Constitutive and trophoblast-specific expression of a class of bovine interferon genes

(bovine trophoblast protein 1/choriocarcinoma/leukocytes/placenta/Sendai virus)

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ABSTRACT The early conceptus in sheep and cattle secretes a low molecular weight protein called ovine and bovine trophoblast protein 1 (TP-1) that is critical for establishment of pregnancy. TP-1 is a type I interferon (IFN) and is most related to IFN-ω. Here we have determined if TP-1 genes are regulated similarly to other type I IFNs. Single day 18 bovine conceptuses secrete $10^6$ units of IFN antiviral activity per hour in culture, amounts $300$ times higher than those produced by Sendai virus-induced leukocytes. Although conceptuses express mRNA for IFN-α, IFN-ω, and TP-1, TP-1 constitutes $>99\%$ of the IFN produced. In contrast, leukocytes produce predominantly IFN-α, although TP-1 mRNA is inducible by Sendai virus to very low levels. TP-1 mRNA is detectable by Northern analysis in conceptuses from early pregnancy but is absent in late gestation placenta and several adult tissues. Transfected bovine TP-1 genes are expressed in human choriocarcinoma (JAR) cells in the absence of any specific stimulus, whereas these cells do not secrete antiviral activity constitutively or after transfection with a bovine IFN-ω gene. The transfected TP-1 gene is not expressed in nontrophoblast cells (mouse L929 and hamster Chinese hamster ovary), however. The 5′ promoter region of the TP-1 gene is sufficient to direct trophoblast-specific expression onto a human growth hormone reporter gene in JAR cells. Deletion of the promoter from -450 to -126 results in a 4- to 5-fold decrease in expression. Together these data demonstrate that the genes for TP-1 are inducible by virus but are expressed preferentially in trophoblast cells and are functionally distinct from IFN-ω genes.

Ovine and bovine conceptuses secrete large amounts of an interferon (IFN) structurally related to the IFN-ω subtype during the preimplantation stage of early pregnancy (1). These IFNs possess full antiviral (2, 3) and antiproliferative (3) activities and are thought to play a critical role in maternal recognition of pregnancy (1, 4). The proteins, originally called ovine and bovine trophoblast protein 1 (TP-1), are encoded by multiple genes (5–7) that appear distinct from those IFN-α and -ω whose cDNA has been cloned from leukocytes following viral infection (8, 9). The TP-1 mRNA have 3′ noncoding regions that are highly conserved across isoforms and species and may constitute a separate subtype (6).

Type I IFN represents a large multigene family comprised of distinct subtypes. IFN-α contains 165 or 166 amino acids and is encoded by at least 15 functional genes in the human and by a minimum of 10–12 genes in the bovine genome (8, 10). IFN-ω contains 172 or 174 amino acids (8, 9, 11, 12), and it too is encoded by multiple human and bovine genes (5, 8). The IFN-ω had been called IFN-αω by Capon et al. (8), but the name IFN-ω(9, 11) is now preferred to distinguish it more clearly from IFN-α. IFN-ω genes may be absent in some species, including the dog (13) and perhaps mouse (10, 14).

IFN-α is a cytokine and possesses potent antiviral and antiproliferative bioactivities (10, 14). Under normal circumstances IFN-α genes are constitutively expressed only at very low levels (15, 16). They are, however, inducible by viral infection coordinately with the structurally related IFN-β, and expression appears to occur in various cell types and tissues (14). The primary mechanisms mediating this induction are transcriptional. All IFN-α promoters contain several repeated motifs of the sequence GAAANN (in which N is any nucleotide), and hexanucleotide repeats of the sequence GAAANN (in which NN is TG, GT, GC, CT, or CC) confer viral responsiveness to heterologous promoters (17–19). Furthermore, a minimal 117-base-pair (bp) region upstream of the transcription start site, including five repeats of the sequence GAAANN, is sufficient to mediate virus inducibility in a human IFN-α1 gene (20). IFN-ω genes are assumed to be regulated similarly to the IFN-α because of conservation within the promoter sequences for these two IFNs (8).

Expression of TP-1 mRNA appears limited to cells of the trophectoderm between day 11 and day 22 in sheep (21, 22) and day 12 through at least 25 in cattle (23) and probably occurs constitutively in the absence of viral infection. However, the mechanisms whereby the trophoblast IFN genes are regulated are unknown. The present studies were initiated to define whether genes for TP-1 share regulatory mechanisms in common with the other type I IFNs. Specifically, the objectives were to determine (i) if trophoblast IFN (TP-1) genes are virus inducible, (ii) if IFN-α and -ω genes are coexpressed with TP-1 in the trophoblast, and (iii) if TP-1 expression is restricted to trophoblast cells.

