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GATA factor activity is required for the trophoblast-specific transcriptional regulation of the mouse placental lactogen I gene

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SUMMARY

The molecular determinants governing tissue-specific gene expression in the placenta are at present only poorly defined, particularly with respect to the regulation of specific hormone genes whose products are vital to embryonic development and the maintenance of a nurturing maternal environment. In continuing our analysis of the trophoblast-specific expression of the mouse placental lactogen I gene, we now demonstrate that the transcription factors GATA-2 and GATA-3 regulate the activity of this gene promoter. These factors are expressed in placental trophoblast cells, with peak levels of the GATA-2, GATA-3 and placental lactogen I mRNAs each accumulating at midgestation. Analysis of a region of the placental lactogen I gene promoter, previously shown to be sufficient for directing trophoblast-specific transcription, revealed the presence of three consensus binding sites for GATA-2 or GATA-3. Both GATA-2 and GATA-3 bind to these sites in vitro and mutation of these sites results in a significant decrease in promoter activity as assayed by transient transfection into the choriocarcinoma-derived cell line Rcho-1, which expresses endogenous GATA-2 and GATA-3. Furthermore, overexpression of GATA factors in Rcho-1 cells stimulates transcription from a co-transfected placental lactogen I gene promoter. Most significantly, expression of GATA-2 or GATA-3 was found to induce transcription from this promoter in transfected non-trophoblast (fibroblast) cells. These data indicate that GATA factors are both limiting and required transcriptional regulatory molecules in placental trophoblasts, and that the tissue specificity of the placental lactogen I gene is determined, at least in part, by GATA-2 and/or GATA-3.

Key words: GATA factor, placenta, placental lactogen, transcription factor, trophoblast

INTRODUCTION

The trophoblast lineage is derived from the extraembryonic trophectoderm, the first differentiated cells arising from the preimplantation mammalian embryo (Ilgren, 1983). These cells are responsible for invasion of the uterine epithelium, resulting in implantation of the embryo (Strickland and Richards, 1992). Trophoblast cells at this stage remain diploid and proliferative until they have migrated away from the inner cell mass, at which point some cells differentiate into giant cells (Müntener and Hsu, 1977; Ilgren, 1983). Giant cells, so designated because of their large size compared to neighboring trophoblast and decidual cells, have amplified, polytene genomes (Varmuza et al., 1988). This cellular transformation begins at the pole opposite the mouse embryo and proceeds laterally to the embryonic pole (Dickson, 1963).

The giant cells are a major source of placental hormones, including members of the prolactin (PRL)/growth hormone (GH) family (Soares et al., 1991). In the mouse, these hormones include placental lactogens I and II (mPL-I and mPL-II, respectively) which bind to the PRL receptor with high affinity (Harigaya et al., 1988; MacLeod et al., 1989), and proliferin and proliferin-related protein (Linzer et al., 1985; Colosi et al., 1988). The serum profiles of PRL and the placental hormones divide gestation into three phases. In the first phase (through day 8 of the 19-20 day gestational period), PRL is the predominant hormone in this family present in maternal serum (Barkley et al., 1978). Synthesis of mPL-I initiates at the peri-implantation stage of gestation (Nieder and Jennes, 1990) in the giant cells (Faria et al., 1991), but the level of this protein in the maternal serum remains low until day 8; the serum concentration of mPL-I then increases rapidly to its peak on day 10 (Ogren et al., 1989). Coincident with this increase in mPL-I concentration, secretion of PRL is drastically reduced (Barkley et al., 1978).

After day 10 of pregnancy, mPL-I levels rapidly decline and remain low until term (Ogren et al., 1989). Since a parallel decrease is observed in the abundance of placental mPL-I mRNA (Colosi et al., 1987), the reduction in circulating mPL-I may be due primarily to a decrease in mPL-I gene transcription. As mPL-I synthesis and secretion decline, mPL-II begins to be produced by the same trophoblast giant cells that synthesize mPL-I (Yamaguchi et al., 1992), marking the beginning of the third wave of lactogenic hormones in pregnancy. Unlike mPL-I, mPL-II levels continue to increase until term (Soares and Talamantes, 1985). These three phases of gestation thus

represent developmentally regulated shifts in hormone expression: a shift from the pituitary to the placenta as the origin of PRL-like hormones; a shift from PRL to PL-I and from PL-I to PL-II as the primary lactogenic hormone; and a shift within trophoblast giant cells from PL-I to PL-II gene expression.

The activation of the mPL-I gene prior to and following implantation suggests that this gene could serve as a useful model for investigating early events in trophoblast differentiation. Furthermore, since an analysis of the expression of the closely related PRL and GH genes has led to the characterization of a transcription factor essential for normal pituitary development (Simmons et al., 1990; Pfäffle et al., 1992), the identification of transcriptional regulators of the mPL-I gene may directly reveal factors that also are important in directing trophoblast differentiation.

We have previously demonstrated that the 274 bp immediately upstream of the mPL-I gene transcriptional start site (Fig. 1) are sufficient to confer trophoblast-specific transcription after transfection into the Rcho-1 cell line and that this region includes two AP-1 sites required for maximal expression (Shida et al., 1993). Here we report that members of the GATA transcription factor family fulfill an essential role in the trophoblast-specific expression of the mPL-I gene and show that these factors may also play a critical role in trophoblast differentiation.

MATERIALS AND METHODS

Cell culture

Rcho-1 rat trophoblast and Jurkat human T lymphoma cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 50 μM β-mercaptoethanol, 1 mM sodium pyruvate and penicillin/streptomycin. Mouse L fibroblasts and mouse C1300 neuroblastoma cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% calf serum and penicillin/streptomycin.

