Trophoblast-Specific Transcription from the Mouse Placental Lactogen-I Gene Promoter

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We have isolated the gene encoding mouse placental lactogen-I and characterized the promoter region of this gene by transient and stable transfection. Promoter sequences extending 274 basepairs (bp) up-stream from the start site of transcription contain all of the elements necessary for maximal expression upon transient transfection into the rat choriocarcinoma Rcho-1 cell line; these Rcho-1 cultures contain both proliferative trophoblast stem cells and terminally differentiated trophoblast giant cells. In stably transfected cell lines, expression from this promoter increases as the percentage of differentiated cells in the culture increases. In contrast to these results in trophoblast cells, the 274-bp promoter as well as a promoter region extending 2700 bp up-stream of the transcriptional start site are unable to drive transcription in a variety of other cell types. Mutational and protein binding analyses indicate that two AP-1 sites are required for maximal expression in Rcho-1 cells, and that the composition of the AP-1 transcription factor may vary as differentiation in the cell culture increases. In addition to these two AP-1 sites, at least one other element appears to be critical for promoter activity in trophoblast cells. (Molecular Endocrinology 7: 181–188, 1993)

INTRODUCTION

The development of the extraembryonic cell lineages in mammals includes the differentiation of multiple placental trophoblast cell types. One of the functions of these trophoblast cells is the synthesis and secretion of hormones that regulate both maternal and fetal physiology. Among these secreted proteins in rodents, ruminants, and primates are members of the PRL/GH family. In rodents, these hormones are found to be synthesized in either the trophoblast giant cells or the spongiotrophoblasts (1); two of these hormones, placental lactogen-I (PL-I) (2, 3) and PL-II (2–6), are synthesized exclusively in the trophoblast giant cells and functionally replace PRL during pregnancy (7).

Both PL-I (8) and PL-II (9) bind to the PRL receptor with high affinity, and both hormones have PRL-like activities (7). Although these biological activities may be as numerous and varied as those induced by PRL (10), some of the primary activities of PL-I and PL-II during gestation are the maintenance of the corpus luteum, the regulation of maternal carbohydrate metabolism in the liver, and the development of the mammary glands for postpartum lactation (7). These two placental hormones also enter the fetal circulation, where they may participate directly in the growth and development of the fetus (7). Synthesis of PL-I is first detected soon after implantation (11), but significant quantities of this hormone are not present in the circulation until midgestation (12). On gestational day 10 in the mouse, PL-I levels peak at approximately 8 μg/ml in the maternal serum, and then rapidly decline (12). In contrast, PL-II synthesis does not commence until midpregnancy, and the concentration of this hormone achieved by gestational day 12 is maintained or gradually increases until term to a level of 0.5–1 μg/ml in maternal serum (7).

The transcriptional factors and genetic elements regulating the specific expression of these hormone genes in placental trophoblast giant cells and those governing the switch from PL-I to PL-II synthesis are unknown. We have characterized a region of the mPL-II gene sufficient for directing transcription specifically in the trophoblast giant cells of transgenic mice, but this region is large (2.7 kilobases), and this approach is limited in dissecting such a large regulatory region (13). One of the major limitations in defining these regulatory components has been the lack of a suitable continuous cell culture system for expression of rodent PL-I and PL-II. This lack of trophoblast cell lines that express differentiated phenotypes has also restricted progress

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toward the more general goals of characterizing the pathways and underlying mechanisms of trophoblast cell differentiation.

The recent development of the Rcho-1 cell line (14) provides a potential cell system for investigating PL gene expression and studying trophoblast differentiation. Rcho-1 cells, derived from a transplantable rat choriocarcinoma (15), differentiate in culture into giant cells that produce both PL-I and PL-II. Conditions in which these cells display a decrease in PL-I expression and, concurrently, an increase in PL-II expression (as seen in giant trophoblast cells *in vivo*) have not been found; these cell cultures may, therefore, prove useful in identifying extracellular regulators or missing intracellular components of the switch from PL-I to PL-II synthesis. In contrast, the synthesis of both of these hormones indicates that these cells do contain the regulatory factors necessary for trophoblast-specific PL gene expression. Thus, we have now used this cell system to define a region of the mouse PL-I (mPL-I) gene promoter that directs transcription specifically in trophoblast cells.