MATERIALS AND METHODS

Tissue and Cell Collection. Day 16, 18, and 19 bovine conceptuses were recovered within 30 min after slaughter by flushing the uterus with Dulbecco’s phosphate-buffered saline. Conceptuses used for collection of RNA were processed individually within 1 h of recovery. Those used for IFN production were placed into serum-free minimum essential medium containing glucose and insulin (24) and incubated at 37°C for 3 h in 5% CO2/95% air, after which time the medium was centrifuged (1000 × g, 10 min) and stored at 4°C until assay.

Leukocytes were isolated from jugular venous blood (50 ml) by collection of theuffy coat and repeated lysis of erythrocytes in 0.83% ammonium chloride. Leukocytes were diluted to $3 \times 10^6$ cells per ml in either RPMI medium containing 10% (vol/vol) fetal bovine serum and 40 µg of

Abbreviations: IFN, interferon; TP-1, trophoblast protein 1; RTPCR, reverse transcription-polymerase chain reaction; hGH, human growth hormone; IRF, IFN regulatory factor; CHO, Chinese hamster ovary.

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gentamycin sulfate per ml (control) or the same medium containing 100 hemagglutination units of Sendai virus per ml (virus control). Leukocyte cultures (17 ml) were incubated at 37°C, 5% CO₂/95% air for 8 h, after which time the medium was processed as described for conceptsus cultures and the cells harvested for RNA.

RNA Preparation and Northern Analysis. Total cellular RNA from bovine conceptuses, control and virus-induced leukocytes, spleen, skeletal muscle, kidney, and liver was prepared by a standard procedure (25). Poly(A)+ RNA from late gestation bovine placenta was purified (26), analyzed on formaldehyde gels, and blotted onto nylon (Biotrans, ICN) membranes (26). Blots were hybridized with a full-length cDNA for bovine TP-1 (bTP-509; ref. 6) and washed by using conditions similar to those for the Southern blots.

Antiviral and Immunoneutralization Antiviral Assays. Media were assayed by using a cytopathic effect reduction assay employing GBK-2 cells infected with vesicular stomatitis virus (3, 27). IFN titers were expressed in units of activity, in which 1 unit was equivalent to that amount of IFN that protected 50% of the GBK-2 cell monolayer from lysis. The assay was validated with the human leukocyte/Sendai IFN-α WHO International Reference Standard (no. Ga 23 902-530) (1 unit = 0.09 international reference unit).

Immunoneutralization assays were performed with polyclonal antisera raised against recombinant bovine IFN-α1 and bovine TP-1 (28). Briefly, dilutions of IFN were preincubated with anti-IFN antisera in 96-well assay plates for 45–60 min prior to the addition of the GBK-2 cells. Immunoneutralization results were expressed as the percentage of residual IFN titer compared with untreated controls.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) of IFN mRNA. Total RNA from bovine conceptuses and leukocytes was analyzed for subtype-specific IFN mRNAs by RT-PCR (29). Briefly, RNA (100 ng) was treated with RNase-free DNase (26), reverse transcribed from a dT15 primer by using avian myeloblastosis virus reverse transcriptase, and then PCR amplified for 60 cycles (94°C, 1 min; 50°C, 1 min; 72°C, 1 min). PCR employed a "3'-sense" primer that was IFN-specific and a "3'-antisense" oligo(dT)15 primer. Since all known type I IFN genes are intronless, it is normally impossible to distinguish PCR products of cDNA and genomic DNA. The use of oligo(dT) as a 3'-antisense primer ensured that the PCR product was the result only of reverse-transcribed poly(A)+ RNA. Three 5'-sense IFN-specific primers used for PCR were made against specific regions of bovine IFN-α1, IFN-α1-8 (8), and TP-1 (bTP-509; ref. 6) 5'-α1 = GCCTGGAGGTTTGCAGACA; 5'-α8 = AGACCTGAAATCACCCTGACATGA; 5'-TP1 = AGACCTTGGTTCTTCTCAG).

PCR products were separated in agarose gels and blotted onto nylon membranes. Since IFN-α subtypes are so highly related none of the 5' PCR primers was expected to be completely specific. Therefore, products of the three IFN-α subtypes were distinguished by employing cDNA probes for IFN-α, IFN-ω, and TP-1 under conditions of high stringency. The probes used were a full-length human IFN-α (30), a 277-bp PCR product representing the 3' noncoding region of bovine IFN-α1 (8), and a 266-bp Bgl II to Sp8 1 fragment representing the 3' noncoding region of ovine TP-1 (oTP-266; refs. 22 and 23). All probes were random prime labeled with [α-32P]dATP (kit from Boehringer Mannheim) and utilized in buffer containing 50% (vol/vol) formamide and 5× sodium citrate/sodium chloride (SSC; ref. 26) at 42°C. Membranes were washed (0.1x SSC at 42–50°C) under conditions in which the hybridization to each IFN subtype did not hybridize to the other subtypes (data not shown).