DNA constructions

The mPL-I (Colosi et al., 1987), mGATA-2 (unpublished; provided by S. Orkin), mGATA-3 (Ko et al., 1991) and CHOB (Harpold et al., 1979) cDNAs were propagated in plasmid vectors containing bacteriophage RNA polymerase promoters. These plasmids were used to generate radiolabeled RNA probes by in vitro transcription or DNA probes by random priming. The CHOB cDNA, which was used to control for the amount and integrity of each RNA sample, hybridizes to ribosomal protein S2 mRNA (Suzuki et al., 1991). The eukaryotic expression construct for GATA-2 contains the human GATA-2 cDNA downstream of the adenovirus major late promoter in the pMT2 vector (Bonthron et al., 1986); the GATA-3 and GATA-3ΔZF (in which the zinc-finger DNA-binding domain of GATA-3 has been deleted – Yang et al., 1994) expression constructs contain the mouse GATA-3 cDNA downstream of the Rous sarcoma virus long terminal repeat. The wild-type 274 bp mPL-I promoter linked to the CAT gene (mPL-I-CAT) has been described (Shida et al., 1993). Site-directed (SDM) mutations in the mPL-I promoter were made by the polymerase chain reaction, utilizing overlapping, complementary oligonucleotide primers that contained the desired mutations. The site-directed mutants that were constructed are shown below, with the wild-type core GATA-2/3-binding sites underlined:

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                    −229              −209
                    •                  •
Wild Type           CAGATGATCTTTCTGATTTGG
                    GTCTACTAGAAAGACTAAACC

SDM(−227/−213)      CAcAaGcTtccTgcagcTTGG
                    GTgTtCgAaggAcgtcgAACC

                    −96          −81
                    •             •
Wild Type           GCTTATCAATAGACAT
                    CGAATAGTTATCTGTA

SDM(−93/−89)        GCTagcCcATAGACAT
                    CGAtcgGgTATCTGTA
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In addition, these two mutations were combined in SDM(–227/–213)/(–93/–89).

Animal care

Timed-pregnant Swiss-Webster mice were obtained from Harlan Breeding Laboratory (Indianapolis, IN), with day 0 defined as the day of appearance of a vaginal plug. Mice were maintained on days of 14 hour light/10 hour darkness, with lights on at 06.00 h, and with food and water freely available. Animals were sacrificed according to procedures approved by the Institutional Animal Care and Use Committee.

RNA purification and filter hybridization

The procedures used for the isolation and hybridization of RNA from cell cultures and mouse tissues have been described previously (Jackson et al., 1986). In brief, total RNA was purified from cell cultures or from placental tissue (separated from both maternal and fetal components) by homogenization in guanidinium thiocyanate solution and centrifugation of the lysate through a CsCl cushion. Equal amounts of total RNA, as determined by optical density at 260 nm, were denatured and applied to formaldehyde-agarose gels. After electrophoresis, the RNAs were transferred and UV-crosslinked to nitrocellulose filters, and then hybridized overnight at 42°C in 50% formamide, 5× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate), 0.1% SDS, 0.1% bovine serum albumin, 0.1% ficoll, 0.1% polyvinylpyrrolidone, 40 μg/ml sonicated salmon sperm DNA, and 3×10^6 cts/minute/ml ^{32}P-labeled mGATA-2, mGATA-3, mPL-I, or CHOB cDNAs. Filters were washed at 50°C in 1× SSC, 0.1% SDS before autoradiography.

In situ hybridization

Dissected tissue was rapidly frozen and stored at –80˚C until use. 20 μm sections were cut, mounted onto gelatin/poly-L-lysine-treated glass slides, and fixed with 5% paraformaldehyde. Anti-sense and sense RNA probes were synthesized in parallel transcription reactions containing α-[^{35}S]UTP with SP6 or T7 RNA polymerase and the mPL-I, mGATA-2, or mGATA-3 cDNAs as templates. Hybridization mixtures of 80 μl containing 1×10^7 cts/minute/ml of the RNA probe in 50% formamide, 300 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.02% bovine serum albumin, 0.02% ficoll, 0.02% polyvinylpyrrolidone, 10% dextran

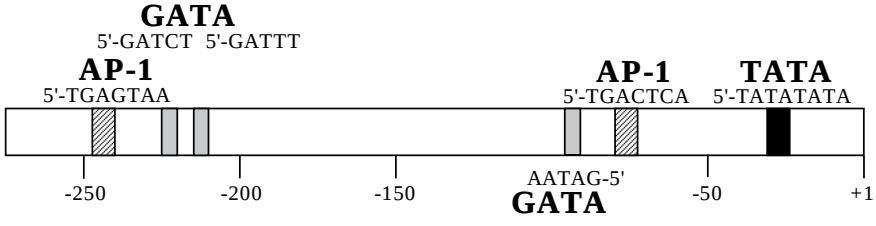


Fig. 1. Schematic diagram of the mPL-I promoter to –274 bp showing the locations of the TATA box (solid), the two AP-1 sites (hatched) and the three GATA factor consensus motifs (stipled).

