

Transcriptional Activation of the Decidual/Trophoblast Prolactin-Related Protein Gene*

KYLE E. ORWIG† AND MICHAEL J. SOARES

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

ABSTRACT

The decidual/trophoblast PRL-related protein (d/tPRP) is dually expressed by decidual and trophoblast cells during pregnancy. We have characterized the proximal d/tPRP promoter responsible for directing d/tPRP expression in decidual and trophoblast cells. We have demonstrated that the proximal 93 bp of d/tPRP 5'-flanking DNA are sufficient to direct luciferase gene expression in primary decidual and Rcho-1 trophoblast cells, but not in fibroblast, undifferentiated uterine stromal cells or trophoblast cells of a labyrinthine lineage. The 93-bp d/tPRP promoter was also sufficient to direct differentiation-dependent expression in trophoblast giant cells. Mutational analysis demonstrated the differential importance of activating protein-1 and Ets regulatory elements (located within the proximal 93

bp of d/tPRP 5'-flanking DNA) for activation of the d/tPRP promoter in decidual *vs.* trophoblast cells. Disruption of the activating protein-1 regulatory element inhibited d/tPRP promoter activity by more than 95% in decidual cells, and approximately 80% trophoblast cells. Disruption of the Ets regulatory element reduced d/tPRP promoter activity by approximately 50% in decidual cells, while inactivating the d/tPRP promoter in trophoblast cells. Protein interactions with the trophoblast Ets regulatory element were shown to be cell type specific and to change during trophoblast giant cell formation. In conclusion, a 93-bp region of the d/tPRP promoter is shown to contain regulatory elements sufficient for gene activation in decidual and trophoblast cells. (*Endocrinology* **140**: 4032–4039, 1999)

DECIDUAL/TROPHOBLAST PRL-related protein (d/tPRP) is member of the PRL family that, as its name suggests, is dually expressed by decidual and trophoblast cells during pregnancy (1–3). Decidual cell expression of d/tPRP is initiated at the time of implantation, is associated with the onset of decidualization, and increases until the uterine decidua regresses at midpregnancy. Trophoblast cell expression of d/tPRP expression begins at midpregnancy and continues until parturition. The coordinated expression of d/tPRP by decidual and trophoblast cells ensures its continual presence from the time of implantation until parturition, suggesting its physiological importance. d/tPRP does not activate the PRL receptor signaling pathway and is thus considered a nonclassical member of the PRL family (4). However, d/tPRP is retained in the uterine environment through an interaction with heparin-containing molecules (4, 5), where it binds specifically to immune cells within the pregnant uterus (5). The precise action of d/tPRP on its target cells has not yet been fully resolved.

Apart from its biological relevance, d/tPRP expression is associated with acquisition of both decidual and trophoblast differentiated phenotypes (1–3). Dissection of molecular pathways leading to gene-specific transcriptional activation

has provided considerable insight into the regulatory factors controlling cell differentiation. Regulators of erythroid, pituitary, and muscle cell differentiation have been identified through analyses of promoters controlling differentiation-dependent gene activation (6–10). Thus, examination of the d/tPRP gene promoter may provide insight into the regulatory pathways controlling both decidual and trophoblast differentiation.

The availability of *in vitro* models has enabled us to investigate transcriptional control mechanisms regulating d/tPRP expression in decidual and trophoblast cells. A primary decidual cell culture system has been established and has proven useful for the evaluation of d/tPRP promoter activities in decidual cells (11). In addition, the Rcho-1 trophoblast cell line, which faithfully recapitulates the trophoblast giant cell developmental lineage (12–14), has proven to be a valuable tool for the characterization of trophoblast-specific gene regulation (11, 15–21). We have previously characterized the d/tPRP promoter and reported that 3960 bp of d/tPRP 5'-flanking DNA were sufficient to direct tissue-specific and differentiation-dependent expression (11). In the present study, we have characterized further the d/tPRP promoter and identified *cis*-regulatory elements that modulate its activity in decidual and trophoblast cells.

Materials and Methods

Animals

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. 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.

Received January 22, 1999.

Address all correspondence and requests for reprints to: Dr. Michael J. Soares, Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, Kansas 66160. E-mail: msoares@kumc.edu.

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

† Supported by a fellowship from the Lalor Foundation and the Kansas Health Foundation. Present address: Laboratory of Reproductive Physiology, School of Veterinary Medicine, Room 100E, University of Pennsylvania, 3850 Baltimore Avenue, Philadelphia, Pennsylvania 19104-6009.

Cell culture

Primary decidual cell cultures were established from deciduoma tissue collected from rats on day 7 of pseudopregnancy, as previously described (11). Cells were initially plated at a concentration of 3×10^6 cells/75-cm² flask in DMEM/MCDB 302 culture medium (all cell culture media were obtained from Sigma Chemical Co., St. Louis, MO) containing 10% FBS (Sigma Chemical Co.). After 20 h, medium and unattached cells were removed and replaced with fresh medium containing 1% FBS. The UI rat uterine stromal cell line was (22) maintained in Ham's F-10/DMEM culture medium supplemented with 10% FBS. The Rcho-1 trophoblast cell line was derived from a rat choriocarcinoma and is capable of differentiating along the trophoblast giant cell lineage (12). Rcho-1 trophoblast cells were routinely maintained in subconfluent conditions with NCTC-135 culture medium supplemented with 20% FBS, 50 μ M 2-mercaptoethanol, and 1 mM sodium pyruvate (12). Rcho-1 cells were induced to differentiate by growing them to near confluence in FBS-supplemented culture medium and then replacing the FBS with 10% horse serum (JRH Biosciences, Lenexa, KS) (13, 14). The HRP-1 trophoblast stem cell line was derived from labyrinthine rat trophoblast cells and was maintained in RPMI 1640 medium containing 10% FBS (23, 24). L929 mouse fibroblast cells were maintained in RPMI 1640 medium containing 10% FBS. All culture media were supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin (Sigma Chemical Co.).

Cloning of d/tPRP promoter-reporter constructs

We previously reported the amplification and cloning of 3960 bp of d/tPRP 5'-flanking DNA into the pGL-2 basic (Promega Corp., Madison, WI) luciferase reporter vector (11). Briefly, the 3960-bp d/tPRP promoter was PCR amplified from a 6.9-kb restriction fragment of the d/tPRP genomic clone using the high fidelity Tth polymerase (Advantage Genomic PCR kit, CLONTECH Laboratories, Inc., Palo Alto, CA). PCR primers were designed so the amplified product contained a *Kpn*I restriction site at the 5'-end and an *Xho*I site at the 3'-end. This allowed directional cloning of the d/tPRP promoter into the *Kpn*I and *Xho*I sites of the pGL-2 basic vector (Promega Corp.).

