

Distinct Regulatory Regions from the Prolactin-Like Protein C Variant Promoter Direct Trophoblast Giant Cell *Versus* Spongiotrophoblast Cell-Specific Expression*

GUOLI DAI, MICHAEL W. WOLFE, AND MICHAEL J. SOARES

Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, Kansas 66160

ABSTRACT

PRL-like protein C variant (PLP-Cv) is a newly identified member of the PRL family. PLP-Cv is specifically expressed in the chorioallantoic placenta by two distinct cell populations: trophoblast giant cells and spongiotrophoblast cells. To gain some insight regarding the control of PLP-Cv gene expression and the regulatory factors controlling trophoblast giant cell and spongiotrophoblast cell lineages, we have initiated a structural and functional analysis of the PLP-Cv promoter. The activities of a series of PLP-Cv promoter constructs, ranging in size from 4.5 kb to 50 bp, ligated to a luciferase reporter have been assessed in the Rcho-1 trophoblast cell line (restricted to trophoblast giant cell differentiation) and in a primary spongio-

phoblast cell culture system after transient transfection. PLP-Cv promoter constructs containing 4.5 kb to 149 bp of 5'-flanking DNA possessed full activity in the trophoblast giant cell model. A region located between -149 and -124 bp upstream of the PLP-Cv transcription start site was found to be essential for activation of the PLP-Cv promoter. Spongiotrophoblast cells required additional PLP-Cv 5'-flanking DNA for full activity. A region located between -2518 and -2242 bp upstream of the PLP-Cv transcription start site significantly enhanced PLP-Cv promoter in spongiotrophoblast cells. In conclusion, mechanisms underlying the activation of the PLP-Cv promoter are different in trophoblast giant cells *vs.* spongiotrophoblast cells. (*Endocrinology* 140: 4691–4698, 1999)

THE PRL family represents a large group of hormones/cytokines expressed by the anterior pituitary, uterus, and/or placenta with important implications on the establishment and maintenance of pregnancy (1, 2). Investigations into the biology and regulation of members of this family have provided insight into signaling mechanisms underlying viviparity. Individual members of the PRL family have been shown to possess classical PRL-like actions, including participation in the control of maternal ovarian and mammary gland development and function (3–5), whereas other members possess nonclassical actions and contribute to modulating vasculature at the maternal-fetal interface (6, 7) and controlling cells of hemopoietic origin participating in immune and inflammatory responses (8–12).

PRL-like protein C variant (PLP-Cv) is a member of a subfamily of PRL family members possessing a six-exon/five-intron gene structure (13) that differs from the prototypical PRL five-exon/four-intron gene structure (14, 15). This subfamily is referred to as the PLP-C subfamily and also includes PLP-C, PLP-D, and PLP-H identified in the rat placenta (16–21), PLP-C α isolated from the mouse placenta (22), and decidual/trophoblast PRL-related protein (d/tPRP), which has been characterized from uterine decidua and placenta of both the mouse and rat (23–27). Biological roles for PLP-C subfamily members during pregnancy are beginning to emerge.

The chorioallantoic placenta prominently contributes to the production of members of the PLP-C subfamily and can be divided into two functional compartments: the junctional zone and the labyrinth zone (28). Hormone/cytokine production is a fundamental role of the junctional zone, whereas nutrient/waste transport characterizes the labyrinth zone. The junctional zone is situated at the interface with the uterine decidua, its development at midgestation is essential for progression of pregnancy, and it is a rich source of PLP-C subfamily members (2, 28, 29). PLP-Cv, PLP-C, PLP-D, PLP-H, and d/tPRP are coordinately expressed by two cell types within the junctional zone of the rat chorioallantoic placenta: trophoblast giant cells and spongiotrophoblast cells (13, 16–21, 27). Information on the regulation of PLP-C subfamily member expression potentially provides insight into the development of both trophoblast giant cell and spongiotrophoblast cell lineages. *In vitro* models of trophoblast giant cell and spongiotrophoblast cell differentiation have been established and provide an effective means for examining trophoblast cell-specific gene regulation (26, 30–34). Using these models, a 2.1-kb PLP-Cv promoter-reporter construct was previously shown to possess trophoblast cell-specific activation (13).

In this report, we extend our functional analysis of the PLP-Cv promoter and demonstrate distinct DNA regulatory regions responsible for trophoblast giant cell *vs.* spongiotrophoblast cell gene activation.

Materials and Methods

Reagents

FBS and donor horse serum (HS) were purchased from JRH Bioscience (Lenexa, KS). All restriction enzymes, polymerases, and DNA ligase were purchased from New England Biolabs, Inc. (Beverly, MA).

Received March 12, 1999.

Address all correspondence and requests for reprints to: Dr. Guoli Dai, Department of Molecular and Integrative Physiology, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, Kansas 66160. E-mail: gdai@kumc.edu.

* This work was supported by grants from the J. B. Reynolds Foundation and the NICHD (HD-02528, HD-20676, HD-29797, and HD-33994).

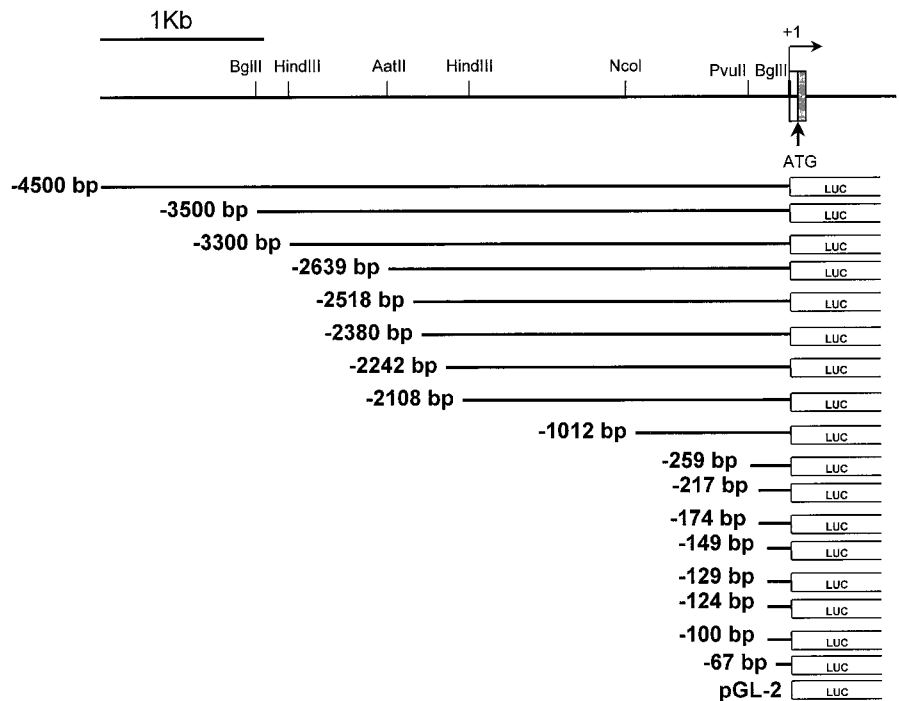


FIG. 1. Design of the PLP-Cv promoter-luciferase reporter constructs. *Top panel*, Restriction map of 4.5 kb of the 5'-flanking region of the PLP-Cv gene. *Bottom panel*, A series of promoter-luciferase constructs generated with restriction fragments or by PCR. PCR-generated constructs were verified by DNA sequencing.