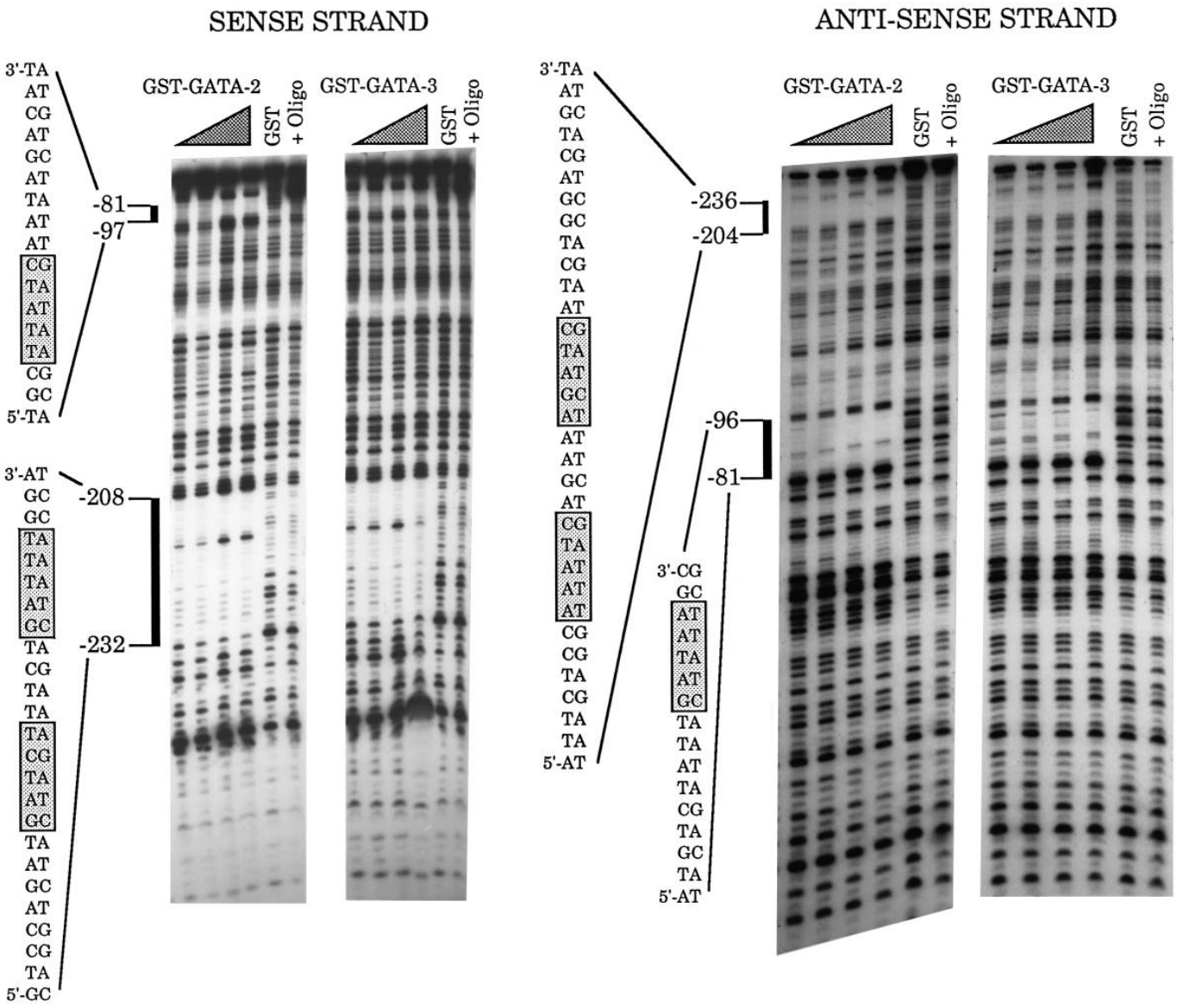


Fig. 2. Localization of GATA factor-binding sites in the mPL-I promoter. A fragment of the mPL-I gene from –274 to +2 was end-labeled on either the sense or anti-sense strand, incubated with increasing amounts of GST-GATA-2 or GST-GATA-3 (from left to right, 2-12 μg of fusion protein) and exposed to DNase I. Controls include reactions supplemented with 8 μg GST instead of the fusion protein, or with 8 μg of the fusion protein along with 200 ng of a double-stranded oligonucleotide containing a consensus binding site for GATA-2 and GATA-3. Reaction products were fractionated by polyacrylamide gel electrophoresis and regions protected from DNase I digestion were detected by autoradiography. The boundaries and sequences of the protected regions on each strand are indicated; shaded boxes are the core GATA factor recognition sequences.

sulfate, 10 mM dithiothreitol, 0.5 mg/ml yeast tRNA and 0.5 mg/ml poly(A) were applied to the tissue sections, and the sections were then overlaid with a coverslip. Slides were hybridized overnight at 47°C in a humidified chamber, then treated with RNase A (20 μg/ml in 2× SSC) at 37°C for 30 minutes, washed in progressively lower concentrations of SSC (from 1× to 0.25×) at 47°C and dehydrated through an ethanol step series (50-100%). The slides were coated with NTB-2 emulsion (Kodak, Rochester, NY) and exposed for two weeks prior to development. Slides were stained with hematoxylin/eosin and photographed using a Nikon optiphot microscope.

DNA transfections and CAT assays

Transient transfections into subconfluent rat Rcho-1 or mouse L cell cultures were performed with cationic lipids, most recently with lipofectamine (GIBCO-BRL). Cells were transferred to 60 mm dishes 24 hours before transfection with 25 μg of lipofectamine and 15-20 μg DNA in 2 ml of serum-free medium. After 18 hours, the medium in each dish was replaced with serum-containing medium and the cultures were maintained an additional 54 hours, at which time the cells were harvested. Cell extracts were prepared by repeated freezing and thawing in 0.25 M Tris-HCl, pH 7.4. Equal amounts of protein from each extract, as determined by dye-binding measurements (BioRad), were then assayed for CAT activity as described (Gorman et al., 1982). Quantification of CAT assays was accomplished using a Molecular Dynamics phosphorimager.

Cell labeling and immunoprecipitation

Cell cultures were incubated for four hours in methionine-free

medium supplemented with 80 μCi/ml [^{35}S]methionine/cysteine (New England Nuclear). Cells were harvested in phosphate-buffered saline and lysed in 1.0% SDS, 40 mM Tris, pH 7.8, 1% β-mercaptoethanol, 100 mM dithiothreitol. The lysate was heated at 100°C for 10 minutes and then diluted with 9 volumes of a solution containing 150 mM NaCl, 1.0% sodium deoxycholate, 1.0% Triton X-100, 10 mM Tris-HCl, pH 7.4 and 1 mM phenylmethylsulfonylfluoride. Samples were then treated with Protein A-Sepharose (Sigma) for 30 minutes at 4°C and then centrifuged; the supernatant was then incubated for 2 hours at 4°C with monoclonal antibodies against human GATA-2 (Nagai et al., 1994) or human GATA-3 (Yang et al., 1994), or with normal serum as a control. A combination of Protein G (Pharmacia) and A Sepharose was used to recover the immune complexes, and the immunoprecipitated proteins were visualized by SDS gel electrophoresis and autoradiography.

DNase I footprinting

A fragment of the mPL-I gene from an *Eco*RI site at −274 to a *Hin*cII site at +2 was subcloned into a plasmid vector. After linearization at either end of this plasmid insert, the DNA was end-labeled by treatment with calf intestinal alkaline phosphatase followed by incubation with T4 polynucleotide kinase and γ-[^{32}P]ATP. The DNA was then cut at the far side of the insert and the promoter fragment labeled at one end was recovered by gel electrophoresis. Labeled DNA (2×10^4 counts/minute) was incubated with purified GST (8 μg), GST-chicken GATA-2 or GST-chicken GATA-3 (from 2-12 μg), or 8 μg GST-GATA-2 or GST-GATA-3 plus 200 ng of an unlabeled double-stranded oligonucleotide, δE4, containing a consensus GATA factor-binding site (Ko et al., 1991). Preparation of the GST-GATA factor fusion proteins has been described previously (Ko and Engel, 1993). Binding reactions were carried out on ice for 45 minutes in a final volume of 20 μl containing 10 mM Tris-HCl, pH 8.0, 100 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 100 μg/ml bovine serum albumin, 5% (v/v) glycerol and 0.5 μg poly(dI:dC). Reactions were then supplemented with 0.02 μg DNase I (Promega) and maintained at room temperature for 90 seconds. The DNA was then precipitated with ethanol, solubilized in 80% formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol and heated at 90°C for 3 minutes before loading on a 7% polyacrylamide sequencing gel. Size markers were Maxam and Gilbert sequencing reactions of the labeled mPL-I DNA fragment. After electrophoresis, the gel was fixed in 10% acetic acid, dried and autoradiographed.

