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Specificity Protein-1 and -3 Trans-Activate the Ovine Placental Lactogen Gene Promoter

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Abstract

The proximal promoter (-383/+16) of the ovine placental lactogen (oPL) gene provides trophoblast-specific expression *in vitro*. Footprint 6 (FP6; -319/-349) lies within this region, and transfection of two-base pair mutations across FP6 into BeWo cells identified potential binding sites for CCAAT-enhancer binding protein (CEBP) and Specificity Proteins (Sp). Transfection of CEBP dominant negative or over-expression constructs did not impact transactivation of the proximal promoter. However, Sp1 and Sp3 over-expression constructs increased ($p \leq 0.05$) transactivation. Additionally, Sp1 and Sp3 short-hairpin RNA constructs reduced ($p \leq 0.01$) transactivation of the proximal promoter. In EMSA supershift assays, Sp1 and Sp3 antibodies were able to inhibit migration of the complexes formed with nuclear extracts from BeWo cells and ovine chorionic binucleate cells (oBNC). Furthermore, Southwestern analysis of oBNC nuclear extracts identified a nuclear protein corresponding with Sp3, identified by Western analysis. In conclusion, these results indicate that Sp1 and Sp3 are capable of interacting with FP6 of the oPL gene proximal promoter and function to enhance its transactivation.

Keywords

sheep; placenta; oPL; transcription; Sp1; Sp3; CEBP

1. 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 (PL). 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., 1995).

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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. Functional placental insufficiency leads to intrauterine growth restriction, a disease affecting $\approx 8\%$ of all pregnancies, which in severe cases requires early delivery of the fetus in order to avoid fetal mortality (Brar & Rutherford, 1988; Pollack & Divon, 1992). Intrauterine growth restriction 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 or providing nutrients to the developing fetus throughout gestation (Regnault et al., 1999).

The transcriptional regulation of the sheep PL (oPL) 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 protein protected regions (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 Pura element (Liang et al., 1999; Limesand and Anthony, 2001; Limesand et al., 2004).

The region encompassing -383/-217 bp of the oPL promoter appears to enhance activation of the minimal promoter (Liang et al., 1999) to convey maximal trophoblast specific transactivation. 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 objective of this research was to identify and analyze the transcription factors interacting with FP6 of the oPL proximal promoter.

Materials and methods

2.1 FP6 block mutation constructs

Block mutations encompassing the GAGGAG sequence of FP6 (-319/-349) in the oPL gene were generated using site directed mutagenesis as previously described by this laboratory (Liang et al., 1999; Limesand and Anthony, 2001). The following overlapping, synthetic oligonucleotides were used in dual PCR: FP6 Δ 1F, 5'-GCA GCG GCC GCC ATT CCA GCA TTC TTG-3'; FP6 Δ 1R, 5'-ATG GCG GCC GCT GCC CTC CTC CA-3'; FP6 Δ 2F, 5'-CTG GCG GCC GCC ATG GCA ACC CAT-3'; FP6 Δ 2R, 5'-ATG GCG GCC GCC AGG GGT CTT CCC T-3'; FP6 Δ 3F, 5'-GGA GCG GCC GCG GAG GAG GGC AT-3'; and FP6 Δ 3R, 5'-TCC GCG GCC GCT CCC TAC TCA GGG AT-3'. After digestion with restriction endonucleases *Kpn*I and *Hind*III (New England Biolabs, Inc., Beverly, MA), the mutated FP6 promoter products were ligated into pGL3 Basic vector (Promega, Madison, WI). The FP6 block mutations Δ 1, Δ 2 and Δ 3 were confirmed using Southern analysis and nucleotide sequencing. An alkaline-lysis procedure, followed by CsCl equilibrium centrifugation, was used to obtain covalently closed circular DNA (Liang et al., 1999; Limesand and Anthony, 2001).