The GH₃ pituitary tumor and L929 cell lines and a Rous sarcoma virus promoter- β -galactosidase (RSV- β -GAL) reporter plasmid were obtained from American Type Culture Collection (Manassas, VA). Transformation-competent *Sure* bacterial cells were acquired from Stratagene (La Jolla, CA). DNA extraction kits were purchased from QIAGEN (Chatsworth, CA). The pGL-2 basic vector and an RSV promoter-luciferase reporter plasmid were purchased from Promega Corp. (Madison, WI). T7 DNA sequencing kits were acquired from U.S. Biochemical Corp. (Cleveland, OH). PCR cloning kits were obtained from Invitrogen (San Diego, CA) and CLONTECH Laboratories, Inc. (Palo Alto, CA). Lipofectamine reagent for transfection was obtained from Life Technologies, Inc. (Gaithersburg, MD). Kits for monitoring β -galactosidase activities were acquired from Tropix (Bedford, MA). Unless otherwise noted, all other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Generation of promoter-reporter constructs

A series of DNA fragments flanking the 5'-end of the PLP-Cv gene was subcloned into *Kpn*I and *Bgl*III cloning sites upstream of the luciferase reporter gene within the pGL-2 basic vector. PCR-generated constructs were verified by DNA sequencing.

Animals and tissue collection

Holtzman rats were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The animals were housed in an environmentally controlled facility, with lights on from 0600–2000 h, and allowed free access to food and water. Timed pregnancies and tissue dissections were performed as previously described (35). Day 0 of pregnancy was defined by the presence of sperm in the vaginal smear. Protocols for the care and use of animals were approved by the University of Kansas animal care and use committee.

Cell culture models

A series of trophoblast and nontrophoblast cell lines was examined for the ability to express PLP-Cv. The Rcho-1 trophoblast cell line was derived from a rat choriocarcinoma and is capable of differentiating along the trophoblast giant cell lineage (30, 31). Rcho-1 trophoblast cells were routinely maintained in subconfluent conditions with NCTC-135 culture medium supplemented with 20% FBS, 50 μ M 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μ g/ml strepto-

mycin (30, 31). Differentiation was induced by growing the cells to confluence in FBS-supplemented culture medium and then replacing the serum supplementation with 10% HS (30, 31, 36). The HRP-1 trophoendodermal stem cell line represents a cell population with labyrinthine trophoblast characteristics (37). HRP-1 trophoendodermal cells were routinely maintained in RPMI 1640 culture medium containing 10% FBS and the above supplements. GH₃ cells were derived from a rat pituitary tumor (38) and were maintained in DMEM supplemented with 10% FBS and antibiotics. L929 cells represent a mouse fibroblast cell line and were maintained in RPMI culture medium supplemented with 10% FBS and antibiotics.

Primary spongiotrophoblast cell cultures were established from junctional zones of day 13 rat chorioallantoic placentas as previously described (33). Tissues were cut into small pieces with iris scissors and dissociated with dispase (4.8 mg/ml) and deoxyribonuclease I (80 U/ml) for 1 h at 37 C with continuous shaking. At the end of the digestion, the suspension of cells and tissue fragments was mixed several times with the aid of a Pasteur pipette and centrifuged. The harvested cells were then resuspended in DMEM supplemented with 10% FBS and filtered through a nylon mesh (74 μ m pore size). The cell suspension was washed and then plated in DMEM supplemented with 10% FBS.

PLP-Cv promoter analysis in trophoblast and nontrophoblast cell types

Promoter-reporter constructs were transiently transfected into Rcho-1 trophoblast, HRP-1 trophoendodermal, GH₃ pituitary tumor, and L929 fibroblast cell lines and primary spongiotrophoblast cell cultures using a liposome-mediated delivery system. Cells from each of the cell lines were seeded in 35-mm tissue culture dishes (3×10^5), grown to approximately 70–80% confluence, and then transfected with 2 μ g of the promoter-luciferase construct, RSV promoter-luciferase (RSV-Luc; positive control), or pGL-2 basic vector (negative control). A RSV promoter- β -galactosidase construct (RSV- β -GAL; 0.5 μ g) was cotransfected and used to evaluate transfection efficiency. Forty-eight hours after transfection, cells were collected, and lysates were prepared. Luciferase activity was measured with a luminometer according to the procedure of Brasier *et al.* (39). β -Galactosidase activities and total protein concentrations in the lysates were determined with a Galacto-Light kit (Tropix) and the protein-dye binding method (40), respectively. In each experiment, transfections for a given promoter construct were performed in triplicate, and experiments were replicated at least three times.