A similar PCR-based strategy was used to generate serial deletion constructs of the d/tPRP promoter by using the same 3'-primer and changing the location of the 5'-primer. Deletion constructs of the d/tPRP promoter ranged in size from 2500 to 37 bp upstream of the transcription start site, and all promoter constructs extended to 38 bp downstream of the transcription start site.

The 93-bp d/tPRP-Luc construct was the smallest promoter fragment that retained activity in primary decidual and Rcho-1 trophoblast cells. This fragment contains several consensus response elements, including those for activating protein-1 (AP-1) and Ets family transcription factors. PCR site-directed mutagenesis was used to generate the mutant constructs, 93-bp Δ AP-1 d/tPRP-Luc and 93-bp Δ Ets d/tPRP-Luc. The putative d/tPRP AP-1 element (TGACTTCTTG) and the putative d/tPRP Ets element (ACATCCGC) were both mutated by insertion of a *Not*I restriction site (GCGGCCGC). The resulting constructs were directionally cloned into *Kpn*I and *Xho*I sites of pGL-2, and their sequences were verified using the dideoxy chain termination method (25) and the ABI Prism 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA). Synthesized oligonucleotides were obtained from Life Technologies, Inc. (Gaithersburg, MD).

Transient/stable transfections and luciferase assays

d/tPRP-Luc constructs were transiently transfected into the primary decidual cells and Rcho-1 trophoblast cells using a liposome-mediated delivery system (Lipofectamine, Life Technologies). Cells (8×10^5 decidual cells or 5×10^4 Rcho-1 cells) were plated in 35-mm culture dishes and transfected with 2 μ g of each of the d/tPRP-Luc constructs. Primary decidual cells were transfected on day 1 of culture; Rcho-1 trophoblast cells were transfected on day 3 of culture corresponding to the time that cells were exposed to differentiating conditions. Forty-eight hours after transfection, cell lysates were prepared, and luciferase assays were performed using the Luciferase Assay System (Promega Corp.). Luciferase activity was determined using a luminometer according to the procedure described by Brasier and co-workers (26). Protein concentrations for normalization were determined using the protein-dye binding method described by Bradford (27).

Rcho-1 trophoblast cells were also stably transfected with the 93-bp d/tPRP-Luc construct (3 μ g) via cotransfection with pSV₂-neo (0.3 μ g; a plasmid providing neomycin resistance). Cells were selected for 2 weeks in the presence of 250 μ g/ml geneticin (G418, Mediatech, Inc., Herndon, VA) as previously described in our laboratory (18). Cellular lysates were collected from proliferative and differentiated stable transfectants and evaluated for luciferase activity as described above.

Electrophoretic mobility shift assay

Rcho-1 trophoblast cells were isolated and washed in cold PBS. Nuclear extracts were prepared according to the procedure previously described by Dignam and co-workers (28). Cells were washed in lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 500 μ M dithiothreitol (DTT), and the protease inhibitors, 0.5 mM phenylmethylsulfonylfluoride, 1 μ g/ml pepstatin A, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 10 μ g/ml soybean trypsin inhibitor]. Cells were then homogenized in lysis buffer containing 0.5% Nonidet P-40, and the homogenate was centrifuged at 2000 \times g. The resulting pellet was washed twice in lysis buffer containing Nonidet P-40, resuspended in extraction buffer [20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl₂, 200 μ M EDTA, 20% glycerol, 5 mM DTT, and protease inhibitors] and incubated on ice for 10 min. The nuclear suspension was centrifuged at 14,000 rpm for 5 min at 4 C, and the resulting supernatant was diluted with an equal volume of dilution buffer [20 mM HEPES (pH 7.9), 50 mM KCl, 200 mM EDTA, 20% glycerol, 500 μ M DTT, and protease inhibitors]. Double-stranded oligonucleotides (18 bp; synthesized by Life Technologies, Inc.) containing the d/tPRP Ets-binding site were labeled with T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, MA) and γ -³²P-labeled ATP (DuPont NEN, Beverly, MA). Typical binding reactions contained 8 μ g nuclear extract, 25 mM HEPES (pH 7.9), 1 mM EDTA, 50 mM NaCl, 0.5 mM DTT, 35 μ M phenylmethylsulfonylfluoride, 200 μ g/ml BSA, 10% glycerol, 1 μ g poly(deoxyinosinic-deoxycytidylic acid), and 25 fmol (2×10^5 cpm) radiolabeled probe and were incubated at room temperature for 30 min. Supershift experiments included the addition of 5 μ l Ets1/2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Binding reactions containing cold oligonucleotide competitor or antibodies were allowed to equilibrate for 15 min before the addition of probe. Nucleoprotein complexes were resolved on 5% polyacrylamide gels in 1 \times TAE (40 mM Tris-acetate and 1 mM EDTA, pH 8.0) and visualized by autoradiography.

RT-PCR

RT-PCR and Southern blotting were used to monitor d/tPRP expression in differentiating Rcho-1 cells. Total RNA was extracted from Rcho-1 cells on various days of culture, essentially as described by Chomczynski and Sacchi (29), using TRIzol (Life Technologies, Inc.). RT reactions were performed using 0.5 μ g oligo(deoxythymidine) primers and 5 μ g total RNA (Superscript Pre-amplification kit, Life Technologies). The resulting complementary DNAs were amplified by PCR using oligonucleotide primers specific for d/tPRP, as previously described in our laboratory (11). The PCR reactions also contained primers that amplified a 244-bp region of rat β -actin to demonstrate equal loading and the integrity of the messenger RNA template (30). Reaction products were fractionated in agarose gels and transferred to a nylon membrane by capillary action. Southern blots were performed using ³²P-labeled d/tPRP complementary DNA and visualized by autoradiography.

Results

Deletion analysis of the d/tPRP promoter

We have previously reported that the 3960-bp d/tPRP promoter directs cell type-specific expression in decidual and trophoblast cells as well as differentiation-dependent expression in Rcho-1 trophoblast cells (11). In this study, we evaluated a series of deletion constructs of the d/tPRP promoter, ranging in size from 3960 to 37 bp upstream of the transcription start site (see Fig. 1A). Promoter constructs were cloned into the pGL-2 basic luciferase reporter plasmid