2.2 Construction of two-base pair mutation constructs

Two-base pair transversion mutations, encompassing FP6 (-349/-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) -383 oPL promoter sequence (Liang et al., 1999). Sixteen forward and sixteen reverse primers containing the mutations were generated starting at -349 and ending at -318 (Table 1). PCR, using *Taq* DNA Polymerase, was employed using the linearized WT -383 pGL3 (Liang et al., 1999) plasmid as the template with a separate reaction for the forward and the reverse primers. The resulting products were then agarose gel purified, annealed together and used as the template, with the pGL3 Basic primers (RV3 and GL2), for the second PCR. After restriction endonuclease digestion (*Kpn*I and *Hind*III) the product was ligated into the pGL3 plasmid using a 1:3 molar ratio of plasmid to PCR insert. Competent DH5 α cells were transformed with the ligation mixture and plasmid DNA was isolated from resulting colonies, digested and separated to confirm the size of the insert. The sequences were subsequently compared to the WT -383 pGL3 sequence using BLAST analysis to confirm that the mutation was correct. Once the mutations were correctly obtained the plasmids were amplified and isolated using a CsCl centrifugation gradient.

2.3 Cell culture and transient transfections

BeWo cells (a human choriocarcinoma cell line) were obtained from American Type Culture Collection (Rockville, MD) and maintained as previously described by Limesand *et al.* (Limesand et al., 2004). Cell passage was kept below 10 after thawing cells for the transient transfection analysis to ensure optimal results. Transient transfections were performed in 6-well dishes at a density of 0.2×10^6 cells/well. Cells were additionally treated for 48 hours with 80 μ M 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 μ g/ml streptomycin (Sigma Chemical Co., St. Louis, MO), 3 mls per well, to cause them to differentiate into syncytiotrophoblasts, producing a more homogenous population (Kudo and Boyd, 2002; Wice et al., 1990).

Transient transfections were performed using Polyfect (Qiagen, Valencia, CA) transfection reagent. All transfection experiments were repeated three times, with each experiment using separate plasmid DNA isolations, and three replicate wells/construct. For each reaction, 5 μ g of pGL3 plasmid DNA (either mutated or WT -383 pGL3), 12 μ l Polyfect, 0.25 μ g of control β -galactosidase expression plasmid (RSV promoter and enhancer, ClonTech Laboratories, Inc., Palo Alto, CA) and F12K culture medium without serum or antibiotics in a total volume of 100 μ l. The polycationic lipid/DNA complexes were allowed to form at room temperature for 15 minutes, at which time the cells were washed twice with 3 mls serum- and antibiotic-free medium. After the 15 minute incubation, 900 μ l of complete F12K medium was added to the Polyfect-DNA mixture and added to the differentiated BeWo cells. The cells were incubated at 37°C for 48 hour and then harvested by lysing. Luciferase and β -galactosidase activities were measured using a Luciferase Assay System (Promega, Madison, WI) and a Galacto-Light Plus Assay kit (Applied Biosystems, Bedford, Massachusetts).

For transient co-transfections with the CEBP- α , - β , and - δ over-expression vectors (Liu et al., 2001), kindly provided by Dr. Norman Curthoys (Colorado State University), 5 μ g of WT -383 pGL3 was added to the cells with either 5 μ g of control plasmid (pGL2), CEBP- α , - β , or - δ plasmids. The dominant negative (DN) co-transfections were performed in the same manner, with the WT -383 pGL3 construct being added to the cells in addition to either control plasmid (pcDNA3.1), A-CEBP (Moitra et al., 1998), generously donated by Dr. Charles Vinson (National Cancer Institute, NIH), or DN-CEBP- α (Pabst et al., 2001), a kind gift from Dr. Daniel Tenen (Harvard Medical School).

For transient co-transfections using the Sp1 and Sp3 over-expression vectors, the Sp1 construct (Naar et al., 1998) was provided by Dr. Robert Tjian, (University of California, Berkeley), and the Sp3 construct (Kennett et al., 1997) was obtained from Dr. Jon Horowitz (North Carolina State University). Over-expression co-transfections were performed using WT -383 pGL3 with either the control plasmid (pcDNA3.1) or the Sp1 or Sp3 expression constructs. The co-transfections were also performed with a construct containing the FP6 (-319/-349; FP6/PRL) sequence in front of the minimal rat prolactin promoter construct (Duval et al., 1999) and the Sp1 or Sp3 expression plasmids. All DNA concentrations remained constant at 5 µg/reaction.