RESULTS

Identification of GATA factor-binding sites in the mPL-I promoter

Previous results indicated that at least one element located between −242 and −188, relative to the start site of transcription, is crucial for mPL-I promoter activity in transfected trophoblast cell cultures (Shida et al., 1993). Within this 54 bp region are the sequences GATCT (from −224 to −220) and GATTT (from −215 to −211). The sequence GATCT has been shown to be a high affinity binding site for GATA-2 and GATA-3 (Ko and Engel, 1993); these binding site selection studies also revealed that the sequence GATTT can form complexes with GATA factors, although this sequence was recovered with a much lower frequency than GATAA or GATCT (Ko and Engel, 1993). Electrophoretic mobility shift (data not shown) and DNase I footprinting (Fig. 2) assays demonstrated that both of these sites in the mPL-I promoter are indeed recognized by GATA-2 and GATA-3. A third high affinity binding site for both GATA-2 and GATA-3 was identified between −97 and −81 (Fig. 2); this region contains the consensus sequence GATAA, which is recognized with high affinity by all members of the GATA factor family (Ko and Engel, 1993). The two upstream sites combined to yield a single extended footprint from −236 to −204, which was approximately twice the size of the downstream footprint, suggesting that both upstream sites are occupied simultaneously in vitro. DNase I cleavage was more pronounced at the nucleotides immediately flanking occupied GATA sites (Fig. 2), indicating that GATA factor-binding at −236 to −204 and at −97 to −81 resulted in the increased exposure of adjacent bases to the nuclease.

Expression of GATA-2 and GATA-3 in trophoblast cells

The binding of GATA-2 or GATA-3 to the presumptive regulatory sites in the mPL-I promoter would be relevant to the trophoblast-specific expression of the mPL-I gene only if these factors are expressed in trophoblast cells. RNA was therefore isolated from the rat trophoblast cell line Rcho-1, which expresses the endogenous rPL-I gene (Faria et al., 1990; Faria and Soares, 1991) and from mouse placentae to determine if either or both of these GATA factor mRNAs were present. GATA-2 and GATA-3 mRNA were detected in both the Rcho-1 and mouse placental RNA preparations (Fig. 3). Although the levels of the GATA-2 and GATA-3 mRNAs are very low in the placenta at day 8 (but evident upon longer exposure), the accumulated levels of these mRNAs were highest at mid-gestation (day 10), coincident with the time of maximal synthesis of mPL-I (Fig. 3). By day 12, the mPL-I mRNA level has declined significantly and this low level was maintained through day 18; the GATA-2 and GATA-3 mRNAs also were found to decrease in amount later in gestation, although both mRNAs were still readily detectable through day 18.

Within the placenta, GATA-2 and GATA-3 mRNAs were found by in situ hybridization to be synthesized in the tro-

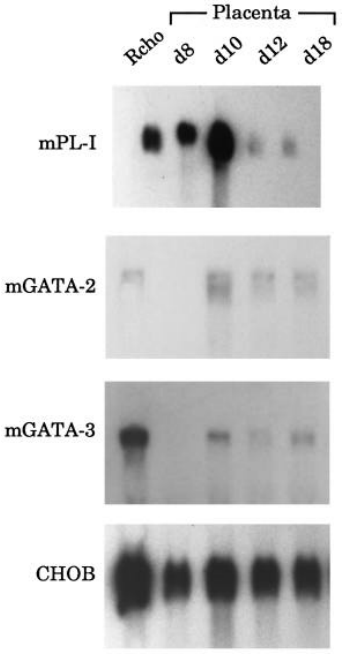


Fig. 3. Expression of GATA-2 and GATA-3 mRNA in trophoblasts. RNA was purified from the rat Rcho-1 trophoblast cell line or from mouse placental tissue isolated at days 8, 10, 12, or 18 of gestation. Total RNA (15 μg/lane) was denatured and fractionated by electrophoresis through a formaldehyde-agarose gel. After transfer to nitrocellulose, these RNAs were hybridized to radiolabeled cDNA probes specific for GATA-2, GATA-3, or mPL-I. Hybridization to the CHO-B cDNA was used to control for variations in RNA loading and transfer.