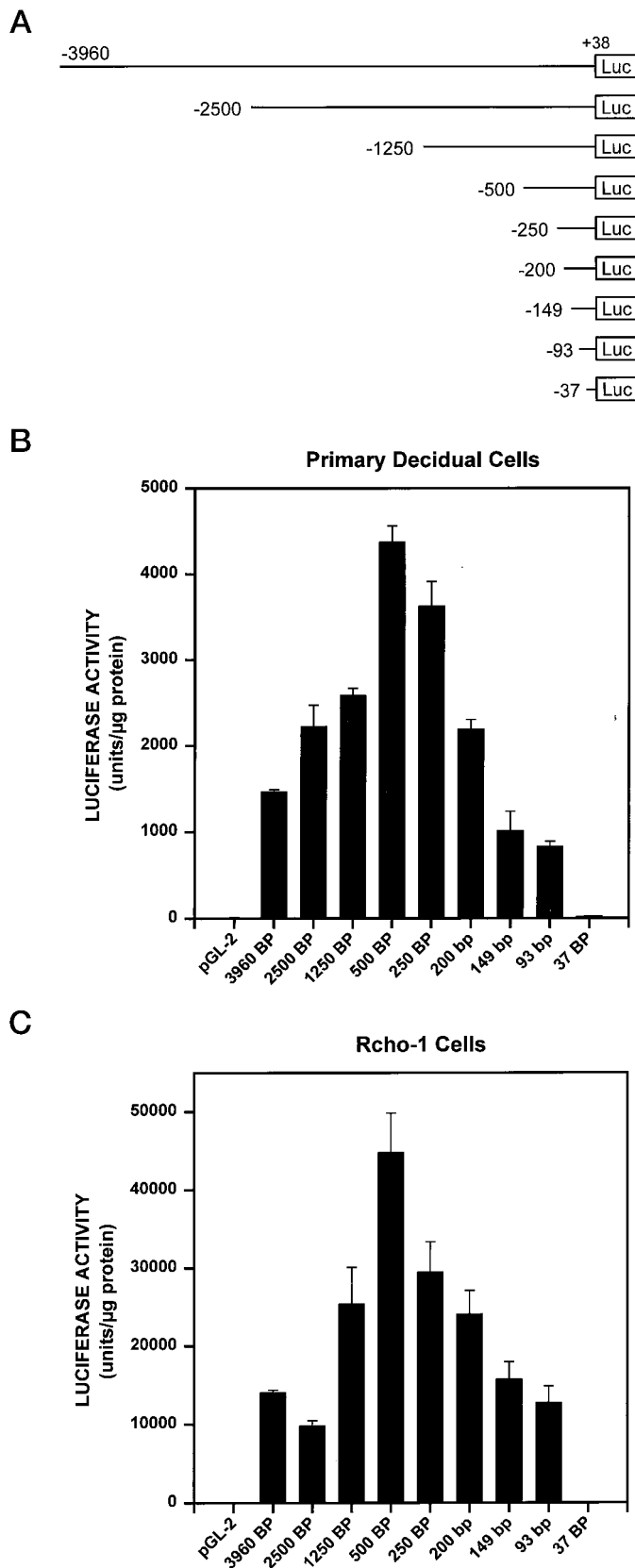


FIG. 1. Deletion analysis of the d/tPRP promoter in decidual and trophoblast cells. A, Schematic representation of d/tPRP promoter-luciferase reporter constructs. Various d/tPRP promoter deletion con-

and transiently transfected into primary decidual cells and Rcho-1 trophoblast cells.

Luciferase assays were performed 48 h after transfection and demonstrated that 93 bp of d/tPRP 5'-flanking DNA were sufficient to activate luciferase gene expression in both decidual and trophoblast cells (Fig. 1, B and C). Similar deletion analysis profiles were observed in both cell types and revealed the presence of potential positive and negative regulatory elements located between -3960 and -93 bp. Luciferase activity was not detectable in either decidual or trophoblast cells when transfected with the 37-bp d/tPRP-Luc construct (Fig. 1, B and C). Similar to our observation with the 3960-bp d/tPRP-Luc construct (11), 93-bp d/tPRP-Luc directed cell type-specific expression that was restricted to primary decidual and Rcho-1 trophoblast cells. No significant luciferase activity was observed in L929 mouse fibroblasts, UI uterine stromal cells, or HRP-1 trophoblast cells (Fig. 2). To determine whether the 93-bp d/tPRP promoter was capable of directing differentiation-dependent expression, 93-bp d/tPRP-Luc was stably transfected into Rcho-1 trophoblast cells. The results depicted in Fig. 3 demonstrate a significant activation of the 93-bp d/tPRP promoter as the Rcho-1 trophoblast cells progressed from a proliferative to a differentiated phenotype. Similar findings were noted with other stably transfected parent lines (data not shown). These results indicate that, like the 3960-bp d/tPRP promoter, the 93-bp promoter is capable of directing the appropriate cell-specific and differentiation-dependent expression.

Mutational analysis of the 93-bp d/tPRP promoter

Evaluation of the 93-bp d/tPRP promoter revealed the presence of several regulatory elements, including a putative AP-1 site, located between -79 and -76 bp, and a putative Ets element, located between -52 and -49 bp upstream of the transcription start site (Fig. 4A). Mutational analysis was used to determine the relative importance of these regulatory elements for d/tPRP expression in decidual and trophoblast cells. A PCR-based strategy was used to introduce a *NotI* restriction site into either the putative AP-1 or putative Ets elements of the d/tPRP promoter (Fig. 4A). The mutant promoter-reporter constructs, 93-bp Δ AP-1 d/tPRP-Luc and 93-bp Δ Ets d/tPRP-Luc, were transiently transfected into primary decidual and Rcho-1 trophoblast cells and evaluated for expression of the luciferase gene.

Mutation of the putative AP-1 response element in the d/tPRP promoter resulted in a 94% reduction in promoter

structs were generated by PCR, using the high fidelity Tth polymerase, and directionally cloned into the pGL-2 luciferase reporter plasmid. All d/tPRP promoter constructs extended 38 bp downstream of the transcription start site. B, d/tPRP-Luc deletion constructs were transiently transfected into primary decidual cell cultures on day 1 of culture using a liposome-mediated delivery system. Cell lysates were collected 48 h after transfection and evaluated for luciferase activity. C, d/tPRP deletion constructs were transiently transfected into Rcho-1 trophoblast cells on day 3 of culture. Rcho-1 cells were maintained in NCTC medium containing 20% FBS until the time of transfection. After transfection, the Rcho-1 cells were switched to NCTC containing 10% horse serum. Cell lysates were collected 48 h after transfection and similarly evaluated for luciferase activity. Each value represents the mean \pm SE of the mean of triplicate measurements.

FIG. 2. The 93-bp d/tPRP promoter directs cell type-specific expression. *Left panel.* The 93-bp d/tPRP-Luc was transiently transfected into L929 mouse fibroblasts, UI uterine stromal cells, and primary decidual cells. *Right panel.* The 93-bp d/tPRP-Luc was transiently transfected into HRP-1 trophoblast stem cells and Rcho-1 trophoblast cells. Cell lysates were collected from all cells 48 h after transfection, and luciferase activities were evaluated as described in Fig. 1. Luciferase activity in each cell type is reported as a ratio of the pGL-2 promoterless vector. Please note that the 93-bp d/tPRP promoter activity positively correlated with endogenous d/tPRP gene expression. Each value represents the mean \pm SE of the mean of triplicate measurements.