RESULTS

Isolation of the mPL-I Gene

The mPL-I gene was isolated from a mouse genomic DNA library by screening with the mPL-I cDNA (16). Genomic fragments containing the first exon and up-stream sequences were subcloned into a plasmid vector under the assumption that the regulatory elements essential for trophoblast-specific expression would be located up-stream of the transcription start site. The sequence of this up-stream region and exon I is shown in Fig. 1; the start site of transcription was mapped by primer extension to a position 63 basepairs (bp) up-stream of the translation initiating ATG in exon 1 (Fig. 2). To determine whether sequences up-stream of this transcriptional start site can direct trophoblast-specific expression, an initial set of constructs was prepared linking the mPL-I up-stream sequences from positions −2700 to +2, −274 to +2, or −64 to +2 to the bacterial chloramphenicol acetyltransferase (CAT) gene. These constructs, designated 5′-2700CAT, 5′-274CAT, and 5′-64CAT, respectively, all contain the mPL-I gene TATA box at position −30.

Transient Transfection of mPL-I Promoter Constructs

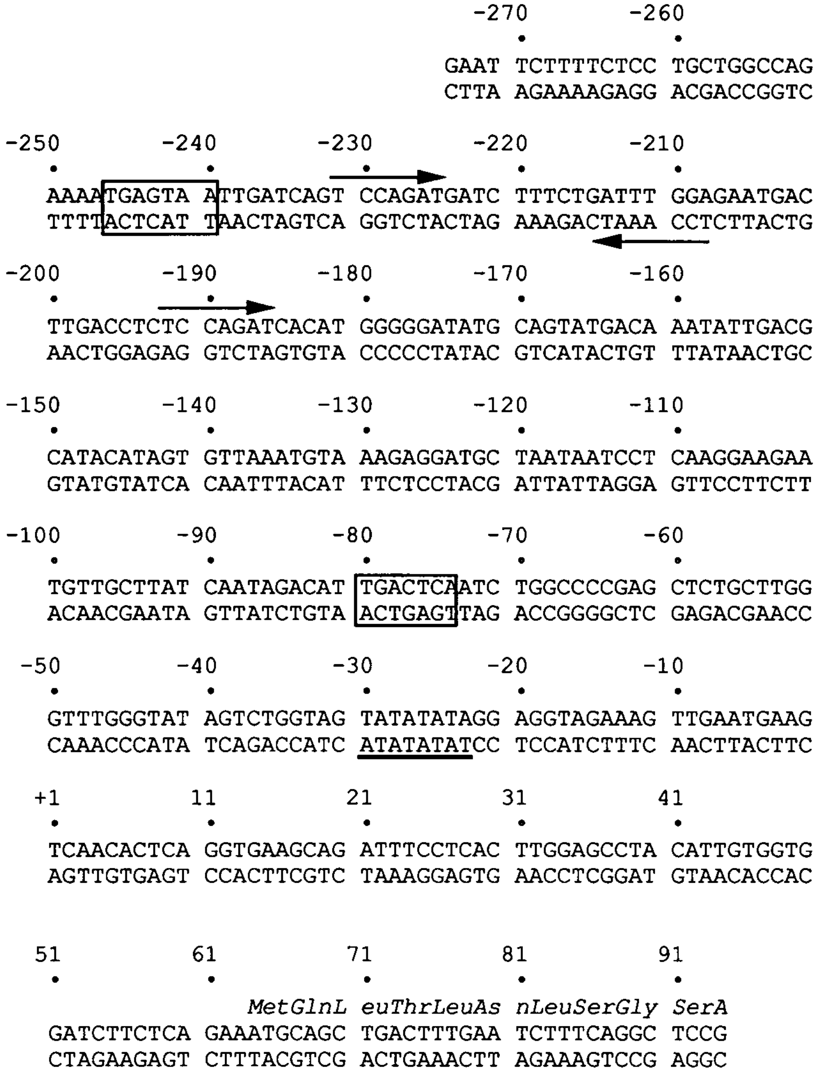
These three promoter constructs were transiently transfected into Rcho-1 cells, and both 5′-2700CAT and 5′-274CAT were found to be active in this cell line (Fig. 3). Moreover, the amount of CAT enzymatic activity detected in Rcho-1 cells transfected with 5′-274CAT was consistently greater than that obtained by transfection of 5′-2700CAT, which may reflect the presence of negatively acting regulatory sequences up-stream of position −274. In contrast to the 5′-274CAT and 5′-2700CAT promoter constructs, 5′-64CAT yielded undetectable CAT activity in transfected Rcho-1 cells. Thus, one or more of the elements required for directing transcription in Rcho-1 cells from the mPL-I gene promoter are located between positions −64 and −274. To determine whether the activity of the −2700 and −274 promoter regions was specific for the Rcho-1 cells, these DNA constructs were also introduced into Chinese hamster ovary (CHO) cells (Fig. 3), monkey Cos-7 cells, and mouse L-cells. In each of these cell lines, no significant transcription could be detected from any of these three promoter constructs. In comparison, the Rous sarcoma virus (RSV) promoter (in RSV-CAT) was highly active in both CHO and Rcho-1 cells (Fig. 3).