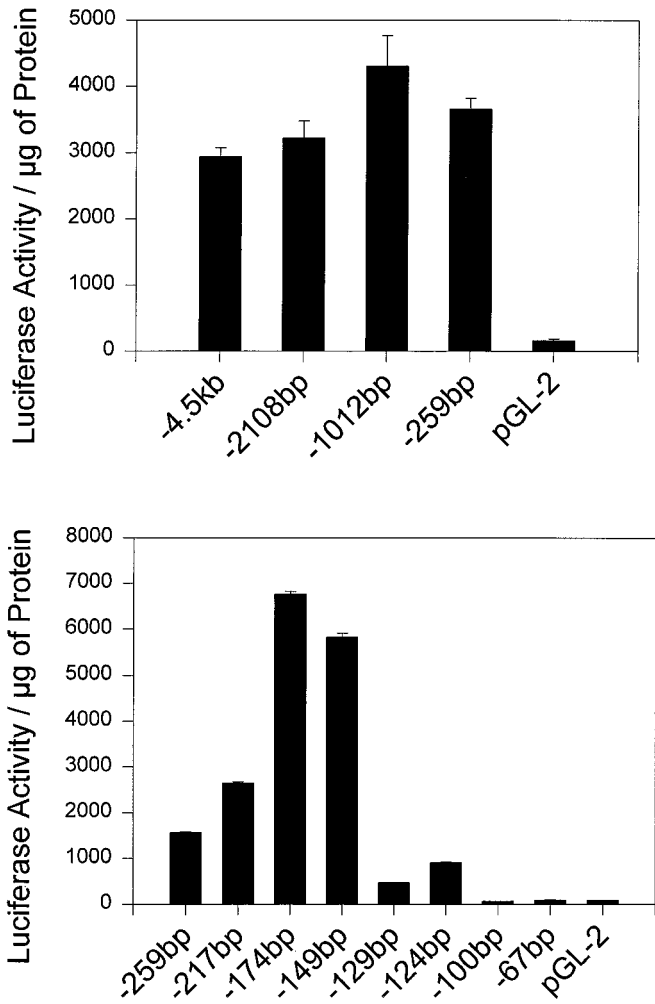


FIG. 2. PLP-Cv promoter activity in trophoblast giant cells. Rcho-1 trophoblast cells represent a cell culture model restricted to trophoblast giant cell differentiation. Rcho-1 trophoblast cells were maintained in NCTC culture medium supplemented with 10% FBS. DNA constructs (2 µg) were transfected into Rcho-1 trophoblast cells (trophoblast giant cell lineage) using a liposome-mediated delivery system on day 3 of culture. RSV-β-Gal (0.5 µg) was cotransfected to evaluate transfection efficiency. Culture medium was changed to NCTC supplemented with 10% HS immediately after transfection. Forty-eight hours after transfection, cell lysates were prepared, and luciferase, β-galactosidase activities, and total protein concentration were determined. Luciferase activities were normalized according to β-galactosidase activity and protein concentration. Each value is the mean ± SEM of triplicate measurements. The initial functional analysis indicated that the -259 bp promoter construct possessed full promoter activity in the trophoblast giant cell culture system (*top panel*). Further deletion analysis is shown in the *bottom panel*. Note that the region between -124 to -100 bp is essential for the activation of the PLP-Cv promoter in trophoblast giant cells.

Results

Aspects of the regulation of trophoblastic PLP-Cv expression were investigated through the analysis of PLP-Cv promoter-luciferase reporter constructs in an *in vitro* model of trophoblast giant cell differentiation and an *in vitro* model of spongiotrophoblast cell differentiation. Promoter-reporter constructs examined are shown in Fig. 1.

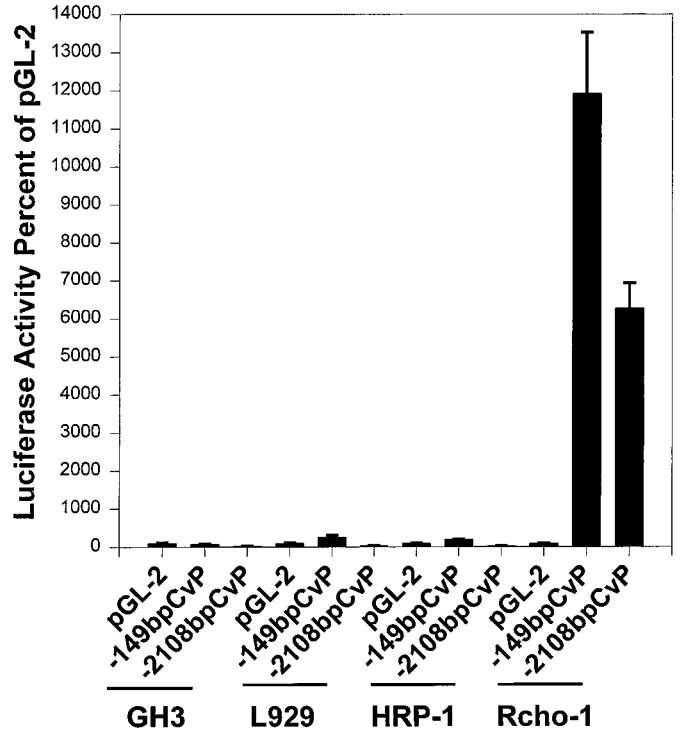


FIG. 3. Trophoblast giant cell-specific activation of the -149 bp PLP-Cv promoter. Trophoblast (Rcho-1, HRP-1) and nontrophoblast (GH₃, L929) cell lines were cotransfected with promoter-luciferase constructs [2 µg each of the promoterless pGL-2 vector, -149 bp PLP-Cv promoter (-149bpCvP), or the -2108 bp PLP-Cv promoter (-2108bpCvP)] and RSV-β-Gal (0.5 µg). Forty-eight hours after transfection, cell lysates were prepared, and luciferase and β-galactosidase activities and total protein concentration were determined. Luciferase activities were normalized according to β-galactosidase activity and protein concentration. Each value is the mean ± SEM of triplicate measurements. Note that activation of the -149 and -2018 bp PLP-Cv promoter constructs was restricted to the Rcho-1 trophoblast giant cell model.

PLP-Cv promoter activity in trophoblast giant cells

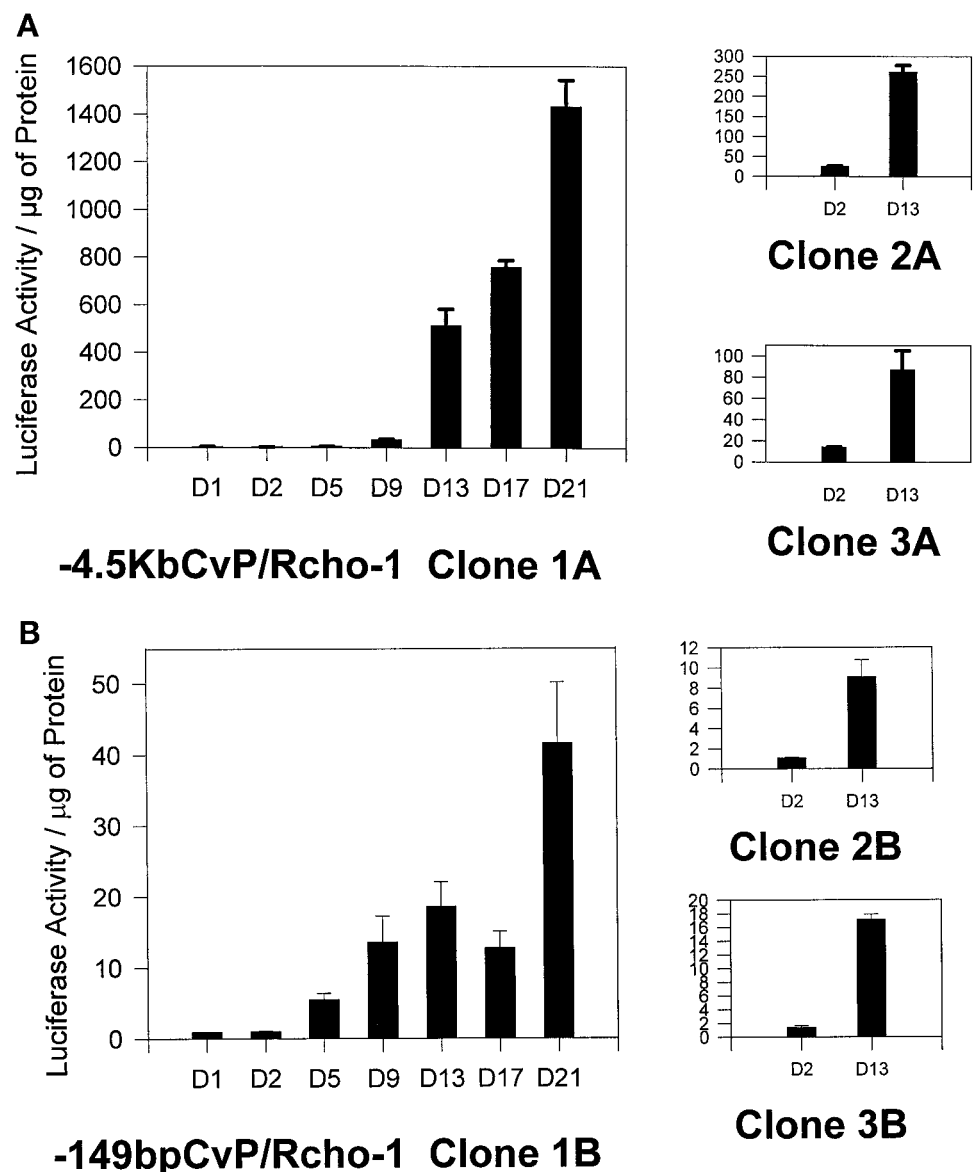
Rcho-1 trophoblast cells can be manipulated to undergo differentiation along the trophoblast giant cell lineage (30, 34) and have proven to be effective tools for studying gene regulation in differentiating trophoblast giant cells (31, 32). PLP-Cv is one of a number of trophoblast giant cell-specific genes that is activated during Rcho-1 trophoblast cell differentiation (28). Initially, activities of PLP-Cv promoter constructs containing 4500, 2108, 1012, and 259 bp of 5'-flanking DNA fused to the luciferase reporter gene were tested in differentiating Rcho-1 trophoblast cells after transient transfection (Fig. 2, *top panel*). Each of the constructs exhibited similar levels of activity that were significantly greater than the pGL2 promoterless construct. A second series of PLP-Cv promoter constructs containing from 67-259 bp of 5'-flanking DNA were fused to luciferase and evaluated in differentiating Rcho-1 trophoblast cells (Fig. 2, *bottom panel*). Within the 259-bp proximal PLP-Cv promoter we identified specific functional regions. A potential repressor/inhibitory region was identified between -217 and -174 bp upstream of the transcriptional start site and potential enhancer regions between -149 and 129 bp and between -124 and -100 bp of the transcriptional start site (Fig. 2, *bottom panel*).