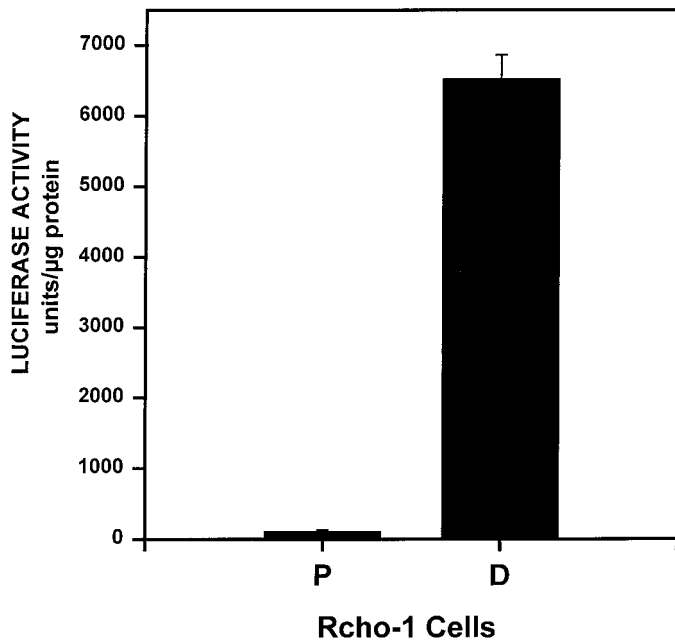
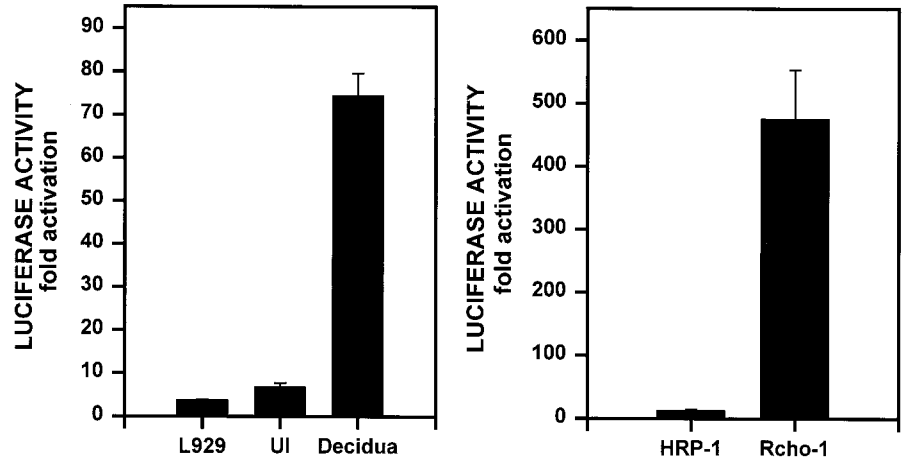


FIG. 3. The 93-bp d/tPRP promoter directs differentiation-dependent expression in Rcho-1 trophoblast cells. Rcho-1 cells were stably transfected with the 93-bp d/tPRP-Luc construct via cotransfection with pSV2-neo (a plasmid providing neomycin resistance). After 2 weeks of selection in the presence of geneticin (G418; 250 μ g/ml), parent lines were plated for analysis. Cell lysates were collected from proliferative (P) and differentiated (D) stably transfected Rcho-1 cells and evaluated for luciferase activity, as described in Fig. 1. Each value represents the mean \pm SE of the mean of triplicate measurements.

activity in primary decidual cells compared with an 80% loss of promoter activity in Rcho-1 trophoblast cells. The Ets mutation of the d/tPRP promoter caused a more modest reduction in promoter activity in primary decidual cells (54%), but totally abolished d/tPRP promoter activity in Rcho-1 trophoblast cells (Fig. 4B).

Trophoblast nuclear proteins bind the putative Ets response element in the d/tPRP promoter

To determine whether Rcho-1 trophoblast cell nuclear proteins could bind the putative d/tPRP Ets regulatory element,

an electrophoretic mobility shift assay was performed using Rcho-1 trophoblast cell nuclear extracts and a radiolabeled oligonucleotide encompassing the putative d/tPRP Ets regulatory element (Fig. 5A). Two predominant DNA-protein complexes were observed. The slower mobility complex (complex I) was a sharp band, whereas complex II was represented by a more diffuse band (or bands) with a faster mobility (Fig. 5B, lane 2). The specificity of protein binding to these complexes was demonstrated by competition with a 400-fold excess of cold d/tPRP Ets oligonucleotide (Fig. 5B, lane 3). Similar competition with d/tPRP Δ Ets (mutant Ets site; see Fig. 5A) did not result in a reduction of protein binding. To demonstrate further that complexes I and II resulted from binding to the putative Ets response element in the d/tPRP promoter, competition experiments were performed using an oligonucleotide containing a functional consensus Ets element from the CD4 promoter, CD4 Ets (see Fig. 5A). Wurster and co-workers have previously demonstrated an interaction between this region of the CD4 distal enhancer and the Ets family member, Elf-1, from Jurkat T cell nuclear extracts (31). The results presented in Fig. 5B, lane 5, demonstrate that CD4 Ets effectively blocked the formation of complexes I and II on d/tPRP Ets, whereas no reduction in complex formation was observed with the mutant, CD4 Δ Ets (Fig. 5B, lane 6). We next attempted to supershift the complex with antibodies recognizing both Ets 1 and Ets 2 (α Ets1/2). No supershifted complexes were observed using the Ets1/2 antibodies (data not shown). Additional high mol wt DNA-protein complexes were detected (Fig. 5B). These complexes were of lesser abundance and did not appear to exhibit marked cell or differentiation-dependent changes.

Cell type-specific binding to the d/tPRP Ets element

The data presented in Fig. 2 indicate that the 93-bp d/tPRP promoter could activate transcription of the luciferase reporter in Rcho-1 trophoblast cells, but not in HRP-1 trophoblast stem cells. Electrophoretic mobility shift assays were performed to compare the DNA-protein binding profiles between Rcho-1 and HRP-1 nuclear extracts and the d/tPRP Ets probe. The results of these experiments demonstrate that the pattern of nuclear proteins binding the d/tPRP Ets oligonucleotide were clearly different between Rcho-1 tropho-