Stable Transfection of mPL-I Promoter Constructs

Clonal Rcho-1 cell cultures contain a mixture of proliferative small cells and terminally differentiated giant cells; under differentiation conditions, the percentage of giant cells in the population and the expression of PL-I increase, indicating that the small cells are probably giant cell precursors (14). To verify that the two active promoter constructs, 5′-2700CAT and 5′-274CAT, were expressed in a manner similar to the endogenous mPL-I gene, these constructs were stably introduced into Rcho-1 cells by cotransfection with the plasmid pSV2-neo and selection for G418 resistance. Several independent, stably transfected cell lines were established in this manner. Two of these cell lines generated with 5′-274CAT (clones 16 and 17) and two transfected with 5′-2700CAT (clones 3 and 19) were then examined for CAT activity within 2 days of plating, when differentiation is minimal, and 1 week after shifting cells from growth medium to differentiation medium, when the percentage of giant cells and expression of endogenous PL genes are high. Although the level of CAT activity varies among the independent clones (most likely due to positional and copy number variations of the transfected DNA), CAT expression from the 5′-2700 promoter and the 5′-274 promoter increased upon cell differentiation in each stably transfected cell line (Fig. 4). Consistent with the transient transfection results, the 5′-64CAT construct was also inactive in stably transfected, differentiated cell cultures (data not shown).

Functional Role of AP-1 Elements within the mPL-I Promoter

Inspection of the mPL-I promoter sequence between positions −64 and −274 reveals two potential AP-1 elements, the target of *jun-jun* homodimers and *jun-fos* heterodimers (17). The proximal AP-1 site (TGACTCA) is located between residues −80 and −74, and the up-stream site (TGAGTAA) lies between positions −246 and −240. To test the importance of these two sequences for transcriptional activity of the mPL-I gene



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                                   -270       -260
                                     •          •
                                GAAT TCTTTTCTCC TGCTGGCCAG
                                CTTA AGAAAAGAGG ACGACCGGTC

-250      -240       -230       -220       -210
  •         •          •          •          •
AAAATGAGTA ATTGATCAGT CCAGATGATC TTTCTGATTT GGAGAATGAC
TTTTACTCAT TAACTAGTCA GGTCTACTAG AAAGACTAAA CCTCTTACTG

-200      -190       -180       -170       -160
  •         •          •          •          •
TTGACCTCTC CAGATCACAT GGGGGATATG CAGTATGACA AATATTGACG
AACTGGAGAG GTCTAGTGTA CCCCCTATAC GTCATACTGT TTATAACTGC

-150      -140       -130       -120       -110
  •         •          •          •          •
CATACATAGT GTTAAATGTA AAGAGGATGC TAATAATCCT CAAGGAAGAA
GTATGTATCA CAATTTACAT TTCTCCTACG ATTATTAGGA GTTCCTTCTT

-100       -90        -80        -70        -60
  •         •          •          •          •
TGTTGCTTAT CAATAGACAT TGACTCAATC TGGCCCCGAG CTCTGCTTGG
ACAACGAATA GTTATCTGTA ACTGAGTTAG ACCGGGGCTC GAGACGAACC

 -50       -40        -30        -20        -10
  •         •          •          •          •
GTTTGGGTAT AGTCTGGTAG TATATATAGG AGGTAGAAAG TTGAATGAAG
CAAACCCATA TCAGACCATC ATATATATCC TCCATCTTTC AACTTACTTC

 +1         11         21         31         41
  •         •          •          •          •
TCAACACTCA GGTGAAGCAG ATTTCCTCAC TTGGAGCCTA CATTGTGGTG
AGTTGTGAGT CCACTTCGTC TAAAGGAGTG AACCTCGGAT GTAACACCAC

 51         61         71         81         91
  •         •          •          •          •
              MetGlnL euThrLeuAs nLeuSerGly SerA
GATCTTCTCA GAAATGCAGC TGACTTTGAA TCTTTCAGGC TCCG
CTAGAAGAGT CTTTACGTCG ACTGAAACTT AGAAAGTCCG AGGC
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Fig. 1. Nucleotide Sequence of the 5′-Flanking Region of the mPL-I Gene

The nucleotide sequence of the mPL-I promoter from the *Eco*RI site at −274 through exon 1 is shown. The start site of transcription is position 1. The two AP-1 sites are *boxed*, the TATA box is *underlined*, and a conserved heptad repeat is marked by the *three arrows*. The coding region begins at position 63, and exon 1 terminates within codon 11 (GCA = alanine).

promoter in trophoblast giant cells, mutations at each of these AP-1 sites were generated. For the down-stream AP-1 site, the sequence TGACTCA was converted to atACTCA in the construct designated SDM −80/−79; for the up-stream AP-1 site, the sequence TGAGTAAT was altered to a *Bgl*II restriction endonuclease site (gagaTctc) to generate SDM −246/−239. Both of these mutations were made within the promoter extending to position −274. The resultant modified promoters were transiently transfected into Rcho-1 cells. Site-directed mutation of either of these AP-1 sites reduced, but did not eliminate, transcription from the mPL-I promoter (Fig. 5).