Transfection of IFN and Reporter Genes. Chinese hamster ovary (CHO), L929, and JAR cells were transfected (31) with 10 μg of pUC19 plasmids bearing the 2.5-kilobase (kb) EcoRI fragment of the bovine IFN-ω1 gene (BoIFN-ω1; ref. 8) or a 2-kb HindIII-Sac I fragment of a bovine TP-1 gene (5). Cotransfection or parallel transfection of the pXGHS plasmid (Nichols Institute, San Capistrano, CA) containing the human growth hormone (hGH) gene under the control of the mouse metallothionein promoter was used to assess transfection efficiency. After addition of the precipitates to cells in 100-mm dishes, cultures were incubated for 12–16 h, washed, and fed 10 ml of fresh medium. Medium was harvested 24 h later and assayed for antiviral activity. Parallel dishes of mock-transfected JAR cells were incubated with 5 hemagglutination units of Newcastle disease virus per ml for 8 h to induce IFN production.

CHO, GBK-2, and JAR cells were transfected with 10 μg of p0GH and equivalent molar amounts of hGH reporter gene constructs as described above. Medium from transfected cells was collected after 24 h and assayed for hGH by means of a specific radioimmunoassay (Nichols Institute).

Plasmids. A reporter construct of the bovine IFN-α1 promoter was prepared by ligating a 1.8-kb, Sp8 I to Nco I fragment of the bovine TP-1 gene 5' region (positions −1800 to +65) that had been blunt ended with mung bean nuclease (26) into the Xba I site of the plasmid p0GH (Nichols Institute). Expression from this plasmid, designated pBTP-1.8GH, was compared with the promoterless p0GH and pXGHS. The deletion mutant plasmid pBTP-450GH was prepared by releasing a HindIII fragment from pBTP-1.8GH, thereby truncating the promoter at position −450. The plasmid pBTP-126GH was prepared by PCR to amplify the promoter from positions −126 to +50. This fragment was subsequently cloned into the Xba I site of p0GH. The fidelity of the PCR was assessed by DNA sequencing. Plasmids for transfection were purified on cesium chloride density gradients (26).

RESULTS

Antiviral Activity Produced by Bovine Conceptuses and Leukocytes. The amount of antiviral activity released by day 18 bovine conceptuses compared with virus-induced leukocytes is shown in Fig. 1A. The modest amount of activity present in culture medium of virus-induced leukocytes is within the range reported by others for small-scale culture of bovine cells (32, 33), although much larger cultures and "priming" may improve yields (34). Conceptuses produced ≈300 times more IFN than leukocytes (up to 105 units per conceptus per hour), when expressed on an equivalent basis. Production of TP-1 is maximal around day 18 in cattle (23, 35), at which time TP-1 is the predominant protein secretory product of the conceptus (35). Approximately 75% of the antiviral activity produced by leukocytes was neutralized by an antisera to recombinant bovine IFN-α1, whereas anti-bTP-1 antisera was without effect (Fig. 1B). In contrast, >99% of the antiviral activity produced by bovine conceptuses was neutralized by the anti-bTP-1 antisera, whereas anti-IFN-α was without effect. These data demonstrate that conceptuses produce significantly more IFN than leukocytes and that the two cell types differ in the predominant subtype of IFN secreted.

RT-PCR of Conceptus and Leukocyte RNA. The immunoneutralization results indicated that leukocytes produced predominantly IFN-α. Presumably the bulk of the residual leukocyte antiviral activity was due to IFN-ω, which represents at least 10–15% of the total IFN produced by human leukocytes (11, 36). To identify and distinguish between the highly related IFN-α, IFN-ω, and TP-1 mRNA, specific oligonucleotide primers were constructed for RT-PCR amplification. PCR products were identified by means of Southern blot hybridization with cDNA probes able to distinguish between the different subtypes. Although leukocytes constitutively contained mRNA for IFN-α, mRNA for IFN-α and
-ω were enhanced following virus induction (Fig. 2). Bovine TP-1 mRNA was also virus-inducible in these cells but the appearance of the signal only became apparent on Southern blots upon prolonged autoradiographic exposures. In contrast, bovine conceptuses expressed much higher amounts of TP-1 mRNA. The presence of at least two hybridizing bands relates to the existence of multiple poly(A) signals in the genes for bovine IFN-α, IFN-ω, and TP-1 (5, 7, 8) and the use of the oligo(dT)$_{15}$ primer during PCR.