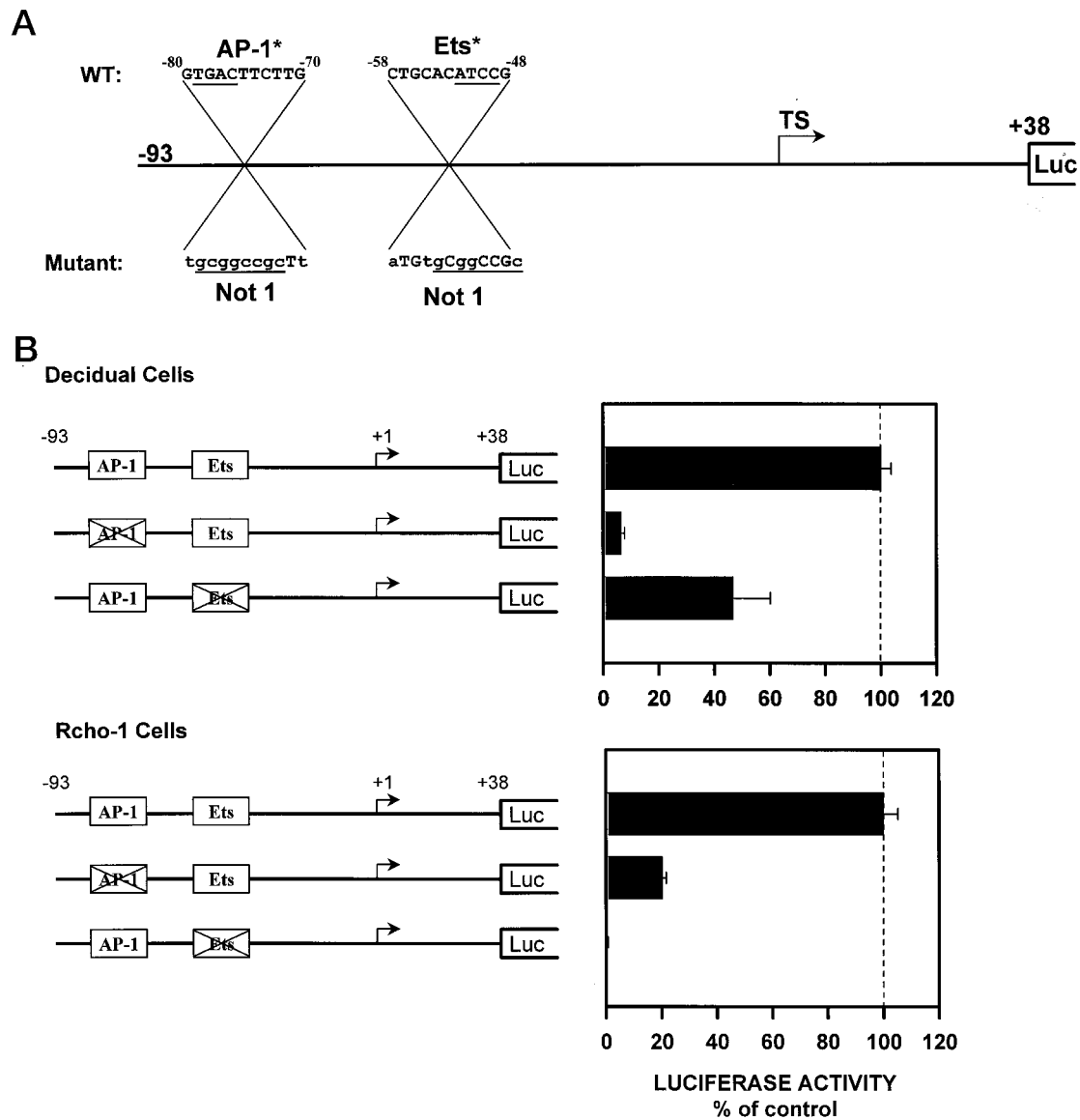


FIG. 4. Functional analysis of the 93-bp d/tPRP promoter in decidual and trophoblast cells. A, Schematic diagram indicating the location of putative AP-1 and Ets family regulatory elements within the 93-bp d/tPRP promoter. Wild-type sequences for d/tPRP AP-1 and d/tPRP Ets are indicated *above the line*. Mutant promoter constructs (sequence *below the line*) were created by introducing a *NotI* restriction site into the d/tPRP AP-1 and d/tPRP Ets elements to create d/tPRP Δ AP-1 and d/tPRP Δ Ets. Lowercase letters in the mutant sequence represent nucleotides that deviate from those in the wild-type sequence. Asterisks indicate that these are putative AP-1 and Ets regulatory elements. B, The 93-bp d/tPRP-Luc, d/tPRP Δ AP-1-Luc, and d/tPRP Δ Ets-Luc constructs were each transiently transfected into primary decidual cells or Rcho-1 trophoblast cells according to the protocols described in Fig. 1. *Top*, Primary decidual cell lysates were collected 48 h after transfection with wild-type or mutant d/tPRP promoter-reporter constructs. Luciferase activities are reported as a percentage of the wild-type 93-bp d/tPRP-Luc control (100%). *Bottom*, Similarly, Rcho-1 trophoblast cell lysates were collected 48 h after transfection with wild-type or mutant d/tPRP promoter-reporter constructs. Each value represents the mean \pm SE of the mean of triplicate measurements.

blast cells (Fig. 6, lane 2) and HRP-1 trophoblast stem cells (Fig. 6, lane 4). These observations may explain why the d/tPRP promoter is inactive in HRP-1 cells.

Binding to the d/tPRP Ets element changes as trophoblast cells differentiate

d/tPRP expression increases during Rcho-1 cell differentiation into trophoblast giant cells (Fig. 7A). To determine whether the nature of nuclear protein binding to the d/tPRP Ets element changes during trophoblast cell differentiation,

electrophoretic mobility shift assay experiments were performed using nuclear extracts from days 2, 5, 9, and 13 of Rcho-1 trophoblast cell culture. The intensity of complex I decreased during trophoblast cell differentiation. In contrast, the intensity of complex II increased as the trophoblast cells differentiated from day 2 to day 13 of culture (Fig. 7B).

Discussion

The objective of the current investigation was to evaluate mechanisms of transcriptional regulation of the d/tPRP gene