To determine if the up-stream site is a target for factors in Rcho-1 cells, in particular AP-1, a double stranded oligonucleotide spanning this sequence was incubated with purified c-*jun* protein or Rcho-1 cell extracts and subjected to electrophoresis. Purified c-*jun* protein did bind this sequence, as did factors present in the Rcho-1 extracts. Furthermore, a consensus AP-1 oligonucleotide (Promega, Madison, WI) was able to compete for binding to this up-stream sequence (Fig. 6). This consensus oligonucleotide, which has the same core sequence as the down-stream mPL-I AP-1 site, also bound AP-1 in Rcho-1 cell extracts when used as the radiolabeled probe (data not shown).

Comparison of the size of the DNA-protein complex that forms with purified c-*jun* protein *vs.* Rcho-1 cell extracts suggested that the complex from Rcho-1 cell extracts is not simply a homodimer of c-*jun*. To identify components of this complex, binding reactions were supplemented with antiserum against c-*jun*, *jun*-B, *jun*-D, or c-*fos*. The c-*fos* antiserum completely blocked complex formation using extracts from either undiffer-

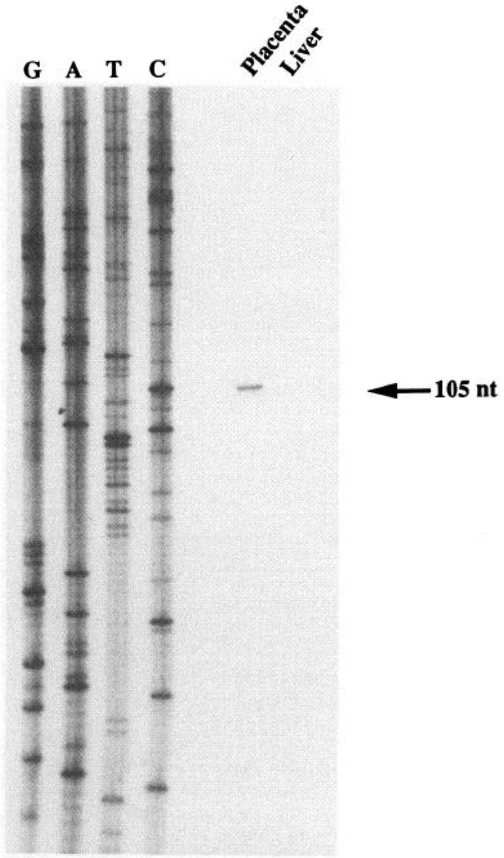

Fig. 2. Mapping of the mPL-I Gene Transcription Start Site

An antisense oligonucleotide complementary to the mPL-I mRNA was end labeled and hybridized to 10 μg total mouse placenta or liver RNA. The primer was extended by reverse transcription, and the product was analyzed by gel electrophoresis alongside a set of sequencing reactions as size standards. The specific extension product of 105 nucleotides (nt) detected with placental, but not liver, RNA is indicated by the *arrow*.

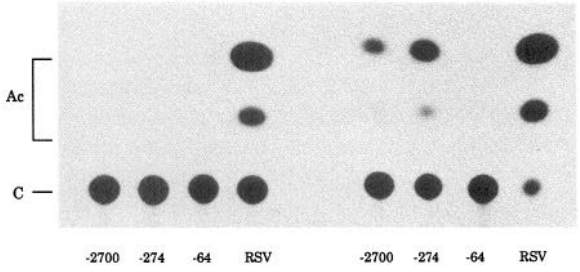

Fig. 3. Activity of the mPL-I Gene Promoter in Transiently Transfected Cell Cultures

CHO (*left*) or Rcho-1 (*right*) cell cultures were transfected with the mPL-I promoter construct 5′-2700CAT, 5′-274CAT, or 5′-64CAT or with RSV-CAT. Equal amounts of protein from the transfected cell lysates were incubated with [^{14}C]chloramphenicol and acetyl-coenzyme-A. Acetylated forms (Ac) were separated from unacetylated chloramphenicol (C) by TLC.

