a FP6 oligonucleotide, when analyzed through electrophoretic mobility shift assays and Southwestern analysis. Transcription factor binding site analysis of the FP6 sequence, specifically the region encompassing the GAGGAG nucleotides, identified potential binding sites for the CEBP and Sp proteins. This coincides with other promoter sequences shown to bind CEBP and Sp proteins (Christy et al., 1989; Kishikawa et al., 2002; Wong and Lee, 2002). Thus, further investigation of this region was necessary.

Functional analysis of the FP6 region was carried out utilizing forskolin treated BeWo cells, a human choriocarcinoma cell line that synthesizes and secretes placental lactogen. Transfection assays using the block mutation constructs (Δ1, Δ2 and Δ3) revealed that mutation of this region, specifically mutations 1 and 2, resulted in significant

decreases in activation compared to the wild type -380 construct, suggesting this region is important in transcriptional activation. Previous analysis showed that the -217 to -380 region of the oPL promoter (encompassing FP6) was necessary for activated transcription in trophoblast cells (Liang et al., 1999). To further define a single contiguous *cis*-element within this FP6 region necessary for transactivation, two-base pair transversion mutations spanning the length of the FP6 sequence, -318/-349, were created. Functional mutation analysis utilizing transfection assays identified the region necessary for maximal transcriptional stimulation. The sequence identified through block mutation analysis and two-base pair mutation analysis as important for transactivation encompasses the region delineated as potentially binding the CEBP, Sp1 and Sp3 sequences. Specifically, the two-base pair mutations that significantly reduced transactivation centered around the region containing the GAGGAG sequence identified in FP6 as functional through block mutation analysis and important for binding a nuclear protein. Although, mutation 2 also resulted in a significant decrease in transactivation, and this lies 5' of the FP6 GAGGAG sequence, it encompasses another potential Sp binding site as well as an Ets site, indicating that other *trans*-acting factors may be involved in stimulating transcription.

EMSA analysis utilizing a radio labeled FP6 oligonucleotide confirmed a specific protein-DNA interaction within the -319/-349 region of the oPL promoter. In addition, anti-sera to both Sp1 and Sp3, but not to CEBP-α, produced specific supershifts when used in conjunction with the FP6 oligonucleotide, suggesting these proteins interact with this region in a sequence-specific manner. In transfection analysis, over-expression of Sp1 and Sp3, but not the CEBP proteins functioned to increase transactivation, while inhibition of Sp1 and Sp3, but not the CEBP proteins, resulted in decreased transactivation. Our results

agree with previous reports indicating that both Sp1 and Sp3 bind to the same sequence and function to activate transcription (Ihn and Trojanowska, 1997; Schanke et al., 1998; Teunissen et al., 2002). These results indicate that Sp1 and Sp3, but not the CEBP proteins, may be interacting with the FP6 region in a sequence-specific manner and functioning to activate transcription.

The Sp1 and Sp3 proteins have been shown to be transcriptional activators in a variety of tissues. Although they are ubiquitous trans-acting factors, they appear to be involved in tissue-specific expression of a number a developmental genes (Kishikawa et al., 2002; Schanke et al., 1998; Wong and Lee, 2002). Sp1 has been shown to be a transcriptional activator while Sp3 can act as either an activator or a repressor of transcription depending on the cell type (Yu et al., 2003). There is also evidence that Sp3 may repress Sp1 mediated transactivation, depending on the ratio of Sp1 to Sp3, the number of binding sites for Sp proteins within the promoter, or the tissue in which they are expressed (Vines and Weigent, 2000; Yu et al., 2003). Sp proteins, specifically Sp1 and Sp3 have been shown to activate pregnancy specific genes in various species, such as the rhesus monkey GH-v gene and human PL3 gene (Fitzpatrick et al., 1990; Jiang and Eberhardt, 1995; Schanke et al., 1998). Sp1 and Sp3 bind to the same sites, GC and GT/A rich sequences. Therefore, Sp1 and Sp3 were good candidates for interacting with the FP 6 region.