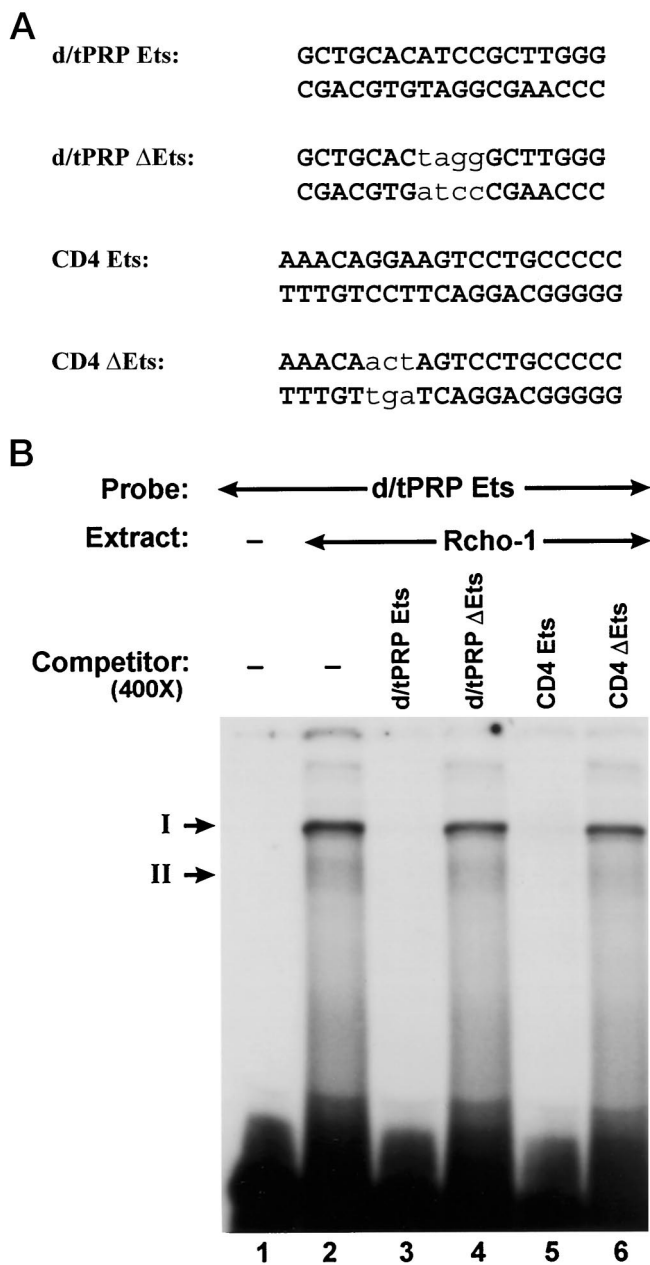


FIG. 5. Electrophoretic mobility shift assays of the d/tPRP Ets element with trophoblast cell nuclear extracts. A, Double stranded oligonucleotides used as a probe (d/tPRP Ets) or competitors in electrophoretic mobility shift assays. CD4 Ets contains a functional Ets element located in the CD4 enhancer that has been shown to bind Elf-1. Lowercase letters in the mutant d/tPRP Ets (d/tPRP ΔEts) and mutant CD4 Ets (CD4 ΔEts) sequences indicate nucleotides that deviate from the wild-type sequences for d/tPRP Ets and CD4 Ets. B, Electrophoretic mobility shift assays containing the ³²P-labeled d/tPRP Ets probe and d9 Rcho-1 nuclear extracts alone (lane 2) or in the presence of a 400-fold excess of cold d/tPRP Ets (lane 3), d/tPRP ΔEts (lane 4), CD4 Ets (lane 5), or CD4 ΔEts (lane 6). Two specifically bound complexes are indicated (I and II). Lane 1 contains free probe in the absence of nuclear extracts.

in both decidua and trophoblast cells and thus gain insight into factors controlling differentiation in both cell lineages. We have demonstrated that the proximal 93 bp of the d/tPRP promoter were sufficient to direct tissue-specific and differ-

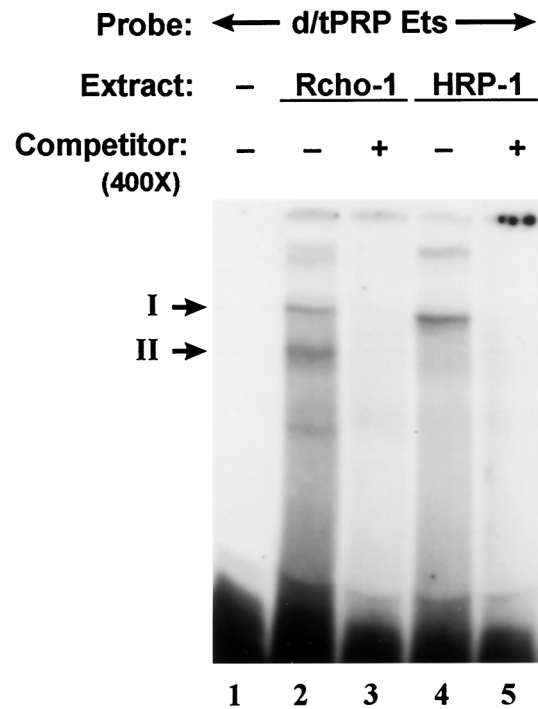


FIG. 6. The binding pattern with d/tPRP Ets is unique to trophoblast cells that express d/tPRP. Electrophoretic mobility shift assays were performed using ³²P-labeled d/tPRP Ets to compare nuclear protein binding profiles between day 9 Rcho-1 cells (that do express d/tPRP) and HRP-1 cells (that do not express d/tPRP). The characteristic Rcho-1 binding pattern, including complexes I and II, is shown in lane 2. Addition of a 400-fold excess of cold d/tPRP Ets abolished binding (lane 3). A different binding pattern was observed with HRP-1 trophoblast stem cells, which do not express d/tPRP (lane 4). Lane 5 includes cold HRP-1 nuclear extracts and a 400-fold excess of cold d/tPRP Ets. Lane 1 contains free probe in the absence of nuclear extracts.

entiation-dependent expression. Examination of this 93-bp region revealed the presence of several putative regulatory elements, including those for AP-1 (-79 to -76) and Ets family (-52 to -49) transcription factors. Mutational analyses revealed the differential importance of the putative AP-1 and Ets elements for the regulation of d/tPRP promoter activity in decidua and trophoblast cells. However, the effect of the AP-1 mutation was much more dramatic in decidua cells, whereas the Ets mutation was more pronounced in trophoblast cells. The presence of a functional AP-1 element immediately upstream of the d/tPRP Ets site is significant, because Ets family members are known to interact with other transcription factors, including AP-1, in their transcriptional activation of a variety of genes (32, 33).

There is limited information available regarding the transcriptional control of decidua-specific genes. Gao and Tseng (34) have reported that the Sp3 protein mediates insulin-like growth factor binding protein-1 expression in decidua cells through interactions with elements located between -2.8 and -2.6 kb of the insulin-like growth factor binding protein-1 5'-flanking DNA. Expression of PRL in the human decidua is directed by a 3-kb promoter region located approximately 6 kb upstream of the pituitary transcription start