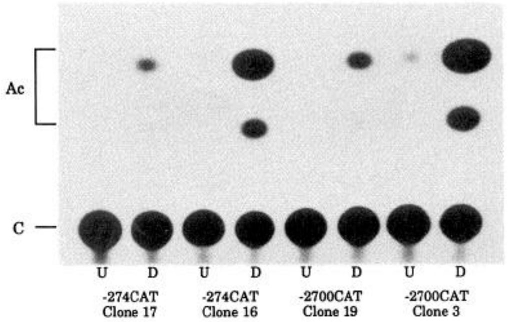

Fig. 4. Activity of the mPL-I Gene Promoter in Undifferentiated and Differentiated Rcho-1 Cell Cultures

Two independent clones of Rcho-1 cells stably transfected with either the mPL-I promoter construct 5′-2700CAT (clones numbered 3 and 19) or 5′-274CAT (clones numbered 16 and 17) were harvested as undifferentiated cell cultures (U) or after differentiation (D). Lysates were prepared from these cultures, and equal amounts of protein were assayed for CAT activity. Acetylated forms (Ac) were separated from unacetylated chloramphenicol (C) by TLC.

entiated (Fig. 7A) or differentiated (Fig. 7B) cells, indicating that most or all of the bound AP-1 detected in this assay is in the form of *jun-fos* heterodimers rather than *jun-jun* homodimers. Addition of c-*jun* antiserum resulted in a supershift of a subset of the protein-DNA complexes formed with undifferentiated cell extracts, suggesting that c-*jun* is present in some, but perhaps not all, of the bound AP-1; with differentiated cell extracts, the c-*jun* antiserum had no observable effect. The *jun*-B antiserum caused a partial reduction in the amount of complex detected with undifferentiated Rcho-1 cell extracts, but nearly complete inhibition of complex formation with differentiated cell extracts. In contrast, anti-*jun*-D had no observable effect, and unlike the other two forms of *jun*, *jun*-D mRNA was not detected in Rcho-1 cells in hybridizations with the corresponding mouse cDNA clone (data not shown).

Additional Elements within the mPL-I Promoter

Comparison of the promoter sequence to a transcriptional element database (Genetics Computer Group, Madison, WI) did not reveal the presence of any previously characterized elements in addition to the AP-1 sites and the TATA box. We, therefore, sought to identify other regions of the −274 promoter that may be involved in trophoblast-specific transcriptional regulation. Mutation of the −274 promoter by deletion to −242 (partway through the up-stream AP-1 site) results in the construct 5′-242CAT with partial activity, while further deletion to −188 (5′-188CAT) renders the promoter inactive and equivalent to the 5′-64CAT construct (Fig. 8). Thus, an essential element for Rcho-1 cell expression appears to be located within a 54-bp region between the up-stream AP-1 site and position −188. Inspection of this region reveals the presence of a duplicated heptad sequence, TCCAGAT, at positions −231 to −225 and −192 to −186; halfway between these repeats, from −208 to −214, is a heptad se-

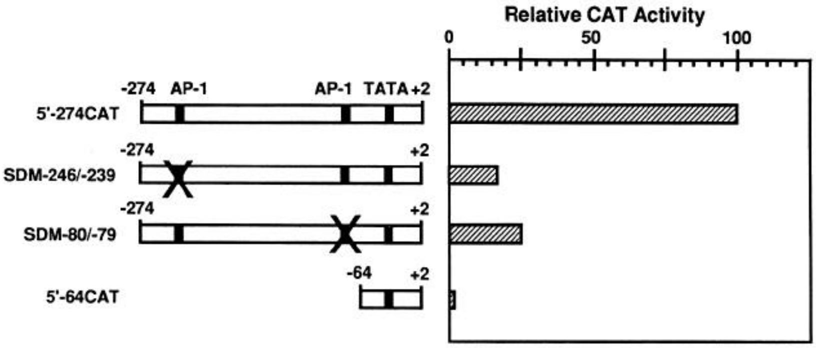

Fig. 5. Transcriptional Activity of mPL-I Gene Promoters Mutated at the AP-1 Sites

Rcho-1 cell cultures were transfected with the mPL-I promoter constructs 5′-274CAT or 5′-64CAT as positive and negative controls, respectively, or with promoter constructs mutated at the up-stream (SDM −246/−239) or down-stream (SDM −80/−79) AP-1 sites. Transfections also included the internal control plasmid $pA_3RSV_{400}LUC$ (25). Equal amounts of protein from the cell extracts were assayed for CAT and luciferase activities. Each value is the mean CAT activity from duplicate or triplicate transfections normalized to luciferase activity.