For Sp RNA interference (RNAi), Sp1 and Sp3 siRNA constructs were purchased from Panomics (Redwood City, CA). The concentration of DNA used in the RNAi experiments was 2.5 µg/reaction. The WT -383 pGL3 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.

All transfection experiments were repeated three times, with each experiment using separate plasmid DNA isolations, and three replicate wells/construct. The luciferase activity for each construct was normalized for transfection efficiency (β-galactosidase activity). The activity obtained for each construct was statistically compared to the control construct (WT -383 pGL3) by least square analysis of variance followed by paired Dunnett tests (Statistical Analysis Systems, Cary, NC). In this analysis, “construct” was the independent variable, blocked by “replicate experiment”, and “normalized activity” was the dependent variable. Data are presented as the mean “normalized activity” ± sem.

2.4 Nuclear protein isolation and separation

Mature ewes were bred at behavioral estrus (day 0) and at 100 days post coitus (dpc), fetal cotyledonary tissue was removed from the placenta and the chorionic binucleate cells (oBNC) were isolated as described previously by our laboratory (Liang et al., 1999; Limesand and Anthony, 2001). BeWo cells were expanded in culture and the nuclear protein isolated as described by Limesand *et al.* (Limesand et al., 2004). The nuclear protein from these cells was subsequently extracted using the procedure of Dignam *et al.* (Dignam et al., 1983).

2.5 Electrophoretic mobility supershift assays

Electrophoretic mobility shift assays (EMSA) were performed as previously described by this laboratory (Liang et al., 1999; Limesand et al., 2004). The synthetic oligonucleotide used for EMSA was FP6 (-345/-325) (5'- ACC CCT GGA GGA GGG CAT GGC -3'; sense strand represented). For supershift analysis, the antibodies (2 µl of rabbit polyclonal crude serum) to Sp1, Sp3 or CEBP-α (Active Motif, Carlsbad, CA) were added to the reaction mixture containing the nuclear protein (20 µg) and the buffer, and allowed to incubate overnight at 4 °C. Following pre-incubation with the antiserum, the unlabeled competitors (100-fold molar excess) were then added to the reaction mixture and incubated at room temperature for 5 minutes, after which the labeled oligonucleotides (10,000 cpm/fmol) 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.

2.6 Western immunoblot analysis

Western analysis was performed as previously described by this laboratory (Limesand et al., 2004) using 50 µg oBNC nuclear extracts or 50 µg nuclear extracts from BeWo cell cultures. Immunoblot detection of Sp1 (sc-14027) and Sp3 (sc-644) was performed by incubating antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) at a concentration of 1:200 (1X TBST

solution) for 2 hours at room temperature with gentle agitation. The membrane was washed 3 × 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.

2.7 Southwestern analysis

The Western immunoblot membranes were 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.

Southwestern analysis was performed on oBNC nuclear proteins using the method described by Limesand *et al.* (2004). 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'. Binding reactions were performed at 4 °C for four hours. The membranes were incubated with either radiolabeled FP6 oligonucleotide alone or in conjunction with 100- or 200- fold molar excess unlabeled FP6 oligonucleotide to determine specificity and, after washing, subsequently exposed to x-ray film.

3. Results

3.1 Functional analysis of FP6

In order to test the functionality of the footprint 6 (FP6) sequence in the transcriptional regulation of the oPL gene, transient transfection analysis in BeWo cells was conducted using FP6 block mutation constructs. All three mutations significantly reduced ($p \leq 0.01$) transactivation through the proximal promoter (data not shown). To determine if a single, contiguous *cis*-element within the FP6 region is responsible for transactivation, 16 two-base pair transversion mutations were created encompassing the FP6 sequence, and the functionality of these mutations was examined in transient transfection analysis in BeWo cells. The results (Figure 1) of these transient transfections identified specific base pairs that, when mutated, significantly decreased activity as compared to WT -383 pGL3. Specifically, mutation constructs 2, 4, 5, 6, 9 and 10 caused significant (4, 5, 6 $p \leq 0.01$; 2, 9, 10 $p \leq 0.05$) decreases in transactivation, whereas mutation constructs 7, 8 and 12 tended ($P \leq 0.10$) to reduce transactivation, when compared to the WT -383 oPL promoter construct. Collectively, these data suggest these bases play an important role in transcriptional regulation of the oPL gene.