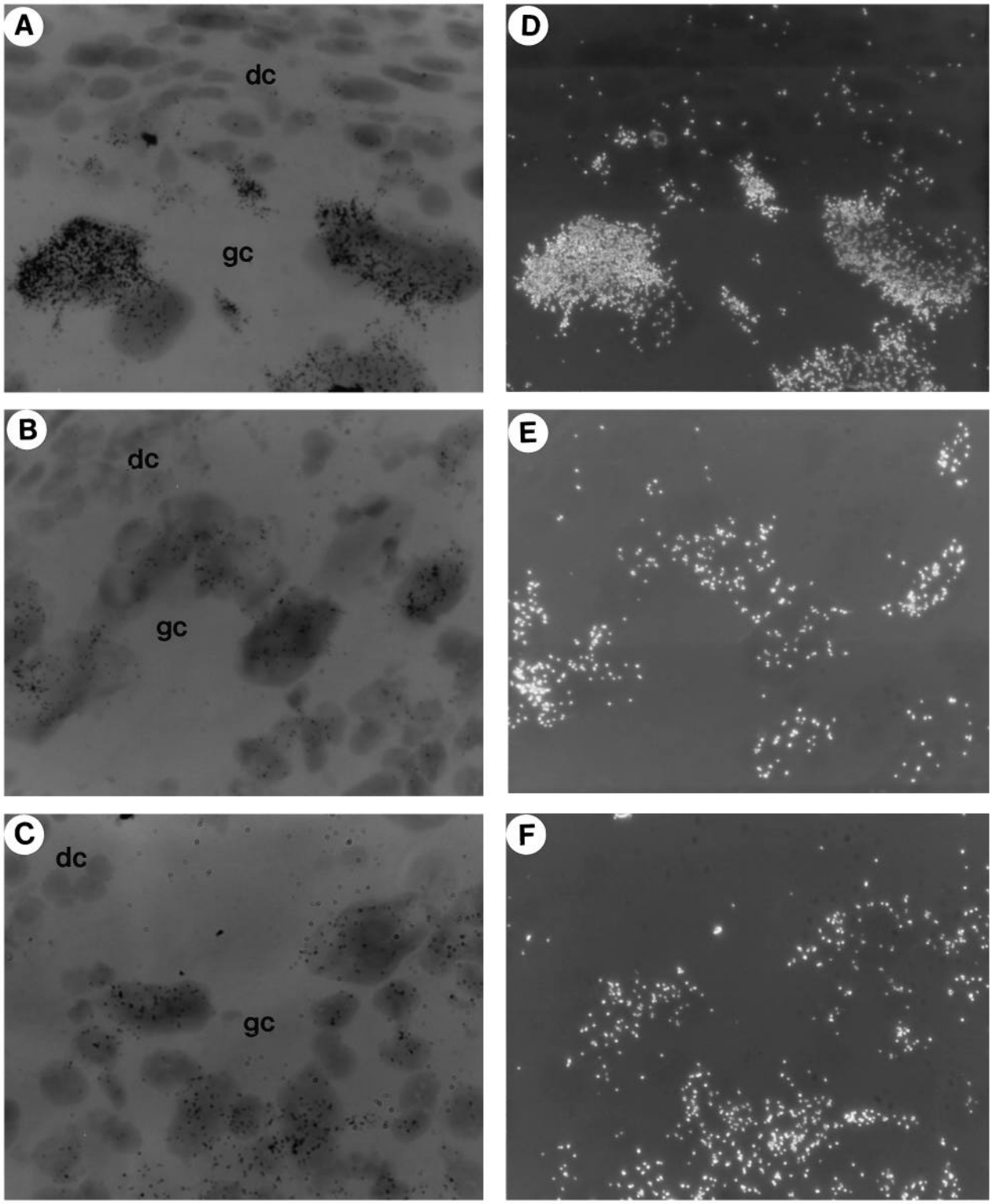


Fig. 4. Localization of GATA-2 and GATA-3 mRNAs to trophoblast giant cells. Mouse placental tissue from day 12 of gestation was sectioned and hybridized to single-stranded RNAs complementary to the mPL-I (A,D), GATA-2 (B,E), or GATA-3 (C,F) mRNA. Silver grains in the bright-field photomicrographs shown on the left (A-C) indicate hybridization to the layer of trophoblast giant cells (gc); hybridization to the GATA-2 and GATA-3 probes can also be seen in smaller trophoblasts underlying the giant cell layer. The corresponding darkfield views are displayed on the right (D-F), with hybridization detected as bright regions. Note the lack of hybridization of all three probes to the maternal decidual cells (dc). Also, no significant hybridization was detected in tissue sections incubated with the corresponding sense strand probes (data not shown).

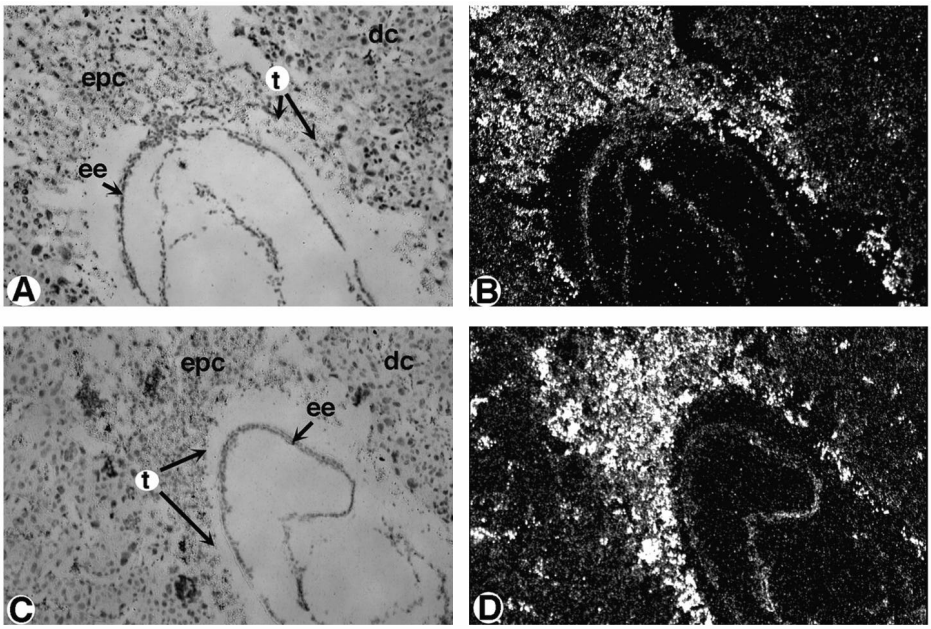


Fig. 5. GATA-2 and GATA-3 synthesis in early gestation. Implantation sites at day 7 of gestation were sectioned and hybridized to radiolabeled anti-sense RNAs to GATA-2 (A,B) or GATA-3 (C,D). The trophoblasts cells (t), ectoplacental cone (epc), extraembryonic ectoderm (ee) and maternal decidual cells (dc) are indicated in the bright-field photomicrographs (A and C). Hybridization to the trophoblasts and cells in the ectoplacental cone is seen as bright regions in the dark-field photomicrographs (B,D). The hybridizing cells have previously been demonstrated to be the cells expressing mPL-I (Nieder and Jennes, 1990; Carney et al., 1993). No significant hybridization was detected with control, sense strand probes (data not shown).

phoblast giant cells, the cells that produce mPL-I (Fig. 4). Expression of GATA-2 and GATA-3 was not found in the maternal decidual tissue, but was detected in trophoblasts of the developing basal and labyrinth regions on day 12 (Fig. 4 and data not shown). Although some of these GATA factor-expressing cells were not positive for mPL-I, these cells may be giant cell precursors since many mPL-I-positive cells were also detected in these regions (data not shown). At earlier times in gestation, expression of GATA-2 and GATA-3 can also be detected in trophoblasts, as well as in the ectoplacental cone (Fig. 5). Based on the hybridization intensity, both on filters and in tissue sections, the concentrations of GATA-2 and GATA-3 mRNAs appear to be similar in the placenta.