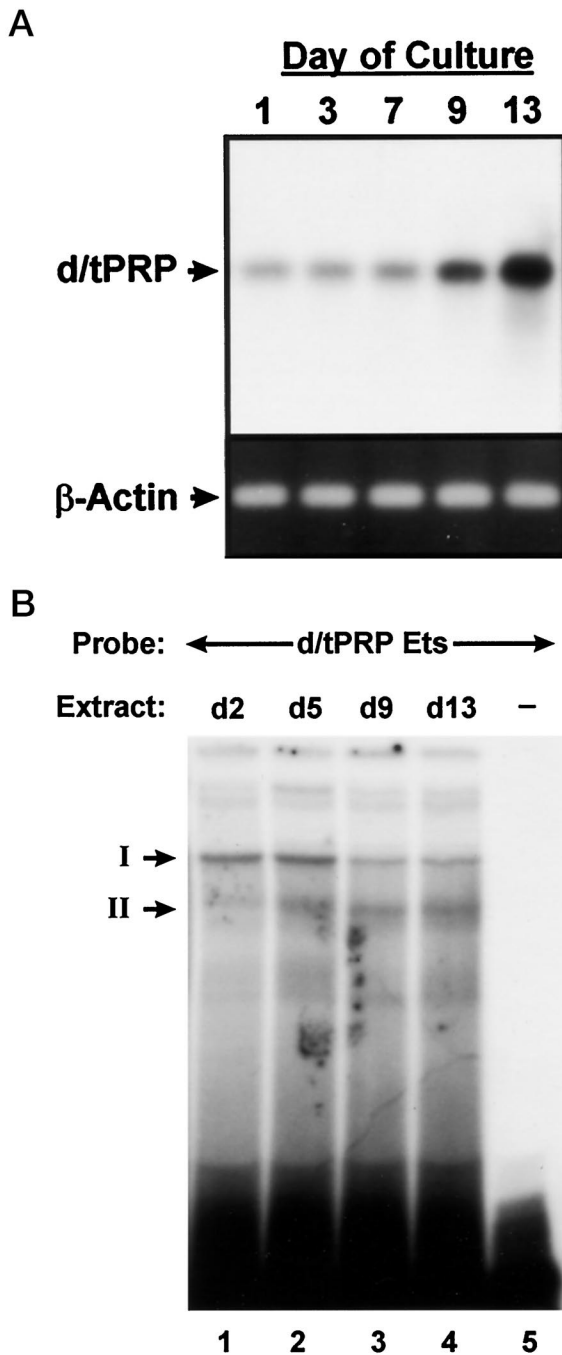


FIG. 7. Developmentally regulated expression of d/tPRP and binding of the d/tPRP Ets regulatory element by Rcho-1 trophoblast nuclear proteins. A, RT-PCR and Southern blot demonstrating that d/tPRP messenger RNA expression increases as Rcho-1 trophoblast cells differentiate from day 1 to day 13 of culture. Each RT reaction contained 5 μ g total RNA. d/tPRP primers distinguish d/tPRP from other members of the rat PRL family. β -Actin primers were included in each reaction to control for equal loading and demonstrate the integrity of the messenger RNA template. B, Electrophoretic mobility shift assays were performed to monitor the nature of complexes I and II, formed between 32 P-labeled d/tPRP Ets and nuclear extracts isolated from Rcho-1 trophoblast cells at various stages of differentiation. The intensity of complex I decreased as trophoblast cells differentiated from day 2 to day 13 of culture. In contrast, the intensity of complex II increased with trophoblast differentiation. Lane 5 contains free probe in the absence of nuclear extracts.

site (35–37). This decidual regulatory region is distinct from the region controlling pituitary PRL expression (36, 37), but the identity of specific factors that *trans*-activate the decidual PRL promoter remains to be determined. Transcription factors, including the basic helix-loop-helix transcription factor, Hand-2, Wilm's tumor-1, and the retinoid X receptor- α , are expressed in uterine decidua (17, 38–41); however, their decidual cell target genes are presently unknown. We have demonstrated that the putative AP-1 and Ets elements play a functionally important role in the regulation of d/tPRP expression in decidual cells. Identification of the decidual proteins interacting with the putative AP-1 and Ets elements may lead to insights regarding the regulation of the differentiated decidual cell phenotype.

The Rcho-1 trophoblast cell model has proven to be an excellent tool for investigating the transcriptional regulation of the placental lactogen-I (PL-I), PL-II, cytochrome P450 side-chain cleavage, PRL-like protein C variant, and d/tPRP genes (11, 15, 16, 18–20). The related RCHO cell line has been used for characterizing the rat PL-II (42) and PRL-like protein A (43) promoters. More specifically, AP-1 and GATA regulatory elements and their associated transcriptional activators have been implicated in the control of PL-I gene activation in differentiating trophoblast cells (15–17, 44). Consistent with these observations, we have demonstrated the importance of a putative AP-1 element within the d/tPRP promoter required for its optimal activation in trophoblast cells. In addition, we have provided new evidence implicating the involvement of an Ets regulatory element in the control of trophoblast cell d/tPRP gene expression.

The Ets family of transcription factors is currently comprised of nearly 30 members that have been described in species ranging from humans to *Drosophila* (32, 45–52). Ets family members are characterized by the presence of a highly conserved Ets DNA-binding domain that recognizes the nucleotide sequence, C/AGGAA/T (48–50). One Ets family member, Ets 2, has recently been implicated in the regulation of trophoblast function (53, 54). However, Ets 2 does not appear to interact with the proximal d/tPRP promoter (present study). The identity of the Ets family member or other class of transcription factor activating the d/tPRP gene via the Ets regulatory element during trophoblast giant cell differentiation remains to be determined.

Acknowledgments

We acknowledge the technical support of Belinda M. Chapman. We appreciate the helpful advice of Drs. Michael Wolfe and Leslie Heckert at the University of Kansas Medical Center. Drs. Arthur Gutierrez-Hartmann and Martine Roussel provided important insight regarding the Ets family.

References

1. Roby KE, 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
2. Gu Y, Soares MJ, Srivastava RK, Gibori G 1994 Expression of decidual prolactin-related protein in the rat decidua. *Endocrinology* 135:1422–1427
3. Rasmussen CA, Orwig KE, Vellucci S, Soares MJ 1997 Dual expression of prolactin-related protein in decidua and trophoblast tissues during pregnancy. *Biol Reprod* 56:647–654
4. Rasmussen CA, Hashizume K, Orwig KE, Xu L, Soares MJ 1996 Decidual prolactin-related protein: heterologous expression and characterization. *Endocrinology* 137:5558–5566