3.2 Identification of transcription factors interacting with FP6

Based on *in silico* transcription factor analysis of the FP6 region, the sequence from -339/-332 (GGAGGAGG) represents a potential binding site for the CCAAT-enhancer binding (CEBP) proteins while the sequence from -340/-325 (TGGAGGAGGGCATGGC) represents a potential binding site for the specificity proteins (Sp). Functional analysis of CEBP proteins was carried out by co-transfection experiments using over-expression constructs for CEBP- α , - β and - δ and the dominant negative constructs A-CEBP and CEBP- α in BeWo cells. Over expression of CEBP - α , - β , - δ (Figure 2) had no enhancement effects on promoter activity as compared to the control constructs, indicating that perhaps CEBP proteins are not involved in activation of transcription of the oPL promoter. However, there was a significant ($p \leq 0.05$) decrease in transactivation with over-expression of the CEBP- δ construct, implying that this protein may interact with the oPL promoter and competes for complex formation by other

transcription factors necessary for activation. Results from the A-CEBP and CEBP- α dominant negative co-transfections (Figure 3) indicated no significant ($p \geq 0.10$) reduction in transactivation, providing further evidence that the CEBP proteins alone are unlikely to activate transcription of the oPL gene.

To determine whether Sp1 and/or Sp3 interact with FP6, BeWo cells were co-transfected with the WT -383 pGL3 construct and either 5.0 μ g of Sp1 or Sp3 expression constructs. Both Sp1 and Sp3 significantly ($p \leq 0.05$) enhanced oPL promoter activity compared to WT -383 pGL3 +control DNA (Figure 4). Co-transfections with the FP6/PRL promoter construct and Sp1 or Sp3 expression vectors indicated that the Sp factors stimulated ($p \leq 0.01$) luciferase expression specifically through interaction with the FP6 sequence (Figure 5). For all of our transfection experiments, we conducted dose titrations in order to determine the amount of construct needed to provide relevant results, considering that BeWo cells express Sp and CEBP proteins. Transfecting 1.0 μ g of either the Sp1 or Sp3 expression constructs did not result in increased reporter activity (data not shown), whereas transfecting twice the amount (10.0 μ g) resulted in repression of activity (data not shown). This was also found with the over-expression of CEBP- δ (Figure 2). We do not know if this repression of activity is due to competition for cofactors, or some other inhibition. Regardless, the increase in activity obtained by transfecting 5.0 μ g of the Sp1 or Sp3 expression constructs resulted in statistically significant increases with both the WT -383 pGL3 (Sp1, 48.6%; Sp3, 13.6%) and FP6/PRL (Sp1, 59.5%; Sp3, 41.0%) reporter constructs. To further confirm that Sp1 and Sp3 were indeed interacting with the FP6 region to stimulate transcription, Sp1 and Sp3 siRNA constructs, a control plasmid, and WT -383 pGL3 were co-transfected into BeWo cells. RNA interference (RNAi) of either Sp1 or Sp3 expression significantly ($p \leq 0.01$) reduced promoter activity of the WT -383 pGL3 construct (Figure 6).

3.3 Verification of Sp interaction with FP6

To further investigate Sp or CEBP protein interactions at FP6, electrophoretic mobility supershift analysis was performed using both BeWo and oBNC nuclear extracts. Antibodies raised against Sp1 and Sp3 resulted in “supershifts” of the radiolabeled FP6 oligonucleotide with both BeWo (Figure 7) and oBNC (Figure 8) nuclear extracts, whereas anti-CEBP- α did not.

Western analysis was carried out using nuclear extracts from oBNC to assess whether Sp1 and Sp3 were present, followed by Southwestern analysis using the same membrane. Sp3 was detected in oBNC nuclear extracts (Figure 9) by Western analysis and a protein with similar mobility was detected by Southwestern analysis, using the radiolabeled FP6 concatamer 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.