Immunoprecipitation of the proteins synthesized in the Rcho-1 trophoblast cell line demonstrated that the GATA-2 and GATA-3 transcripts are both functional, since they are translated into the products of the expected size (Fig. 6). In these experiments, Jurkat (human T lymphoma) and C1300 (mouse neuroblastoma) cells were used as positive controls for GATA-3 and GATA-2 expression, respectively (George et al., 1994). In contrast, immunoprecipitation of mouse L cell lysates indicated that neither GATA-2 nor GATA-3 protein was synthesized at a detectable level in these fibroblast cultures (Fig. 6). Thus, L cells could provide a convenient cellular background to analyze mPL-I promoter activity in the absence of endogenous GATA-2 and GATA-3 factors. This result, coupled with our previous finding that the mPL-I promoter was inactive upon transient transfection into L cells (Shida et al., 1993), suggested that GATA factors may be required for mPL-I promoter activity.

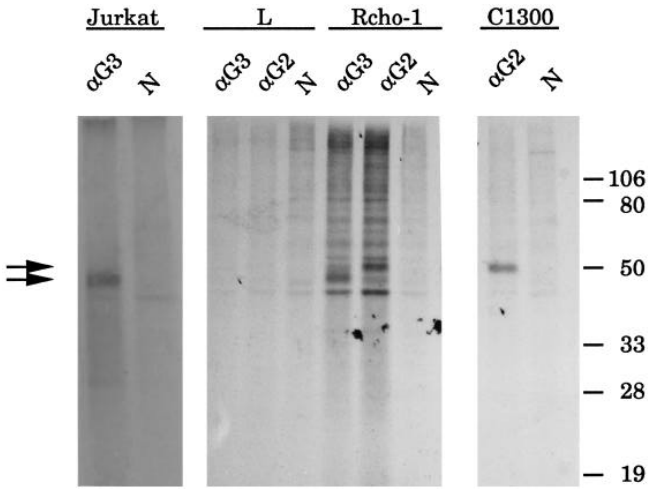


Fig. 6. GATA-2 and GATA-3 proteins are sythesized in trophoblast cell cultures. Human Jurkat T lymphoma cells, mouse C1300 neuroblastoma cells, rat Rcho-1 trophoblasts and mouse L fibroblasts were metabolically labeled with [^{35}S]methionine/cysteine. Cell lysates were then immunoprecipitated with monoclonal antibodies generated against GATA-2 (αG2) or GATA-3 (αG3) or with normal serum. Recovered proteins were detected by electrophoresis and autoradiography. The positions of the GATA-2 protein (in the C1300 and Rcho-1 samples) and the GATA-3 protein (in the Jurkat and Rcho-1 samples) are indicated by the upper and lower arrows, respectively. Although the background is higher in the Rcho-1 samples, GATA-2 and GATA-3 were detected in these cells, as can be seen by the prominent bands of the expected mobility that are present specifically in the anti-GATA-2 or the anti-GATA-3 immunoprecipitates.

GATA factors stimulate the mPL-I promoter in trophoblast cells

To test the possibility that GATA factors might stimulate transcription from the mPL-I promoter, an expression construct for GATA-3 was co-transfected with a plasmid containing the mPL-I promoter linked to the chloramphenicol acetyltransferase (CAT) reporter gene into Rcho-1 trophoblast cell cultures. Suboptimal amounts of the mPL-I-CAT construct were used, such that CAT activity was expressed only at very low levels in cultures transfected with the mPL-I-CAT plasmid

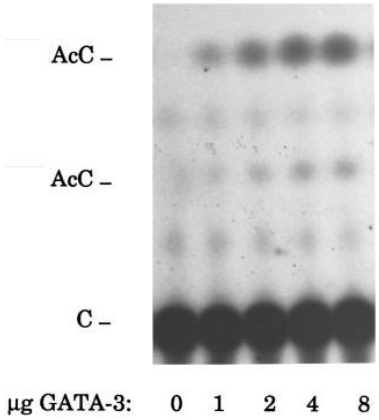


Fig. 7. Stimulation of mPL-I promoter activity by GATA-3 in trophoblast cells. Rcho-1 cells were co-transfected with 2 μg of mPL-I-CAT DNA and variable amounts of the GATA-3 expression construct. The total amount of DNA in each transfection was kept constant at 20 μg by inclusion of plasmid vector DNA. Lysates of the transfected cultures were assayed for CAT activity, which was visualized as the conversion of chloramphenicol (C) to acetylated forms (AcC).

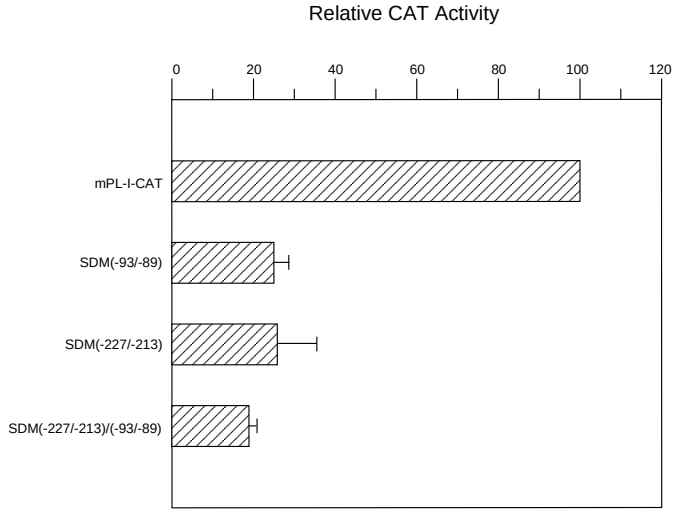


Fig. 8. The GATA factor-binding sites in the mPL-I promoter are required for maximal activity. Rcho-1 cells were transiently transfected with 20 μg of wild-type mPL-I-CAT DNA or with the mPL-I-CAT construct mutated at the downstream (SDM–93/–89), upstream (SDM–227/–213), or both downstream and upstream GATA factor sites. Transfected cultures were harvested and assayed for CAT enzymatic activity, which was quantified by phosphorimage analysis. Data represent the mean ± standard error from four independent transfections.

alone (Fig. 7). As increasing amounts of the co-transfected GATA-3 expression construct were added, transcription from the mPL-I promoter correspondingly increased (Fig. 7). Thus, GATA-3 can stimulate transcription from the mPL-I promoter, even in cells that express endogenous GATA factors, suggesting that GATA factors are present in limiting amounts (relative to the co-transfected mPL-I-CAT plasmid) in transfected trophoblast cells.