Previously, we determined that the -2108 bp PLP-Cv promoter reporter construct possessed trophoblast cell-specific activation (13). In the following experiment, we evaluated whether the 149 -bp minimal PLP-Cv promoter retained trophoblast cell-specific activation. Activities of the promoterless pGL2, 149 -bp minimal PLP-Cv promoter-luciferase, and 2108 -bp PLP-Cv promoter-luciferase constructs were assessed in GH₃ lactotrophs, L929 fibroblasts, HRP-1 labyrinthine trophoblast cells, and Rcho-1 trophoblast cells. Only the Rcho-1 trophoblast cells are capable of expressing the endogenous PLP-Cv gene (13). Both PLP-Cv promoter constructs showed significant activation above the pGL2 control construct only in the Rcho-1 trophoblast cells (Fig. 3).

Expression of the endogenous PLP-Cv gene is activated as Rcho-1 trophoblast cells progress from a proliferative undifferentiated state to a differentiated trophoblast giant cell phenotype (13). Consequently, we examined whether the -4.5 kb or the -149 bp PLP-Cv promoter constructs con-

tained regulatory regions capable of responding to signals inducing trophoblast giant cell differentiation. PLP-Cv promoter constructs were stably transfected into Rcho-1 cells, and clones were isolated and evaluated during their progression from the proliferation to differentiation states. Three Rcho-1 trophoblast cell clones containing the -4.5 kb and -149 bp PLP-Cv promoter constructs exhibited a differentiation-dependent pattern of activation (Fig. 4). This differentiation-dependent gene activation is associated with genes characteristic of the trophoblast giant cell phenotype [placental lactogen-I (31), cytochrome P450 side-chain cleavage enzyme (32), d/tPRP (26), and matrix metalloproteinase-9 (41)], but not all genes active in trophoblast cells (e.g. thymidine kinase, 32). The activity of the -149 bp PLP-Cv promoter was appreciably less than the activity of the -4.5 kb PLP-Cv promoter. Differences in the short and long promoter activities are probably attributable at least in part to the location of DNA integration within the Rcho-1 tropho-

FIG. 4. Differentiation-dependent PLP-Cv promoter activation in trophoblast cells. Rcho-1 trophoblast cells were stably transfected with -4.5 kb (-4.5 KbCvP) or -149 bp (-149 bpCvP) promoter-luciferase constructs. The pSV2 neo plasmid providing neomycin resistance was co-transfected, and cells were selected for 2 weeks with G418 ($250 \mu\text{g/ml}$). Three clonal cell lines for each construct were isolated. The time course for activation of the -4.5 kb PLP-Cv promoter in clone 1A (top panel) and that for activation of the -149 bp PLP-Cv promoter in clone 1B (bottom panel) are shown. Cell lysates were prepared on days 1, 2, 5, 9, 13, 17, and 21 of culture and evaluated for luciferase activity. Day 1 represents the day the cells are initially plated. Cells from both days 1 and 2 of culture are in a proliferative state, whereas days 5–21 of culture represent various stages of differentiation. The behavior of additional representative clones during proliferative (D2) and differentiated (D13) states are shown in the smaller panels to the right of the time course (clones 2A and 3A, -4.5 kb PLP-Cv promoter, top right panels; clones 2B and 3B, -149 bp PLP-Cv promoter, bottom right panels). Each value is the mean \pm SEM of triplicate measurements. Note that both the -4.5 kb and -149 bp PLP-Cv promoters were activated in a differentiation-dependent manner.



blast cell genome. The longer promoter probably provides more insulation and is less affected by integration site, whereas activities of the shorter promoter are probably more significantly influenced by the site of DNA integration and in this case may be more negatively impacted. There also appeared to be a trend for earlier differentiation-dependent activation of the -149 bp PLP-Cv promoter *vs.* the -4.5 kb PLP-Cv promoter. Although, this observation suggests the existence of an upstream site contained within the -4.5 kb PLP-Cv promoter that represses the rate of differentiation-dependent activation, it will be necessary to more precisely evaluate the time-course behavior of several clones for each promoter construct before this issue is resolved.

PLP-Cv promoter activity in spongiotrophoblast cells

In addition to trophoblast giant cells, PLP-Cv is expressed in spongiotrophoblast cells confined to the junctional zone of the chorioallantoic placenta (13). To determine whether the PLP-Cv promoter constructs active in Rcho-1 trophoblast cells were also active in spongiotrophoblast cells, we examined their behavior in primary spongiotrophoblast cells after transient transfection. The PLP-Cv promoter-reporter constructs behaved differently in spongiotrophoblast *vs.* Rcho-1 trophoblast cells (see Figs. 2 and 5). Two patterns of activity were apparent (Fig. 5): 1) constructs representing -2108 to -259 bp of the PLP-Cv promoter behaved similarly and were significantly activated above the pGL2 control; and 2) constructs representing -4500 to -2639 bp of the PLP-Cv promoter exhibited similar activity that collectively was 3–5 times greater than the activity of the -2108 to -259 bp PLP-Cv promoter constructs (Fig. 5, *top panel*). The spongiotrophoblast-specific enhancing activity located between -2639 and -2108 bp of the PLP-Cv promoter was further evaluated. Activities of an additional set of PLP-Cv promoter-luciferase reporter constructs indicated that the spongiotrophoblast-specific enhancer activity resided within -2518 and -2242 bp upstream of the PLP-Cv transcription start site (Fig. 5, *bottom panel*). Thus, the PLP-Cv promoter contains regulatory regions specific for activation in the two different trophoblast lineages responsible for their expression.