To further analyze the ability of Sp1 and Sp3 to interact with the FP6 region, immunoblot analysis was performed, with both Western and Southwestern assays, utilizing HeLa, Jurkat and binucleate cell nuclear extracts. Western analysis with Sp3 anti-sera identified specific proteins in all nuclear extracts with an apparent M_r of 105 - 120 kDa. This coincides with previous reports of Sp1/Sp3 proteins (Kishikawa et al., 2002; Wong

and Lee, 2002). In order to confirm these proteins, as identified through Western blot analysis, the same membranes were stripped and used in Southwestern assays. Unlabeled FP6 oligonucleotide specifically competed with binding for the protein identified with the Sp3 antibody. The same experiments (data not shown) were performed using anti-sera to the Sp1 protein however, the results did not agree with those observed with the Sp3 protein. Although, Sp1 and Sp3 bind to the same sequence, and Sp1 was able to stimulate transcription in functional assays and supershift nuclear proteins in EMSA analysis, these Western and Southwestern results suggest that Sp3 may be the primary protein interacting with the FP6 region. The Sp1 anti-sera did not clearly delineate the protein binding in the nuclear extracts as shown with anti-sera to Sp3. As the immunoreactive binding observed with Sp1 was not the same as that observed with Sp3, in the Southwestern and Western analysis, this indicates that the protein interacting with FP6 may be Sp3.

Further evidence supporting an interaction with the Sp1/Sp3 proteins and the FP6 region was obtained using functional co-transfection assays. Expression constructs for both Sp1 and Sp3 were co-transfected into froskolin treated BeWo cells with the -380 oPL promoter construct. Over-expression of both Sp1 and Sp3 resulted in significant increases in transactivation compared to the wild-type promoter construct. These results indicated that Sp1 and Sp3 were indeed functional in BeWo cells and that they both acted as transcriptional activators of the oPL proximal promoter. Furthermore, co-transfection analysis utilizing siRNA constructs for both Sp1 and Sp3 caused significant decreases in activation (60% and 80%, respectively). Together these results suggest that Sp1 and Sp3 may play an important role in stimulating transcription in the oPL gene through a specific interaction with the FP6 region of the oPL promoter.

Although, the functional co-transfection asays showed that over-expression of Sp1 and Sp3 increased transactivation, and inhibition of either protein decreased activation, further analysis revealed that Sp3 may be the *trans*-acting factor interacting with FP6. Activation of the oPL promoter with over-expression of Sp1 was higher than that observed with Sp3. An explanation for this may be that Sp3 is expressed at a higher concentration endogenously and is therefore closer to maximal expression than Sp1. Therefore, over-expression of Sp3 would not result in an increase as dramatic as that seen with Sp1, providing further evidence that Sp3 may be the primary protein interacting with the FP6 region. Likewise, inhibition of Sp3 through siRNA co-transfections would produce the greater effect on transactivation as shown in our experiments. Another explanation could be that when Sp1 is inhibited, endogenous Sp3 compensates for this inhibition by binding to and stimulating transcription. Various reports have shown that Sp1 and Sp3 can act either synergistically to stimulate transcription, or may in fact compete for binding when both are present (Bouwman et al., 2000; Wong and Lee, 2002; Yu et al., 2003).

Sp1 and Sp3 have been shown to interact with promoters in monkey, human, rat and mouse, specifically with those genes involved in differentiation (Gao and Tseng, 1996; Schanke et al., 1998; Teunissen et al., 2002; Wong and Lee, 2002). Both Sp1 and Sp3 have been shown to activate transcription. Both trans-acting factors bind to GC rich sequences such as GGGGCGGGGC (Kadonaga JT, 1988) and although, Sp3 has a dual role as a transcriptional repressor in some cell types, both appear to be involved in activation of genes. In our experiments Sp3 appears to be the primary protein involved in activation of the oPL promoter and functions to stimulate transcription. Therefore, we propose that Sp3

specifically binds to the footprint 6 region of the oPL proximal promoter and functions as a transcriptional activator of the oPL gene.

Future Directions

Based on the data presented above, it appears that Sp3 may be the *trans*-acting factor interacting with the FP6 region of the oPL promoter. Although Sp1 does bind to FP6 and appears to function as a transcriptional activator in co-transfection analysis, it does not appear to interact with the nuclear proteins found in binucleate cell extracts as does Sp3. However, it is possible that Sp1 may bind to the same site as Sp3 and perhaps function in conjunction with Sp3 to transactivate the oPL gene. Therefore, it would be beneficial to follow up with additional experiments utilizing both Sp1 and Sp3 to further delineate their individual roles in transcriptional regulation of oPL. Over-expression experiments utilizing a cell line lacking endogenous Sp3 could provide additional information on the abilility of Sp3 to specifically transactivate the oPL gene promoter. However, there is no available mammalian cell line lacking Sp3 at this time. Utilizing a non-mammalian cell line deficient in endogenous Sp3, while it might provide data regarding addition of exogenous Sp3, might also provide incorrect information because the oPL promoter may not be acting in a trophoblast-specific manner.