If the GATA factors are acting directly to stimulate transcription from the mPL-I promoter, then functional GATA-binding sites (those identified by DNase I footprinting) should be required for full promoter activity. Mutation of either the upstream footprint region (extending over two GATA sites from –227 to –213 and designated SDM–227/–213) or the downstream GATA site (from –93 to –89 and designated SDM–93/–89) significantly reduced promoter activity in transfected Rcho-1 cells (Fig. 8; see the Materials and Methods for a detailed description of the mutations). As expected, mutation of all three binding sites in the construct SDM(–227/–213)/(–93/–89) also resulted in a promoter with low activity (Fig. 8); transcription from this mutant promoter was similar to that from a 5′ deletion construct containing only the TATA element (data not shown). Thus, GATA-2 and GATA-3 stimulate mPL-I transcription directly by binding to two regions within the proximal 274 bp promoter, and the upstream and the downstream binding sites each function as important elements regulating mPL-I gene expression.

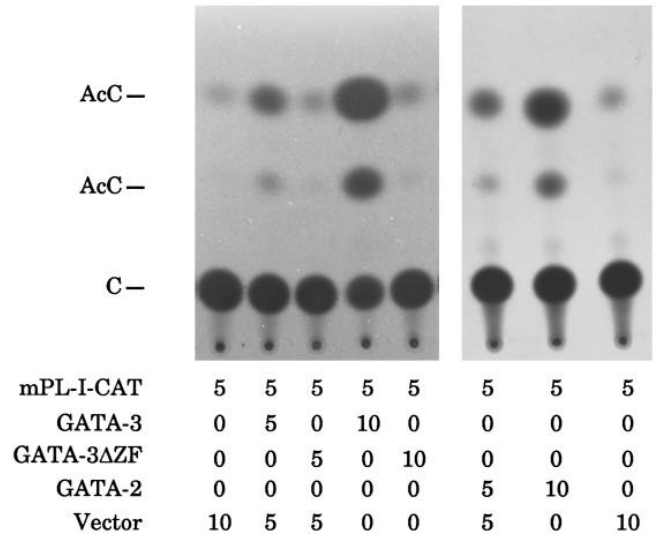
Stimulation of mPL-I promoter activity by GATA factors in fibroblasts

Since a GATA factor (presumably acting in concert with other ubiquitous factors such as AP-1; Shida et al, 1993) is apparently essential for mPL-I transcription, we next considered the more stringent possibility that expression of GATA-2 or GATA-3 in non-trophoblast cells might be sufficient to convert these cells into a trophoblast-like environment which would be permissive for mPL-I promoter activity. Mouse L cells, which lack detectable GATA-2 and GATA-3 proteins (Fig. 6), were therefore co-transfected with mPL-I-CAT and either the GATA-2 or GATA-3 expression construct. Both GATA-2 and GATA-3, but not a form of GATA-3 lacking the zinc finger DNA-binding motif (GATA-3ΔZF; Yang et al, 1994), stimulated transcription from the mPL-I promoter in mouse L cells (Fig. 9). Thus, the expression of active GATA-2 or GATA-3 is both necessary and sufficient to induce transcription from a co-transfected trophoblast-specific gene promoter in fibroblast cells.

DISCUSSION

Differentiation of the extraembryonic trophectoderm into the various trophoblast cell types is expected to involve a coordinated program of gene expression dictated by trophoblast-specific transcription factors or trophoblast-specific combinations of transcription factors. In this study, we have shown that two members of the GATA family of transcription factors, GATA-2 and GATA-3, are essential contributors to the trophoblast-specific expression of the mPL-I gene. The initial identification of these two transcription factors (Yamamoto et al., 1990) followed the characterization of a related factor (now designated GATA-1) that binds the core sequence element, GATA, in erythroid gene promoters and enhancers in various species (Evans and Felsenfeld, 1989; Tsai et al., 1989; Trainor et al., 1990; Zon et al., 1990). At least five members of this multi-gene family of transcription factors have been reported and each member displays a unique tissue-restricted expression pattern (Yamamoto et al., 1990; Arceci et al., 1993; Kelley et al., 1993).

GATA-1 expression appears to be restricted to the hematopoietic lineage (Evans and Felsenfeld, 1989; Tsai et al., 1989; Yamamoto et al., 1990; Joulin et al., 1991) and the testis



mPL-I-CAT	5	5	5	5	5	5	5	5
GATA-3	0	5	0	10	0	0	0	0
GATA-3ΔZF	0	0	5	0	10	0	0	0
GATA-2	0	0	0	0	0	5	10	0
Vector	10	5	5	0	0	5	0	10

Fig. 9. Induction of transcription from the mPL-I promoter in non-trophoblast cells. Mouse L cells were co-transfected with 5 μg of mPL-I-CAT DNA and 5 or 10 μg of an expression construct for GATA-2, GATA-3, or a mutant GATA-3 lacking the zinc-finger DNA-binding domain. The amount of DNA in each transfection was brought to 15 μg by addition of plasmid vector. Lysates of the transfected cell cultures were assayed for conversion of chloramphenicol (C) to acetylated forms (AcC).

(Ito et al., 1993). In contrast, GATA-4 factors have been detected in the heart (Arceci et al., 1993; Kelley et al., 1993). Both GATA-2 and GATA-3 are expressed in cells of the erythroid lineage, as well as in distinct subsets of central and peripheral nervous system neurons (Leonard et al., 1993; Kornhauser et al., 1994; George et al., 1994). GATA-2 expression has also been detected in the adult kidney, whereas GATA-3 is the only member of this transcription factor family that has been found in T lymphocytes (Leonard et al., 1993). In T cells, GATA-3 is required for the transcription of the T cell receptor α, β, γ and δ genes (Ko et al., 1991; Ho et al., 1991; Redondo et al., 1990, 1991; Joulin et al., 1991), as well as for maximal transcription from the human immunodeficiency virus long terminal repeat (Yang and Engel, 1993). We show here that both GATA-2 and GATA-3 are also expressed in placental trophoblast giant cells; we have not ruled out the possibility that additional members of the GATA factor family might also be expressed in trophoblasts.