Nucleotide sequence of 5'-flanking region of the PLP-Cv gene

Two regions within the PLP-Cv 5'-flanking sequence important for trophoblast giant cell and spongiotrophoblast cell-specific expression have been identified. The spongiotrophoblast-specific region is located between -2518 and -2242 bp (Fig. 6, *light shading*), whereas the trophoblast giant cell-specific region is located between -149 and -100 bp (Fig. 6, *dark shading*). Collectively, these two DNA regulatory regions contribute to the pattern of PLP-Cv gene expression in the placenta during gestation.

Discussion

In the present report, we have investigated aspects of the regulation of the PLP-Cv gene in trophoblast giant cells and spongiotrophoblast cells. Two distinct regulatory regions

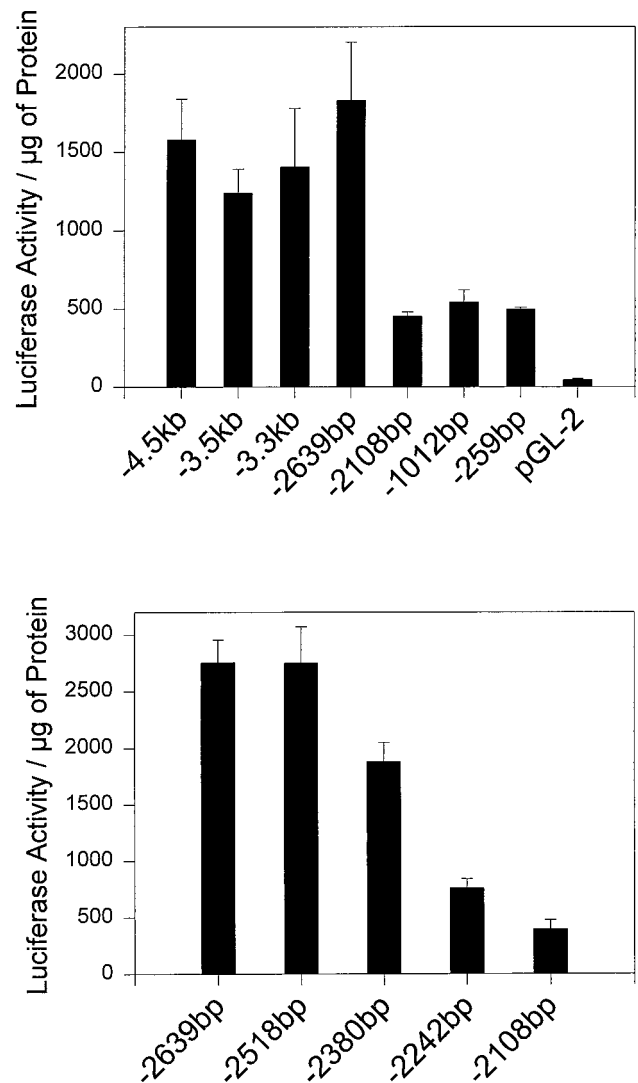


FIG. 5. PLP-Cv promoter activities in spongiotrophoblast primary cultures. The junctional zone on day 13 of gestation was isolated, enzymatically digested, and plated into six-well plates. After 48 h of culture in DMEM-10% FBS culture medium, constructs ($2 \mu\text{g}$) were transfected using a liposome-mediated delivery system. RSV- β -Gal ($0.5 \mu\text{g}$) was cotransfected to evaluate transfection efficiency. Forty-eight hours after transfection, cell lysates were prepared, and luciferase and β -galactosidase activities and total protein concentration were determined. Luciferase activities were normalized according to β -galactosidase activity and protein concentration. Each value is the mean \pm SEM of triplicate measurements. Note that the essential region for activation of the PLP-Cv promoter in spongiotrophoblast cells is located between -2639 and -2108 bp (*top panel*). Further deletion analysis (*bottom panel*) narrowed this region to 277 bp (-2518 to -2242).

within the PLP-Cv promoter were identified. One region, located between -149 and -100 bp, directs trophoblast giant cell-specific and differentiation-dependent expression of the PLP-Cv gene. The other region, located between -2518 bp and -2242 bp, enhances spongiotrophoblast cell-specific expression of PLP-Cv. Trophoblast giant cells and spongiotrophoblast cells represent two distinct lineages of trophoblast cells (28). Each lineage is regulated by a distinct genetic pathway (29, 42–44), and it is apparent from this report that