While our data support the ability of Sp3 to interact with the FP6 region of the oPL promoter, the functional experiments were performed using a heterologous cell line (BeWo). Future functional analysis could be conducted using primary binucleate cell cultures. While the data are not shown here, we have evidence that binucleate cells can be

successfully transfected using an adenoviral vector system. Thus, Sp3 over-expression and siRNA experiments could be performed with this method of transfection using sheep chorionic binucleate cells. This would allow the functionality of the Sp3 *trans*-acting factor to be assessed in a system closer to that of the whole animal. These experiments could provide routes through which genetic therapies could more easily be applied to abnormal pregnacies such as IUGR.

Summary

The proximal promoter (-380/+16) of the ovine (o) PL gene provides trophoblast-specific expression *in vitro*. Footprint 6 (FP6; -319/-349) lies within this region, and block mutations spanning FP6 inhibit transactivation in BeWo (human choriocarcinoma) cells. Our current focus was to identify and functionally characterize the *trans*-acting factors interacting with FP6. Two-base pair transversion mutations were created spanning the length of FP6 (-318/-349), and transiently transfected into forskolin treated BeWo cells. Significant decreases ($p \leq 0.05$) in activity were identified with these mutation constructs within regions of the FP6 sequence that encompassed potential binding sites for CCAAT-enhancer binding protein (CEBP) and Specificity Proteins. Transfection of A-CEBP and CEBP-α dominant negative constructs, which respectively inhibit binding of CEBP proteins and CEBP-α specifically, did not significantly inhibit transactivation of the wild-type -380/+16 promoter construct. Furthermore, co-transfection of CEBP over-expression constructs (CEBP-α, CEBP-β and CEPB-δ) with the wild-type promoter revealed that these constructs did not function to increase transactivation. However, CEBP-β over-

expression resulted in a significant decrease in transactivation, indicating that this protein may bind to the oPL promoter construct and inhibit binding of another transcription factor. Additionally, in supershift analysis using BNC, HeLa and Juarkat cell nuclear extracts with a FP6 oligonucleotide, antisera to the CEBP-α protein did not result in a supershift. These results indicate that the CEBP proteins, specifically CEBP-α, may not interact with the FP6 region to activate transcription of the oPL gene.

In contrast, co-transfection assays with Sp1 and Sp3 over-expression constructs and the -380 oPL promoter construct significantly ($p \leq 0.01$ and $p \leq 0.05$, respectively) increased transactivation in BeWo cells. Furthermore, co-transfections with these Sp1 and Sp3 over-expression constructs and a FP6 minimal prolactin promoter construct resulted in significant ($p \leq 0.01$) increases in transactivation, providing evidence that these *trans*-acting factors specifically interact with the FP6 region. Additionally, co-transfections with Sp1 and Sp3 siRNA constructs caused significant ($p \leq 0.01$) decreases in transactivation compared to the wild type -380 promoter construct, indicating they are necessary for activation of the oPL promoter. In competitive electrophoretic mobility supershift assays, antibodies raised against Sp1 and Sp3 were able to inhibit migration of the complexes formed from labeled oligonucleotide sequences derived from FP6, and nuclear extracts from BNC and BeWo cells, indicating these proteins may be interacting with the FP6 region. Western analysis showed that Sp3, but not Sp1, is present in nuclear extracts of HeLa, Jurkat and BNC cells. Furthermore, Southwestern analysis with a FP6 oligonucleotide identified a nuclear protein with an apparent M_r of 105,000 - 120,000 specifically binding the FP6 region which corresponds with the Sp3 protein identified via Western analysis. Together these results indicate that Sp3 may be the primary protein

interacting with FP6 rather than Sp1. Although Sp1 was able to functionally influence transcription in the tranfection assays, this may in fact be due to the ability of Sp1 to inhibit Sp3 binding as both Sp1 and Sp3 bind to the same sequence. Therefore, it appears that Sp3 may be the nuclear protein interacting with the FP6 region and functioning in a stimulatory manner. In conclusion, these results indicate that Sp3 is capable of interacting with the FP6 region of the ovine placental lactogen gene proximal promoter and may function to enhance its transactivation.

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