The initial hypothesis that GATA-2 or GATA-3 is essential for mPL-I gene expression was based on the identification of potential binding sites for these factors in the −242 to −188 region of the mPL-I gene promoter, a region previously found to be required for transcriptional activity in transfected trophoblast cells (Shida et al., 1993). The present studies have demonstrated that GATA-2 and GATA-3 can bind to two sites within this region of the mPL-I promoter, as well as to a third, more proximal, site in vitro. The two sites that lie between −242 and −188 have the core sequences GATCT and GATTT. These sequences are unlikely to represent GATA-1-binding sites, since selection of random oligonucleotides for those recognized by GATA-1 yielded almost exclusively sequences containing the erythroid consensus motif GATAA (Ko and Engel, 1993). Both of these sites are occupied in vitro even at low concentrations of GATA-2 or GATA-3 protein, suggesting that these two sites are of high affinity, perhaps due to cooperative binding. Protein-protein interaction may be facilitated between GATA factors bound to adjacent sites: oligonucleotides containing two adjacent GATA recognition motifs have been shown to display increased affinity for these factors when compared to oligonucleotides that have single binding sites (Ko and Engel, 1993). The two sites in the mPL-I promoter are spaced such that both are predicted to lie on the same face of the DNA helix (approximately 10 bp apart in the sequence GATCTTTCTGATTT), similar to the arrangement of the T cell receptor δ gene enhancer GATA-binding sites (Redondo et al., 1990; Ko et al., 1991).

The third binding site for GATA-2 and GATA-3 detected by DNase I footprint analysis contains the core motif GATAA between −90 and −94. The extent of the promoter protected by GATA-2 or GATA-3 from nuclease digestion at this site is 16-17 nucleotides, compared to the 25-33 nucleotides protected at the upstream GATA sites. This result suggests that only a single GATA factor binds to this downstream element, consistent with the analysis of the DNA sequence in that region. Both this downstream GATA-binding site and the upstream, adjacent GATA sites are essential for promoter activity, since mutation of either region reduced transcription from the mPL-I promoter in transfected trophoblasts to low levels. The importance of these sites for promoter function further supports the idea that GATA-2 and GATA-3 stimulate mPL-I gene transcription directly by binding to the gene promoter. The finding that over-expression of either of these factors in Rcho-1 trophoblast cells increased transcription from a co-transfected mPL-I promoter further suggests that GATA-2 and GATA-3 might not only be essential for mPL-I gene expression, but that they may be limiting factors that determine the rate of transcription of this gene.

Consistent with a role for GATA-2 and GATA-3 in trophoblast-specific gene expression was the finding that both of these factors are synthesized in trophoblast cells. Indeed, expression of these factors appears to be relatively high in trophoblast giant cells, the cells that actively synthesize the placental hormone, mPL-I. Furthermore, GATA-2 and GATA-3 mRNAs are present in trophoblasts on day 7 of gestation, preceding the large increase in mPL-I secretion. The levels of the GATA-2, GATA-3 and mPL-I placental mRNAs all peak at mid-gestation relative to total placental RNA, suggesting that the amount of GATA-2 or GATA-3 present in giant cells may dictate the rate of mPL-I synthesis. Of course, the levels of active GATA factors in these cells may not correspond precisely to the levels of the GATA factor mRNAs, since translational and post-translational regulation may also be important.

Another member of the PRL/GH family in the mouse, proliferin, is co-expressed with mPL-I in trophoblast giant cells (Lee et al., 1988) and preliminary experiments indicate that transcription of the proliferin gene promoter is also stimulated by GATA factors upon co-transfection into Rcho-1 cells (data not shown). The proliferin gene promoter contains the sequence 5′-GATTTGTTTTAGTCA from −239 to −225 (Linzer and Mordacq, 1987), in which a potential GATA-2/3-binding site (single underlining) is adjacent to an AP-1 site (double underlining; Mordacq and Linzer, 1989). The proximity of the GATA and AP-1 sites, and the distance of these sites from the start of transcription, in the proliferin gene are similar to the arrangement of the upstream AP-1 and

GATA sites in the mPL-I promoter (Fig. 1). In addition, this region of the proliferin gene is flanked by potential GATA recognition motifs at −281 to −285 (GATAG; Ko and Engel, 1993) and at −161 to −157 (GATAA). The idea that a combinatorial association of transcription factors is required to direct trophoblast-specific gene expression might explain how GATA-2 and GATA-3 participate in this regulation, since these factors are not restricted to the trophoblast lineage. Certainly, the presence of GATA-2 or GATA-3 is not sufficient to drive expression of the mPL-I gene, since the mPL-I gene is transcriptionally silent in non-trophoblast cell types that express these factors. However, the addition of GATA-2 or GATA-3 to the repertoire of transcription factors normally present in mouse L fibroblasts was sufficient to induce transcription from the mPL-I gene promoter. Thus, GATA-2 or GATA-3 again appear to be limiting factors in providing an environment for trophoblast-specific gene expression. Whether or not regulatory factors in addition to AP-1 and GATA-2 or GATA-3 that are present both in mouse L cells and trophoblasts are also essential for mPL-I gene expression remains an unanswered question at this time.

GATA factors may regulate a broad program of trophoblast gene expression. The continued presence of GATA-2 and GATA-3 mRNAs in the placenta in late gestation (when mPL-I synthesis has declined) suggests a role for these factors in the expression of other genes in trophoblast cells, as well. One gene expressed in the placenta that has been analyzed in detail is the human α-glycoprotein subunit gene (Fenstermaker et al., 1990; Steger et al., 1991) and recent results indicate that placental expression of this gene also requires GATA factors (Steger et al., 1991; P. Mellon, personal communication). If GATA-2 and GATA-3 are involved in the activation of a battery of trophoblast-specific genes, then it seems likely that these transcription factors play a crucial role in trophoblast cell differentiation. Studies in which the GATA-2 (S. Orkin, personal communication) and GATA-3 (M. Lindenbaum, M. Leonard, P. Pandolfi, F. Grosveld and J.D. Engel, unpublished observations) genes are disrupted in mice may soon enable this question to be addressed.

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