CGCCCTCATCCATTATCCAAGTTGATTAGAAATCATGGAA

-2925 ATCAAAAATCATGAAGAAAAATAACATGGCAATAATGAGAAAATAGATCAAGGAATAAAAAAGA
-2860 AAAGATACAGCTTACATTAGTATTTGGAAAGGGGTTGGGATTTAGCTCAGCAGTAGACGCCTAG
-2795 CAAGTGAAGGCCCTGGGTTTCAGTCCCGAGCTCCGAAAAAAGGAAAAACAAAAAGCATTAGTA
-2730 GTTGGCAATTATCTCTCTCTTTTACTCATCTGTATACTACTCTCTAATGTAGATAGGGTCATG
-2665 GTGATGTATGCAAAGAAAAATGTAGACGTCACATAAAGTTTGCAGTGTCTGTTCAGTTTATG
-2600 AAAATTATGTTTAAACATGTTGTTGAAAAATAAAATAGTCTGTTGGGAGGAGATTACTAATCCAGTC
-2535 AGAGCCCTTTCTAAAAATATTGTGGACAGATAAACCACTAGACAGCATAAGCAAGCAAGAAGT
-2470 TATGGAATGCTAAAAGACTTCTGAAGAGACAAAAGTTTTCAGGAGTCTGAATGGAACCAATFCCC
-2405 TTAGCAAAAATCAACCTCTTAATGATCTTATGTTGGTCTGAAAAAGAAACAACCTAAAGAGTACAG
-2340 AGGGATGCTTTTAGATTATAAGTTTGCACAGAAAGTAATGATCTCATCCATGCTACTCCACACAG
-2275 TCTTATGATTTGGAAGAAACCACTCATATAACCTCAAGGCTTGTTCATTCTGTGTAATACCTTTG
-2210 CTTATCTGTGAATAATGTCCATGTGGATGCAGAATCATCAAGTCACTTTGTTTTGTCTAGACGCA
-2145 GGTGACAGTAGGCTGCAAAAAGTGGTGAACCTTCTGTTAAGCTTCTGTTTCATAAAATCCTACATCT
-2080 ACACATTTACTCATTGGTAAAAAACTAAAAGGATTTCTACAGTCAGCTTAAAGAACTTTGA
-2015 GAGTCTAGGCTATAGATATTATATATTGTCTCTGTCTGTCTGTAGTACCCAAAGGTAATCAGAT
-1950 CACAGTACAAGTGGTTGAAATGACCACATGCTTGCTAGGAATTGACTTCAGGACTTCTGGAAGA
-1885 ATAGTCATTGCTGTCAACCCTGTGAGTTATCCCTGACCCCAACATGTGCAAGGTATATGCTCA
-1820 CAGATAGTGCATATTAGCCAAAAGAACAGATATTCATGATACAATTTTCAGGCCATATGAATCT
-1755 GAATAATAAACCAAGGACCAAGTATGGACAGTTCAGTCCCACTTAGTAGTAGGAACATATAATTAC
-1690 AGAAGTCAGAGTGAAGGAAACACCTGGGTAGGAGAAGGTAATGGTATGGGTAAAGGAGGGATAT
-1625 TAAATATAGAAAGATACATGACAGAATCCCAGAGAGCTGGATAATGAATCTAAATATGCAGGAGT
-1560 AGGGAGTGGGAGACAGTGAAGGACCCAGTACTCAATGGGAGTACTATAGGCTGAAATACCCA
-1495 ACACCTGGGAGATGTAAGCTAAAGAGATCACCTCCAATAGATAGTATACAGCCAGTTGAGTCA
-1430 TATAGACACCCATTCTTCAAAAATTTTATGATACAGAATTTTTCCTATCTAATAAAATACAGGGACA
-1365 AAAATTTACCAGAGACTAAAAGAAAGTCCATGCAATGAGTCCACTTAGGATCAATACCATGAAG
-1300 TCACACCAAATATGACACTACTACTGACCTAATGTTGTTCTTGCAGACAGGACACATGGCTGT
-1235 CCTCTGAGAGGCTCTACCAGCAACTGACTGAGTCAAGTCTGAGTCTTACAGCCAAGTAATGGAC
-1170 TGAGGTCTGGAACACGTTGGAAGAGTTAGGGGTAAGATTGAAGGAGCTGCAATATATGGTAACCC
-1105 CCGAAGGAAGAACAATGCTTTTCTAATCAATGACATCTCTGACCTCCCAAGCCTAAGCCACCA
-1040 ACCAAAGAGCATAATAGAGTACACTGTCCATGGCCCCCTAGACATATGTACTTGAGGAATGTCT
-975 TGTTTTGACAGAATGAGAGAGATTGTGCCTAACCTGTAGAAAATTTGATGCTCCAGAGTAGTTTGT
-910 GATGCTGGGATAAGGTTGGTGGGCTGGGTTTGGGAAATCACCTCAATAGAAAGGGGGATG
-845 GAATGATGAACCTCAGTATGGTTGAAGGGCAATGTGGGTAACCATTTGCAATGTAAATAAATAAAC
-780 AATTTAATAAAAAGGAAATGAAATATGTGTAGCAAAGGGATCCCGAGAATCACATTATATGGA
-715 ATTAATCTCTGATATGTCAATGACCTGTAGATCAGAGTACTTTCCCAAGTTATGTTACAGT
-650 AGGCTTCTAAAATCCCTCATGATACTCTTATAGCTGATTTTTTTTTGTATCAATATTAGACCCAA
-585 CTGATTTGACTTCATTTTCTAATCAGGAAGTATTTCTTTCAACAATATTATGATATTTGTTTG
-520 CATTGAGTTAGAAGATCCCTCAATGTTGATTAGATTCCAGACTAGAGCCACTAATTCGTTAG
-455 CCTTTAAAGAATTCATAACAATATAGATAAATTTACTAATGCTATGATACGTTGGTCAAATATTCA
-390 AGGAAGACTATGTCGCTATTAATAAATACTAGGAAAAGATGAAACAAAGAATGTTAGAATCTAA
-325 GCAATGGGACGTAATCATGAACAATGATTTCCTCATGATCCGACAAGATGATCTCAATAAGGCA
-260 GCTGAGGTGGCTTCAGAGAGTTCAGATATAATTTGCACAAGGATCAACATGTAGGAACAACCAA
-195 GAGAAAACATGCAGACAGATATTAAGTATGATGTAACAAAACAGTTAATCATCCCCTAAAGATATA
-130 TGACATGGTTGATGAAAGACTATTTCTATAAAGTTACTTCTGTTGTGCTGTCGCTGCTG
-65 AGGGTTATCTACTAGAATATATAGGATCAGGAACTAAGTGAATGTACAATTCAGTGAACCTAGA
+1 TCTTCAGAGACAGAACTCATCTGCTTAGGAACCTCCTCAGTGAATG

Fig. 6. Nucleotide sequence of the 5'-flanking region of the PLP-Cv gene. Two regions within the PLP-Cv 5'-flanking sequence important for spongiotrophoblast cell *vs.* trophoblast giant cell expression have been identified. The spongiotrophoblast-specific region is located between -2518 and -2242 bp (*light shading*), whereas the trophoblast giant cell-specific region is located between -149 bp and -100 bp (*dark shading*). The translation start codon is denoted by a box.

mechanisms controlling transcriptional activation of the PLP-Cv gene are cell specific.

Trophoblast giant cell gene regulation

Trophoblast giant cells represent a differentiated population of trophoblast cells arising via a process referred to as endoreduplication (28). These cells are involved in the biosynthesis of peptide and steroid hormones. Rcho-1 trophoblast cells have been extensively characterized and possess attributes ideal for studies on trophoblast giant cell-specific gene regulation. They can be manipulated to proliferate or differentiate. Differentiation is restricted to the trophoblast giant cell lineage and is accompanied by the activation of an array of genes associated with the differentiated trophoblast giant cell phenotype (30, 32, 36, 41).

Promoter regions for members of the PRL family (PL-I, PL-II, PLP-A, PLP-Cv, d/tPRP), cytochrome P450 genes encoding for enzymes involved in placental steroidogenesis, and gelatinase B have each been the subject of analysis in the Rcho-1 trophoblast cell model (13, 26, 31, 32, 41, 45-49; present study). Regulatory regions of various lengths have been identified that are responsible for cell- and differentiation-dependent gene activation. One of the first genes activated during trophoblast giant cell differentiation is PL-I (50). Characterization of the PL-I gene promoter has been the most extensive. Within the proximal 274 bp of the PL-I promoter, activating protein-1 and GATA elements have been identified that are essential for control of transcription (31, 51, 52). Consensus activating protein-1 and GATA elements also appear in the -149 to -100 regulatory region of the PLP-Cv

promoter. The role of these elements in the trophoblast giant cell-specific transcriptional control of the PLP-Cv gene remains to be determined.