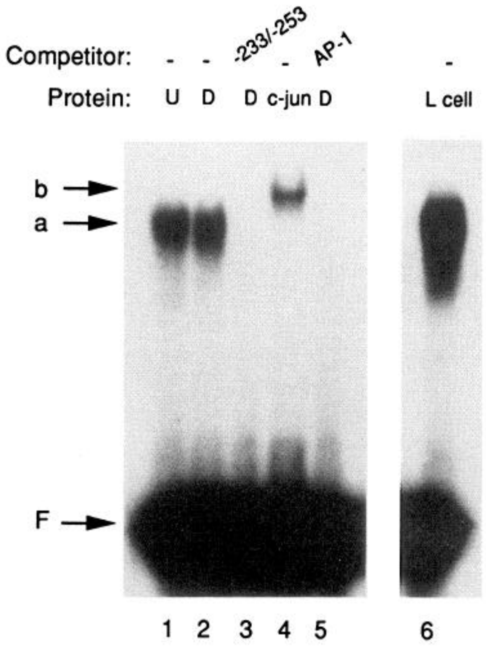

Fig. 6. Binding of AP-1 to the mPL-I Gene Promoter

An end-labeled, double stranded oligonucleotide (−233/−253) encompassing the up-stream AP-1 site was incubated with 10 μg extract from undifferentiated (U) or differentiated (D) Rcho-1 cell extracts, 5 μg purified c-*jun* protein (Promega), or 10 μg extract from mouse L-cells. Double stranded competitor DNAs were the unlabeled probe or the unlabeled consensus AP-1 oligonucleotide (Promega) and were added in a 100-fold molar excess. Specific complexes formed with the cell extracts (a) or purified c-*jun* protein (b) are indicated, along with free (F) probe DNA.

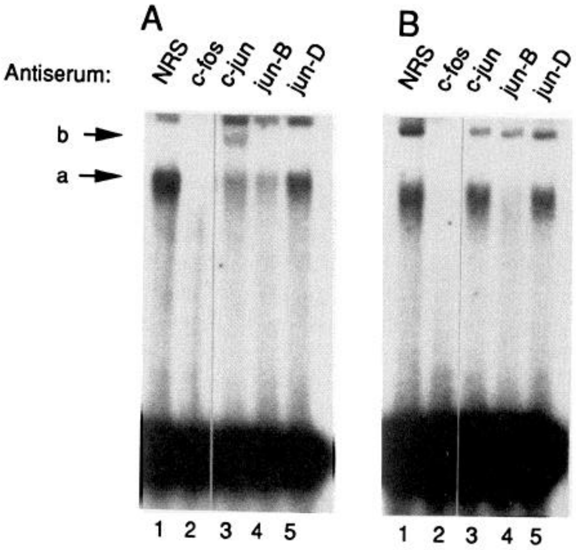

Fig. 7. Composition of AP-1 Associated with the mPL-I Gene Promoter

An end-labeled, double stranded oligonucleotide encompassing the up-stream AP-1 site was incubated with 10 μg extract from undifferentiated (A) or differentiated (B) Rcho-1 cell extracts. Binding reactions were supplemented with normal rabbit serum (NRS) or antiserum against c-*fos*, c-*jun*, *jun*-B, or *jun*-D. The specific complex (a) and the supershifted complex resulting from addition of the antiserum against c-*jun* (b) are indicated by the *arrows*; an even larger complex appears to be a nonspecific effect of rabbit serum, since it is detected with normal rabbit serum.

quence in the opposite orientation that differs by 1 bp (TCCAaAT; Fig. 1).

DISCUSSION

The current studies demonstrate that the rat choriocarcinoma cell line Rcho-1 provides a valuable system for studying trophoblast cell-specific gene expression. The mPL-I gene promoter has strong transcriptional activity in this cell background, but no detectable activity in any of the nontrophoblast cell lines that were examined. Sequences up-stream of the mPL-I gene transcription start site extending to −274 and −2700 are both effective at driving expression of a linked reporter gene in Rcho-1 cells, although the −274 promoter apparently has greater activity. Thus, the −274 to 2 region appears

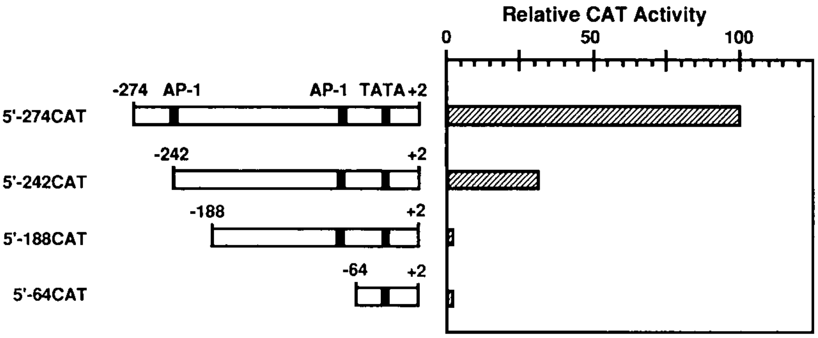

Fig. 8. Transcriptional Activity of mPL-I Gene Promoter Deletion and Site-Directed Mutants