5. Wang D, Ishimura R, Müller H, Dai G, Soares MJ, Target cells for decidual/trophoblast prolactin-related protein within the uteroplacental compartment. 80th Annual Meeting of The Endocrine Society, New Orleans LA, 1998 (Abstract P3-460)
6. Evans T, Felsenfeld G 1989 The erythroid-specific transcription factor Eryf1: a new finger protein. *Cell* 58:877-885
7. Tsai SF, Martin DI, Zon LI, D'Andrea AD, Wong GG, Orkin SH 1989 Cloning of a cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. *Nature* 339:446-451
8. Bodner M, Castrillo JL, Theill LE, Deerinck T, Ellisman M, Karin M 1988 The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. *Cell* 55:505-518
9. Ingraham HA, Chen RP, Mangalam HJ, Elsholtz HP, Flynn SE, Lin CR, Simmons DM, Swanson L, Rosenfeld MG 1988 A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. *Cell* 55:519-529
10. Weintraub H, Davis R, Tapscott S, Thayer M, Krause M, Benezra R, Blackwell TK, Turner D, Rupp R, Hollenberg S 1991 The myoD gene family: nodal point during specification of the muscle cell lineage. *Science* 251:761-766
11. Orwig KE, Dai G, Rasmussen CA, Soares MJ 1997 Decidual/trophoblast-related protein: characterization of gene structure and cell-specific expression. *Endocrinology* 138:2491-2500
12. 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
13. 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
14. Hamlin GP, Soares MJ 1995 Regulation of DNA synthesis in proliferating and differentiating trophoblast cells: involvement of transferrin, transforming growth factor- β , and tyrosine kinases. *Endocrinology* 136:322-331
15. Shida MM, Ng YK, Soares MJ, Linzer DI 1993 Trophoblast-specific transcription from the mouse placental lactogen-I gene promoter. *Mol Endocrinol* 7:181-188
16. Ng YK, 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
17. 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
18. 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
19. Yamamoto T, Chapman BM, Clemens JW, Richards JS, Soares MJ 1995 Analysis of cytochrome P-450 side-chain cleavage gene promoter activation during trophoblast cell differentiation. *Mol Cell Endocrinol* 113:183-194
20. Dai G, Liu G, 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
21. Lin J, Linzer DIH 1998 Identification of trophoblast-specific regulatory elements in the mouse placental lactogen-II gene. *Mol Endocrinol* 12:418-427
22. Piva M, Flieger O, Rider V 1996 Growth factor control of cultured rat uterine stromal cell proliferation is progesterone dependent. *Biol Reprod* 55:1333-1342
23. 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
24. De SK, Larsen DB, Soares MJ 1995 Trophoblast stem cell-derived extracellular matrices: absence of detectable entactin and presence of multiple laminin species. *Placenta* 16:701-718
25. Sanger F, Nicklen S, Coulson AR 1977 DNA sequencing with chain-termination inhibitors. *Proc Natl Acad Sci USA* 74:5463-5467
26. 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
27. 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
28. Dignam JD, Lebovitz RM, Roeder RG 1983 Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* 11:1475-1489
29. Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156-159
30. Koos RD, Olson CE 1989 Expression of basic fibroblast growth factor in the rat ovary: detection of mRNA using reverse transcription-polymerase chain reaction amplification. *Mol Endocrinol* 3:2041-2048
31. Wurster AL, Siu G, Leiden JM, Hedrick SM 1994 ELF-1 binds to a critical element in a second CD4 enhancer. *Mol Cell Biol* 14:6452-6463
32. Wasylyk B, Hagman J, Gutierrez-Hartmann A 1998 Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. *Trends Biochem Sci* 23:213-216
33. Bassuk AG, Leiden JM 1995 A direct physical interaction between Ets and AP-1 transcription factors in normal human T cells. *Immunity* 3:223-237
34. Gao J, Tseng L 1996 Distal Sp3 binding sites in the hIGFBP-1 gene promoter suppress transcriptional repression in decidualized human endometrial stromal cells: identification of a novel Sp3 form in decidual cells. *Mol Endocrinol* 10:613-621
35. DiMattia GE, Gellersen B, Duckworth ML, Friesen HG 1990 Human prolactin gene expression. *J Biol Chem* 265:16412-16421
36. Berwaer M, Martial JA, Davis JRE 1994 Characterization of an up-stream promoter directing extrapituitary expression of the human prolactin gene. *Mol Endocrinol* 8:635-642
37. Gellersen B, Kempf R, Telgmann R, DiMattia GE 1994 Nonpituitary human prolactin gene transcription is independent of Pit-1 and differentially controlled in lymphocytes and in endometrial stroma. *Mol Endocrinol* 8:356-373
38. Hollenberg SM, Sternglanz R, Cheng PF, Weintraub H 1995 Identification of a new family of tissue-specific basic helix-loop-helix proteins with a two-hybrid system. *Mol Cell Biol* 15:3813-3822
39. Srivastava D, Cserjesi P, Olson EN 1995 A subclass of bHLH proteins required for cardiac morphogenesis. *Science* 270:1995-1999
40. Zhou J, Rauscher FJ, Bondy C 1993 Wilms' tumor (WT1) gene expression in rat decidual differentiation. *Differentiation* 54:109-114
41. Mangelsdorf DJ, Borgmeyer U, Heyman RA, Zhou JY, Ong ES, Oro AE, Kakizuka A, Evans RM 1992 Characterization of three RXR genes that mediate the action of 9-*cis* retinoic acid. *Genes Dev* 6:329-344
42. 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
43. Vuille JC, 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
44. Ma GT, Roth ME, Groskopf JC, Tsai FY, 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
45. Watson DK, McWilliams MJ, Lapis P, Lautenberger JA, Schweinfest CW, Papas TS 1988 Mammalian *ets-1* and *ets-2* genes encode highly conserved proteins. *Proc Natl Acad Sci USA* 85:7862-7866
46. Karim FD, Urness LD, Thummel CS, Klemsz MJ, McKercher SR, Celada A, Van Beveren C, Maki RA, Gunther CV, Nye JA 1990 The ETS-domain a new DNA-binding motif that recognizes a purine-rich core DNA sequence. *Genes Dev* 4:1451-1453
47. Wasylyk C, Gutman A, Nicholson R, Wasylyk B 1991 The c-Ets oncoprotein activates the stromelysin promoter through the same elements as several non-nuclear oncoproteins. *EMBO J* 10:1127-1134
48. Seth A, Ascione R, Fisher RJ, Mavrothalassitis GJ, Bhat NK, Papas TS 1992 The *ets* gene family. *Cell Growth Differ* 3:327-334
49. Macleod K, Leprince D, Stehelin D 1992 The *ets* gene family. *Trends Biochem Sci* 17:251-256
50. Wasylyk B, Hahn SL, Giovane A 1993 The Ets family of transcription factors. *Eur J Biochem* 211:7-18
51. Graves BJ, Petersen JM 1998 Specificity within the ets family of transcription factors. *Adv Cancer Res* 75:1-55
52. Dittmer J, Nordheim A 1998 Ets transcription factors and human disease. *Biochim Biophys Acta* 1377:1-11
53. Yamamoto H, Flannery ML, Kupriyanov S, Pearce J, McKercher SR, Henkel GW, Maki RA, Werb Z, Oshima RG 1998 Defective trophoblast function in mice with a targeted mutation of Ets2. *Genes Dev* 12:1315-1326
54. Ezashi T, Ealy AD, Ostrowski MC, Roberts RM 1998 Control of interferon- γ gene expression by Ets-2. *Proc Natl Acad Sci USA* 95:7882-7887