Spongiotrophoblast cell gene regulation

Rodents possess another population of differentiated cells involved in the biosynthesis of peptide hormones, spongiotrophoblast cells. Some insights concerning spongiotrophoblast cell-specific gene regulation have been derived from analysis of promoter-reporter constructs for the 4311 gene, a spongiotrophoblast cell-specific gene, in transgenic mice (53). Calzonetti and co-workers (53) were successful in identifying 5'-flanking DNA from the 4311 gene sufficient to direct expression in trophoblast cells of transgenic mice. A 340-bp region between -3740 and -3400 was sufficient to promote spongiotrophoblast-specific expression of a β -galactosidase reporter gene. In the present report, using a spongiotrophoblast cell culture system (33) to evaluate PLP-Cv promoter activity, we also found a potential upstream enhancer region that was associated with maximal transcriptional activation. Some similarities between the 4311 gene regulatory region and the putative PLP-Cv spongiotrophoblast cell-specific enhancer are evident. Spongiotrophoblast-specific elements shared between the 4311 and PLP-Cv genes have yet to be identified.

Overview

In conclusion, activation of the PLP-Cv promoter is different in trophoblast giant cells *vs.* spongiotrophoblast cells. Consequently, proteins interacting with DNA regulatory regions responsible for trophoblast giant cell *vs.* spongiotrophoblast cell PLP-Cv promoter activation are probably downstream components of different signaling pathways. Differential controls of PLP-Cv transcription imply some significance of the cellular source of the PLP-Cv protein. Each cell type may be sensitive to distinct extracellular signals or cues, and their production of PLP-Cv may be part of unique homeostatic control mechanisms. Alternatively, trophoblast giant cells and spongiotrophoblast cells are situated in distinct locations within the chorioallantoic placenta and are known to differentially glycosylate proteins (54). Glycosylation influences the bioactivity of members of the PRL family (55, 56). Thus, cell type-specific pathways controlling PLP-Cv transcription may emanate from a need 1) for distinct homeostatic mechanisms involving PLP-Cv, 2) to deliver PLP-Cv to specific extracellular compartments, and/or 3) for a requirement to generate PLP-Cv isoforms with different biological properties.

Acknowledgments

We thank Belinda M. Chapman and Bing Liu for technical assistance, and Drs. Kyle E. Orwig, Thomas Peters, and Leslie Heckert for valuable advice during the course of these studies.

References

- Soares MJ, Faria TN, Roby KF, Deb S 1991 Pregnancy and the prolactin family of hormones: coordination of anterior pituitary, uterine, and placental expression. *Endocr Rev* 12:402-423
- Soares MJ, Dai G, Orwig KE, Peters TJ, Müller H 1998 The uteroplacental prolactin family and pregnancy. *Biol Reprod* 58:273-284
- Thordarson G, Villalobos R, Colosi P, Southard JN, Ogren L, Talamantes F 1986 Lactogenic response of cultured mouse mammary epithelial cells to mouse placental lactogen. *J Endocrinol* 109:263-274
- Colosi P, Ogren L, Southard JN, Thordarson G, Linzer DIH, Talamantes F 1988 Biological, immunological, and binding properties of recombinant mouse placental lactogen-I. *Endocrinology* 123:2662-2667
- Galosy SS, Talamantes F 1995 Luteotropic actions of placental lactogens at midpregnancy in the mouse. *Endocrinology* 136:3993-4003
- Jackson D, Volpert OV, Bouck N, Linzer DIH 1994 Stimulation and inhibition of angiogenesis by placental proliferin and proliferin-related protein. *Science* 266:1581-1584
- Linzer DIH 1995 Placental angiogenic and anti-angiogenic factors. *J NIH Res* 7:57-58
- Robertson MC, Gillepsie B, Friesen HG 1982 Characterization of the two forms of rat placental lactogen (rPL): rPL-I and rPL-II. *Endocrinology* 111:1862-1866
- Robertson MC, Cosby H, Fresnoza A, Cattini PA, Shiu RPC, Friesen HG 1994 Expression, purification, and characterization of recombinant rat placental lactogen-I: a comparison with the native hormone. *Endocrinology* 134:393-400
- Dai G, Imagawa W, Liu B, Levan G, Szpirer C, Kwok SCM, Soares MJ 1996 Rcho-1 trophoblast cell placental lactogens: complementary DNAs, heterologous expression, and biological activities. *Endocrinology* 137:5020-5027
- Müller H, Liu B, Croy BA, Head JR, Hunt JS, Dai G, Soares MJ 1999 Uterine natural killer cells are targets for a trophoblast cell-specific cytokine, prolactin-like protein-A. *Endocrinology* 140:2711-2720
- Lin J, Linzer DIH, Regulation of erythropoiesis by mouse placental prolactin-like proteins. 80th Annual Meeting of The Endocrine Society, New Orleans LA, 1998 (Abstract P3-463)
- Dai G, Liu B, Szpirer C, Levan G, Kwok SCM, Soares MJ 1996 Prolactin-like protein-C variant: complementary deoxyribonucleic acid, unique six exon gene structure, and trophoblast cell-specific expression. *Endocrinology* 137:5009-5019
- Chien Y-H, Thompson EB 1980 Genomic organization of rat prolactin and growth hormone genes. *Proc Natl Acad Sci USA* 77:4583-4587
- Cooke NE, Baxter JD 1982 Structural analysis of the prolactin gene suggests a separate origin for its 5' end. *Nature* 297:603-606
- Ogilvie S, Bui WC, Olson JA, Shiverick KT 1990 Identification of a novel family of growth hormone-related proteins secreted by rat placenta. *Endocrinology* 126:3271-3273
- Deb S, Roby KF, Faria TN, Larsen D, Soares MJ 1991 Identification and immunochemical characterization of a major placental secretory protein related to the prolactin-growth hormone family, prolactin-like protein-C. *Endocrinology* 128:3066-3072
- Deb S, Roby KF, Faria TN, Szpirer C, Levan G, Kwok SCM, Soares MJ 1991 Molecular cloning and characterization of prolactin-like protein-C complementary deoxyribonucleic acid. *J Biol Chem* 266:23027-23032
- Conliffe PR, Farmerie WG, Charles GD, Bui WC, Kelly PA, Simmen RCM, Shiverick KT 1994 Expression and characterization of recombinant rat placental prolactin-like protein C. *Mol Cell Endocrinol* 106:121-130
- Iwatsuki K, Shinozaki M, Hattori N, Hirasawa K, Itagaki S-I, Shiota K, Ogawa T 1996 Molecular cloning and characterization of a new member of the rat placental prolactin (PRL) family PRL-like protein D (PLP-D). *Endocrinology* 137:3849-3855
- Iwatsuki K, Oda M, Sun W, Satoshi T, Ogawa T, Shiota K 1998 Molecular cloning and characterization of a new member of the rat placental PRL family, PRL-like protein H (PLP-H). *Endocrinology* 139:4976-4983
- Dai G, Chapman BM, Liu B, Wang D, Orwig KE, White RA, Preuett B, Soares MJ 1998 A new mouse member of the prolactin-like protein-C subfamily: structure and expression. *Endocrinology* 139:5157-5163
- Roby KF, Deb S, Gibori G, Szpirer C, Levan G, Kwok SCM, Soares MJ 1993 Decidual prolactin-related protein. Identification, molecular cloning, and characterization. *J Biol Chem* 268:3136-3142
- Lin J, Poole J, Linzer DIH 1997 Three new members of the mouse prolactin/growth hormone family are homologous to proteins expressed in the rat. *Endocrinology* 138:5541-5549
- Orwig KE, Ishimura R, Müller H, Liu B, Soares MJ 1997 Identification and characterization of a mouse homolog for decidual/trophoblast prolactin-related protein. *Endocrinology* 139:5511-5517
- Orwig KE, Dai G, Rasmussen CA, Soares MJ 1997 Decidual/trophoblast prolactin-related protein: characterization of gene structure and cell-specific expression. *Endocrinology* 138:2491-2500
- Rasmussen CA, Orwig KE, Vellucci S, Soares MJ 1997 Dual expression of prolactin-related protein in decidual and trophoblast tissues during pregnancy. *Biol Reprod* 56:647-654
- Soares MJ, Chapman BM, Rasmussen CA, Dai G, Kamei T, Orwig KE 1996 Differentiation of trophoblast endocrine cells. *Placenta* 17:277-289
- Guillemot F, Nagy A, Auerbach A, Rossant J, Joyner A 1994 Essential role of Mash-2 in extraembryonic development. *Nature* 371:333-336
- Faria TN, Soares MJ 1991 Trophoblast cell differentiation: establishment, characterization, and modulation of a rat trophoblast cell line expressing members of the placental prolactin family. *Endocrinology* 129:2895-2906
- Shida MM, Ng Y-K, Soares MJ, Linzer DIH 1993 Trophoblast-specific tran-