4. Discussion

The placental lactogens are members of the growth hormone (GH)/prolactin (PRL) gene family, and may regulate fetal growth by altering maternal and fetal metabolism following their secretion from terminally differentiated trophoblast cells at the maternal-fetal interface (Anthony et al., 1995, 1998). Ovine PL is synthesized within a subpopulation of the chorionic epithelium, the binucleate cells (Kappes et al., 1992), throughout gestation, and is secreted into both the maternal and fetal vasculature. Previous DNase I protection analysis identified several protein-DNA interactions within the proximal promoter of the oPL gene (Liang et al., 1999), one of which was designated FP6 (-319/-349). Through mutational analysis of FP6, the sequence GAGGAG was shown to be functional and necessary for maximal trophoblast-specific transactivation of the oPL promoter. In our current study, FP6 was analyzed by block

mutations and two-base pair transversion mutations to further define the active element. Our findings revealed potential interactions with CEBP or Sp transcription factors at FP6. In addition to the mutational analysis we used over-expression constructs, dominant-negative constructs, RNAi and electrophoretic mobility supershifts to demonstrate that the *cis*-acting element within FP6 interacts with Sp's rather than CEBP's. Furthermore, Western and Southwestern analysis using oBNC, the source of oPL, nuclear extracts indicate that Sp3 is the predominant transcription factor interacting with FP6.

Previous investigation of the transcriptional regulation of the oPL gene revealed the lack of a functional TATA box and maximal reporter transactivation in choriocarcinoma cells being conferred within the proximal -383 bp of 5'-flanking sequence (Liang et al., 1999). Within the -383 bp proximal promoter, functional sites of interaction have been identified for GATA-2 (Liang et al., 1999), AP-2 α (Limesand and Anthony, 2001) and Pura (Limesand et al., 2004). Our current studies demonstrate that the Sp proteins, Sp1 and Sp3, are able to interact with FP6 (-319/-349) located within the proximal promoterregion required for maximal trophoblast expression. Sp1 and Sp3 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 of developmental genes (Kishikawa et al., 2002; Schanke et al., 1998; Wong and Lee, 2002).

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 PL gene (Fitzpatrick et al., 1990; Jiang and Eberhardt, 1995; Schanke et al., 1998), both members of the same gene family as oPL. 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). 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). There is 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). While both Sp1 and Sp3 were shown to interact with FP6 in electrophoretic mobility supershift analysis of BeWo and oBNC nuclear extracts, and the use of over-expression and RNA interference constructs demonstrated functional interaction of both Sp1 and Sp3 with FP6 in BeWo cells, Western and Southwestern analysis of oBNC nuclear extracts suggested that Sp3 is the predominant factor in the sheep placenta. Western analysis of oBNC nuclear extracts with anti-Sp3 identified a predominant isoform with an apparent M_r of 105 - 120 kDa, coinciding with previous reports of Sp3 (Kishikawa et al., 2002; Wong and Lee, 2002). Southwestern analysis of the same membranes identified FP6 binding the same apparent protein identified with the Sp3 antibody. The same experiments (data not shown) were performed using anti-Sp1, but failed to clearly delineate Sp1 interaction with FP6. The inability to identify Sp1 interactions with FP6 by the combination of Western and Southwestern analysis does not rule out the ability of Sp1 to transactivate the oPL gene promoter at FP6, but may simply result from the loss of shared epitopes between human and sheep Sp1 when the oBNC nuclear proteins were electrophoresed through a denaturing gel.