Rcho-1 cell cultures were transfected with 5′-274CAT or 5′-64CAT as positive and negative controls, respectively, or with the mPL-I promoter 5′-deletion constructs 5′-242 or 5′-188. Equal amounts of protein from the cell extracts were assayed for CAT activity and normalized to the luciferase internal control. Each value is the mean of duplicate transfections.

to contain all of the elements necessary for maximal expression in these trophoblast cell cultures. The ability of the mPL-I promoter region to direct cell type-specific transcription is in contrast to the human PL gene, which requires an enhancer down-stream of the gene for expression in human choriocarcinoma cells (18).

Included in the 274-bp promoter region of the mPL-I gene are two AP-1 sites, both of which are required for full promoter activity. The up-stream AP-1 site is located at −240 to −246, approximately the same position as the AP-1 site (at −225 to −231) in the related proliferin gene promoter (19); both the mPL-I and proliferin genes (5) are active specifically in trophoblast giant cells, with maximal levels of both of these mRNAs present on gestational day 10 (16, 20). The locations of these AP-1 elements are also similar to the position of a cAMP response element (at −248 to −255) in the mPL-II gene promoter (13). Since AP-1 and factors that act through a cAMP response element have overlapping activities (21), AP-1 and related factors may be critical for transcription of each of the placental members of the PRL-related gene family *in vivo*.

Since AP-1 is a ubiquitous transcription factor complex, the lack of mPL-I promoter activity in nontrophoblast cells indicates that two functional AP-1 elements in conjunction with a TATA box are not necessarily sufficient for promoter activity. One possible explanation for this finding is that other sequences in the −1 to −274 region function as repressors, perhaps blocking AP-1 activity. This is seen in the proliferin gene promoter, in which sequences adjacent to the AP-1 site can affect AP-1 function and promoter activity (19, 22) (Mordacq, J. C., M. Athaide, and D. I. H. Linzer, unpublished observations). Another possibility is that the mPL-I gene AP-1 sites interact preferentially with a subset of the many AP-1 factor forms, and that these interactive forms are present at higher levels in Rcho-1 cells than in other cell types. The AP-1 forms in undifferentiated Rcho-1 cell extracts that interact with the mPL-I up-stream site appear to be primarily c-*fos*/c-*jun* and c-*fos*/*jun*-B heterodimers, while in differentiated cells, the form of AP-1 bound to this site is mostly the c-*fos*/*jun*-B heterodimer; this change in the bound form of AP-1 upon differentiation may be important for the activity of this promoter in trophoblast giant cells.

Although the AP-1 sites are required for maximal promoter activity, it seems unlikely that these elements, which are recognized by factors present in many cell types, are sufficient for dictating trophoblast-specific expression of the mPL-I promoter. At least one other element does appear to be required for transcription from the mPL-I gene promoter, lying within a 54-bp region between the up-stream AP-1 site and position −188, as defined by 5′-deletion mutagenesis of the promoter.

Unlike the *in vivo* situation, differentiated Rcho-1 cells continue to synthesize PL-I even after extended culture times (14). Thus, this system has not yet revealed what signals lead to the deactivation of PL-I gene expression and the coincident switch from PL-I to PL-II synthesis. The isolation of stably transfected cell lines that activate the mPL-I gene promoter upon cell differentiation may prove useful in analyzing the effects of various extracellular factors on the transcriptional activity of the mPL-I gene promoter, potentially enabling factors to be identified that are involved in the deactivation of the mPL-I gene during the latter half of gestation. The switch in expression of the PL genes also represents a useful molecular marker for trophoblast cell differentiation. The current classification of trophoblast cells largely by morphology (*e.g.* giant cells) underscores the need to define more subtle molecular differences between these cells that lead to distinct trophoblast cell functions in pregnancy. Analysis of the expression of the PL genes may lead to characterization of the genetic elements and transcription regulatory factors that are involved in a broader program of trophoblast gene expression and cellular differentiation.

MATERIALS AND METHODS

Isolation of mPL-I Genomic Clones

A bacteriophage library containing BALB/c mouse genomic DNA (kindly provided by Laurie Jackson-Grusby) was screened by hybridization to mPL-I cDNA, and bacteriophage DNA was purified from several hybridizing clones. The DNA inserts were restriction mapped and further hybridized to a fragment of the mPL-I cDNA that includes only the 5′-region. A subclone of the genomic DNA containing approximately 3 kilobases of up-stream mPL-I sequence as well as the first exon and the first intron was isolated and used for further analysis.