- scription from the mouse placental lactogen-I gene promoter. *Mol Endocrinol* 7:181–188
32. **Yamamoto T, Roby KF, Kwok SCM, Soares MJ** 1994 Transcriptional activation of cytochrome P450 side chain cleavage enzyme expression during trophoblast cell differentiation. *J Biol Chem* 269:6517–6523
 33. **Lu X-J, Deb S, Soares MJ** 1994 Spontaneous differentiation of trophoblast cells along the spongiotrophoblast pathway: expression of the placental prolactin gene family and modulation by retinoic acid. *Dev Biol* 163:86–97
 34. **Peters TJ, Chapman BM, Soares MJ**, Trophoblast differentiation: an in vitro model for trophoblast giant cell development. In: Tuan RS, Lo CW (eds) *Developmental Protocols*. Totowa: Humana Press, in press
 35. **Soares MJ** 1987 Developmental changes in the intraplacental distribution of placental lactogen and alkaline phosphatase in the rat. *J Reprod Fertil* 79:93–98
 36. **Hamlin GP, Lu X-J, Roby KF, Soares MJ** 1994 Recapitulation of the pathway for trophoblast giant cell differentiation in vitro: stage-specific expression of members of the prolactin gene family. *Endocrinology* 134:2390–2396
 37. **Soares MJ, Shaberg KD, Pinal CS, De SK, Bhatia P, Andrews GK** 1987 Establishment of a rat placental cell line expressing characteristics of extraembryonic membranes. *Dev Biol* 124:134–144
 38. **Tashjian AH, Yasumura Y, Levine L, Sato GH, Parker ML** 1968 Establishment of clonal strains of rat pituitary tumor cells that secrete growth hormone. *Endocrinology* 82:342–352
 39. **Brasier A, Tate J, Habener J** 1989 Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. *BioTechniques* 7:1116–1121
 40. **Bradford MM** 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:249–254
 41. **Peters TJ, Albieri A, Bevilaqua E, Chapman BM, Crane L, Hamlin GP, Seiki M, Soares MJ** 1998 Differentiation-dependent expression of gelatinase B/MMP-9 in rat trophoblast cells. *Cell Tissue Res* 295:287–296
 42. **Cross JC, Flannery ML, Blonar MA, Steingrimsson E, Jenkins NA, Copeland NG, Rutter WJ, Werb Z** 1995 Hxt encodes a basic helix-loop-helix transcription factor that regulates trophoblast cell development. *Development* 121:2513–2523
 43. **Firulli AB, McFadden DG, Lin Q, Srivastava D, Olson EN** 1998 Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1. *Nat Genet* 18:266–270
 44. **Riley P, Anson-Cartwright L, Cross JC** 1998 The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. *Nat Genet* 18:271–275
 45. **Vuille J-C, Cattini PA, Bock ME, Verstuyf A, Schroedter IC, Duckworth ML, Friesen HG** 1993 Rat prolactin-like protein-A partial gene and promoter structure: promoter activity in placental and pituitary cells. *Mol Cell Endocrinol* 96:91–98
 46. **Yamamoto T, Chapman BM, Clemens JW, Richards JS, Soares MJ** 1995 Analysis of cytochrome P450 side-chain cleavage gene promoter activation during trophoblast cell differentiation. *Mol Cell Endocrinol* 113:183–194
 47. **Yamamoto T, Chapman BM, Johnson DC, Givens CR, Mellon SH, Soares MJ** 1996 Cytochrome P450 17 α -hydroxylase gene expression in differentiating rat trophoblast cells. *J Endocrinol* 150:161–168
 48. **Lin J, Linzer DIH** 1998 Identification of trophoblast-specific regulatory elements in the mouse placental lactogen II gene. *Mol Endocrinol* 12:418–427
 49. **Shah P, Sun Y, Szpirer C, Duckworth ML** 1998 Rat placental lactogen II gene: characterization of gene structure and placental-specific expression. *Endocrinology* 139:967–973
 50. **Faria TN, Deb S, Kwok SCM, Talamantes F, Soares MJ** 1990 Ontogeny of placental lactogen-I and placental lactogen-II expression in the developing rat placenta. *Dev Biol* 141:279–291
 51. **Ng Y-K, George KM, Engel JD, Linzer DIH** 1994 GATA factor activity is required for the trophoblast-specific transcriptional regulation of the mouse placental lactogen I gene. *Development* 120:3257–3266
 52. **Ma GT, Roth ME, Groskopf JC, Tsai F-Y, Orkin SH, Grosfeld F, Engel JD, Linzer DIH** 1997 GATA-2 and GATA-3 regulate trophoblast-specific gene expression in vivo. *Development* 124:907–914
 53. **Calzonetti T, Stevenson L, Rossant J** 1995 A novel regulatory region is required for trophoblast-specific transcription in transgenic mice. *Dev Biol* 171:615–626
 54. **Manzella SM, Dharmesh SM, Cohick CB, Soares MJ, Baenziger JU** 1997 Developmental regulation of a pregnancy-specific oligosaccharide structure, NeuAca2,6GalNAc β 1,4GlcNAc on select members of the rat placental prolactin family. *J Biol Chem* 271:4775–4782
 55. **Markoff E, Sigel MB, Lacour N, Seavey BK, Friesen HG, Lewis UJ** 1988 Glycosylation selectively alters the biological activity of prolactin. *Endocrinology* 123:1303–1306
 56. **Lee S-J, Nathans D** 1988 Proliferin secreted by cultured cells binds to mannose 6-phosphate receptors. *J Biol Chem* 263:3521–3527