Previously, we (Limesand et al., 2004) demonstrated the functional interaction of Pura, a single-stranded DNA binding protein, with the oPL proximal promoter. A Pura-Sp1 interaction has been demonstrated for the myelin basic protein gene (Tretiakova et al., 1999), augmenting its transactivation. Pura and Sp1/Sp3 share adjacent binding sites within the smooth muscle actin enhancer (Sukanya et al., 2004) and Sp1 and Pura physically interact in a transforming growth factor β 1 sensitive manner. Pura and Sp1 were shown to interact in the absence of DNA, suggesting that such a heterodimer may form at either a Pura or Sp element to augment transcription (Tretiakova et al., 1999). An element in the human PL promoter that is identical

to the ovine $Pur\alpha$ element (Limesand et al., 2004) lies in the -152/-142 bp region that contributes to the transactivation of the human PL gene (Fitzpatrick et al., 1990). These investigators determined that the -142/-129 bp region of the human PL gene contained a noncanonical Sp1 element that, when deleted, had a more dramatic effect on transactivation than did deletion of the -152/-142 bp region. As the $Pur\alpha$ and Sp1 elements in the human PL gene promoter are adjacent to each other, they may confer a combined SPUR element similar to that described by Subramanian *et al.* (2004) for the smooth muscle α -actin gene promoter. In the current studies we did not investigate the interaction of $Pur\alpha$ with Sp1 or Sp3, and their respective elements are not directly adjacent in the oPL promoter. However, such an interaction, along with potential interactions with GATA-2 and AP-2 α , are likely required to nucleate the preinitiation complex on this TATA-less promoter and elicit maximal transactivation.

In conclusion, we have determined that Sp proteins, Sp1 and Sp3, interact with a previously described DNA sequence within the 5'-flanking sequence of the oPL gene, identified by DNase I analysis using oBNC nuclear proteins. This *cis*-acting element resides within the region required for maximal transactivation within BeWo cells, and its mutation results in significant diminution of oPL promoter activity. The combination of Sp1/Sp3, AP-2 α , GATA-2 and $Pur\alpha$ appear to be required for activity of the oPL proximal promoter, and while none of these transcription factors are expressed only in trophoblast cells, their interaction, along with interactions with other *trans*-acting factors and/or co-regulators, likely provide for oBNC-specific expression of the placental lactogen gene.

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Figure 1.

Two-base pair transversion mutations of FP6 transfected into forskolin treated BeWo cells. The FP6 sequence is shown below the graph with the corresponding mutation number under each base-pair. Activity was calculated as luciferase/ β -galactosidase and expressed as “normalized activity”. The data are presented as means $\pm$ sem. † Denotes activity reduction ($P \leq 0.10$) for mutations 7, 8 and 12, when compared to WT -383 pGL3 (-383). * Denotes activity reduction ($p \leq 0.05$) for mutations 2, 9 and 10, when compared to WT -383 pGL3 (-383). ** Denotes activity reduction ($p \leq 0.01$) for mutations 4, 5 and 6, when compared to WT -383 pGL3 (-383).

Figure 2.

Activity of CEBP- α , CEBP- β and CEBP- δ over-expression constructs co-transfected with WT -383 pGL3 in forskolin treated BeWo cells. Luciferase reporter activity was normalized against β -galactosidase activity, and the normalized activity for each over-expression construct was compared against the activity of WT -383 pGL3 (383/pGL2). The data are presented as means $\pm$ sem. * Designates a reduction ($p \leq 0.05$) in activity for the CEBP- δ co-transfection.

Figure 3.

Activity of WT -383 pGL3 when co-transfected with either a specific CEBP- α dominant negative (DN-CEBP α) or a general CEBP (A-CEBP) dominant negative construct. Luciferase reporter activity was normalized against β -galactosidase activity, and the normalized activity for each dominant negative construct was compared against the activity of WT -383 pGL3 (383/pcDNA). Data are presented as means $\pm$ sem. The activity was not significantly impacted by either CEBP dominant negative construct.

Figure 4.

Activity of WT-383 pGL3 when co-transfected with constructs expressing Sp1 and Sp3. The empty pcDNA 3.1 vector was used as a control. Results are expressed as β -galactosidase normalized activity, and data are presented as means $\pm$ sem. *Designates increased ($p \leq 0.05$) transactivation as a result of over-expressing Sp3. **Designates increased ($p \leq 0.01$) transactivation as a result of over-expressing Sp1.

Figure 5. Activity of the FP6/PRL construct co-transfected with Sp1 and Sp3 expression constructs. The empty pcDNA 3.1 vector was used as a control. Results are expressed β -galactosidase normalized activity, and data are presented as means $\pm$ sem. **Denotes increased activity ($p \leq 0.01$) as a result of over-expressing either Sp1 or Sp3.