Primer Extension Analysis

An antisense 25-mer oligonucleotide complementary to a region near the 5′-end of the mPL-I cDNA (positions 81–105) was synthesized and end labeled with [γ-^{32}P]ATP. This probe was hybridized to 10 μg total RNA, prepared by centrifugation of guanidinium thiocyanate lysates of day 10 pregnant mouse placenta or liver through a CsCl cushion. Reverse transcriptase and deoxynucleotides were then added, and the resultant product was analyzed by electrophoresis on a 6% polyacrylamide sequencing gel and autoradiography.

Mutagenesis and Plasmid Construction

The initial set of promoter fragments containing various amounts of 5′-flanking sequence was generated by restriction endonuclease digestion. A cloning site at the 3′-end of the promoter was generated by cleavage with *Hin*cII (GTCAAC with the G at position −1), which allowed for fusion at position 2 to the CAT gene and the simian virus-40 signals for splicing and polyadenylation. Additional 5′-promoter deletions were produced by the polymerase chain reaction (PCR). Each up-stream PCR oligonucleotide primer included a restriction site at the 5′-end to simplify cloning of the product; the down-stream antisense oligonucleotide corresponded to a sequence in the CAT gene. The sequences of all PCR fragments were determined to verify that no other mutations occurred. Site-directed mutagenesis was also accomplished using PCR. Complementary mutant primers were separately combined with an up-stream or down-stream primer and the promoter DNA template, and the overlapping mutant PCR products were isolated. These fragments were then combined with only the up-stream and down-stream primers for a second round of PCR. The resultant full-length mutant promoters were recloned and sequenced.

Cell Culture and Transfections

Undifferentiated Rcho-1 cell cultures were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 50 μm β-mercaptoethanol, 1 mM sodium pyruvate, and 10 U/ml each of penicillin and streptomycin. To promote differentiation, cultures were grown to confluence, shifted into NCTC-135 medium, supplemented as described above, and maintained for 2 days to 2 weeks. Mouse L-cells, monkey Cos-7 cells, and CHO cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum and 10 U/ml each of penicillin and streptomycin.

Transfections of Rcho-1 cells were carried out by mixing 10 μg mPL-I-CAT or RSV-CAT (23) plasmid DNAs with Lipofectin (GIBCO-Bethesda Research Laboratories, Gaithersburg, MD) according to the manufacturer's instructions. For plasmids differing significantly in size, the mass amount of DNA was varied so that equal moles of DNA were transfected. Cultures were transfected in 6-cm dishes and harvested 2 days later. All other cell types were transfected using diethylaminoethyl-dextran (24). For quantitations of promoter activity, 2 μg of the plasmid pA$_3$RSV$_{400}$LUC (25) were included in cotransfections as an internal control. However, transfections quantifying mPL-I promoter activity were also conducted in parallel without this internal control due to observed competition between the RSV and mPL-I promoters. This internal control was also used to ensure that transfections with mPL-I promoter constructs lacking activity were successful. CAT assays were performed as previously described (26), using 60 μg cell extract; luciferase assays were conducted with 30 μg cell extract, as previously described (27), and used to normalize CAT activity values.

Stably transfected cell lines were generated by cotransfecting Rcho-1 cell cultures in 10-cm dishes with 30 μg mPL-I-CAT plasmid DNA and 2.5 μg pSV2neo DNA (28). Transfections were carried out using lipofectin, and stably transfected colonies were selected by growth in the presence of 250 μg/ml G418; the optimal G418 concentration for selection was determined by initially selecting test colonies with different amounts of G418. Several independent, stably transfected cell lines were generated with each mPL-I promoter contruct. These cell lines were then analyzed for expression of the transfected DNA either under subconfluent (undifferentiated) conditions or after being maintained for 1–2 weeks at confluence in NCTC-135 medium (differentiated conditions).

Cell Extracts and Electrophoresis Mobility Shift Analysis

Whole cell extracts from Rcho-1 and L-cells were prepared according to the method of Zimarino and Wu (29). Double stranded oligonucleotides were radiolabeled by incubation with [γ-^{32}P]ATP and T4 polynucleotide kinase and purified by gel electrophoresis. For binding reactions, 1–2 × 10^4 cpm were incubated in a 20-μl final volume of 10 μg whole cell extract, 5 μg poly(dI-dC), 1 mM EDTA, 25 mM HEPES (pH 7.8), 5 mM MgCl$_2$, 75 mM KCl, 2 mM dithiothreitol, and 5% glycerol. Unlabeled competitor oligonucleotides were added in a 100-fold molar excess, or 2 μl antiserum against *c-jun*, *jun*-B, or *jun*-D (30), or against *c-fos* were added at the beginning of the binding reaction. Bound and free DNAs were separated by 5% polyacrylamide gel electrophoresis and detected by autoradiography.

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