Figure 6.

Activity of WT -383 pGL3 following co-transfection of Sp1 and Sp3 siRNA constructs. The WT -383 pGL3 promoter construct was co-transfected with a control plasmid (the empty siRNA vector pU6 + 27) or the siRNA plasmids for Sp1 or Sp3. Results are expressed as β -galactosidase normalized activity, and data are presented as means $\pm$ sem. **Denotes decreased activity ($p \leq 0.01$) as a result of co-transfecting either Sp1 or Sp3 siRNA constructs.

Figure 7.

Electrophoretic mobility supershift analysis performed using BeWo cell nuclear extracts and a radiolabeled FP6 oligonucleotide. This analysis was used to determine whether BeWo cell-derived Sp1, Sp3, or CEBP- α proteins would bind the FP6 region of the oPL promoter. The radiolabeled oligonucleotide encompassed -325/-345 bp of FP6. A shift in mobility was observed with BeWo nuclear extracts, and addition of 100-fold molar excess unlabeled FP6 oligonucleotide was able to effectively inhibit binding. Antiserum to Sp1 and Sp3, but not CEBP- α , was able to supershift the nuclear protein-oligonucleotide complex.

Figure 8.

Electrophoretic mobility supershift analysis performed using oBNC nuclear extracts and a radiolabeled FP6 oligonucleotide. This analysis was used to determine whether oBNC-derived Sp1, Sp3, or CEBP- α proteins would bind the FP6 region of the oPL promoter. The radiolabeled oligonucleotide encompassed -325/-345 bp of FP6. A shift in mobility was observed with oBNC nuclear extracts, and addition of 100-fold molar excess unlabeled FP6 oligonucleotide was able to effectively inhibit binding. Antiserum to Sp1 and Sp3, but not CEBP- α , was able to supershift the nuclear protein-oligonucleotide complex.

Figure 9.

Western and Southwestern analysis of oBNC nuclear proteins, using the Sp3 antibody and FP6 radiolabeled oligonucleotide. The membrane on the left shows a Western assay using Sp3 antiserum. The arrow marks the nuclear protein, with an apparent M_r of ≈ 105 - 120 k, identified by the Sp3 antibody. The membrane was then subjected to Southwestern analysis, shown in the center, with the radiolabeled FP6 oligonucleotide, identifying the same apparent protein detected by the Sp3 antiserum. The membrane on the right was incubated with the radiolabeled FP6 oligonucleotide and 200-fold molar excess unlabeled FP6 oligonucleotide. The unlabeled FP6 oligonucleotide efficiently competed with the radiolabeled FP6 oligonucleotide for

binding with the nuclear protein identified in both Western and Southwestern analysis, as shown by the arrow.

Table 1

Primers used in generating two-base pair transversion mutations in FP6

Mutation #	Forward Primers	Reverse Primers
MUT 1	cctgagtagg CT agaccctgg	ccaggggtct AG cctactcagg
MUT 2	tgagtaggga TC acccctggag	ctccaggggt GA tccctactca
MUT 3	agtagggaag TG ccctggagga	tcctccaggg CA ctccctact
MUT 4	tagggaagac GG ctggaggagg	cctcctccag CC gtcttccta
MUT 5	gggaagacc GA ggaggagggc	gccctctcc TC gggcttccc
MUT 6	gaagaccct CC aggaggcat	atgccctct GG aggggtctc
MUT 7	agaccctgg TC gagggcatgg	ccatgccctc GA ccaggggtct
MUT 8	accctggag CT gggcatggca	tgccatccc AG ctccaggggt
MUT 9	ccctggagga CC gcatggcaac	gttgccatgc GG tcctccaggg
MUT 10	ctggaggagg CG atggcaacc	gggttgccat CG cctcctccag
MUT 11	ggaggagggc TA ggcaaccat	atgggttgcc TA gccctctcc
MUT 12	aggaggcat CC caaccattc	gaatgggttg GG atgccctct
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	atggcaacc TA tccagcattc	gaatgctgga TA ggggttgccat