IFN-α and -ω subtype mRNAs were expressed in conceptuses in amounts roughly equivalent to those found in virus-induced leukocytes (Fig. 2). Coexpression of these IFN subtypes with TP-1 occurred in all conceptuses examined.

**Tissue Distribution of TP-1 Transcripts.** To determine if “uninduced” expression of TP-1 is restricted to cells of the early conceptus, RNA from several tissues was analyzed by Northern blotting (Fig. 3). Bovine TP-1 mRNA was abundant in RNA from day 18 conceptuses but was absent in placental tissue (cotyledon) from late gestation and several adult tissues (spleen, liver, muscle, and kidney). Within the conceptus itself, TP-1 mRNA is localized and restricted to trophodermal cells in blastocyst stage embryos (22, 23).

**Trophoblast Cell-Restricted Expression of a Transfected TP-1 Gene.** Neither the TP-1 nor IFN-ω gene was expressed when transfected into nontrophoblast cells such as mouse L929 and hamster CHO (data not shown). In contrast, the TP-1 but not the IFN-ω gene was expressed when it was introduced into JAR cells, a human trophoblastic tumor (choriocarcinoma) line, as assessed by assay of antiviral activity in the culture medium (Table 1). Mock-transfected JAR cells do not normally secrete antiviral activity unless they are induced—i.e., by Newcastle disease virus (Table 1). The antiviral activity produced by JAR cells transfected with the TP-1 gene was completely neutralized by anti-bTP-1 antiserum.

**Fig. 1.** Antiviral activity present in pools of culture medium of Sendai virus-induced leukocytes (VIL CM) and day 18 bovine conceptuses (BC CM; $n = 7$). Total activity (A) was measured based on culture of leukocytes for 8 h after viral induction and of conceptuses for 3 h. To compare the relative amount of IFN production values are expressed in log$_{10}$ units of antiviral activity per milligram of RNA isolated for each mass of tissue or cells cultured. Immunoneutralization of antiviral activity was used to assess the relative contribution of bovine IFN-α (IFN-α1) and bovine TP-1 (bTP) to the total activity (B). Results are presented as the amount of residual antiviral activity remaining after preincubation of the sample with normal rabbit serum (NRS) or with antiser against recombinant bovine IFN-α1 or recombinant bovine TP-1.

**Fig. 2.** RT-PCR analysis of RNA extracted from bovine conceptuses (BC) or from control (CL) and Sendai virus-induced (VIL) leukocytes. PCR products were identified on Southern blots that were hybridized with probes for IFN-α, IFN-ω, or TP-1. (Upper) Analysis of a single day 19 conceptus for all IFN subtypes. (Lower) Analysis of two day 16 and three day 18 conceptuses for IFN-ω and TP-1 only. The lanes for bovine TP-1 were overexposed to reveal the faint bands in the VIL lanes.

**Fig. 3.** Northern blot analysis of tissues for TP-1 mRNA. Total RNA was loaded except where indicated. Lanes from left to right: (i) day 18 bovine conceptus (2 μg), (ii) late gestation bovine cotyledon [1 μg of poly(A)$^+$], (iii) day 100 ovine cotyledon (20 μg), (iv) bovine spleen (20 μg), (v) bovine liver (20 μg), (vi) bovine skeletal muscle (20 μg), (vii) bovine kidney (20 μg). The autoradiograph was exposed for 6 days but bands at the expected size of 1.1 kb were present only in the conceptus lane.

Table 1. Production of antiviral activity by JAR cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antiviral activity, units per dish</th>
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<tbody>
<tr>
<td>Transfection</td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td>0</td>
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<tr>
<td>Bovine IFN-ω1</td>
<td>0</td>
</tr>
<tr>
<td>Bovine TP-1</td>
<td>300</td>
</tr>
<tr>
<td>NDV induction</td>
<td>1800</td>
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Cells were transfected with plasmids bearing either the IFN-ω1 (EcoRI fragment of BoIFN-ω1; ref. 8) or TP-1 (HindIII–Sac I fragment; ref. 5) gene or mock-transfected and induced with Newcastle disease virus (NDV). Medium was collected 24 h after recovery from transfection or 8 h after NDV induction. Results reflect the mean secretion of antiviral activity by duplicate plates of transfected cells.

The TP-1 5′ Promoter Region Is Sufficient for Trophoblast-Specific Expression. To determine whether the 5′ flanking region was sufficient for the expression of the bovine TP-1 gene in trophoblast cells, a 1.8-kb fragment upstream of the translation start site was inserted into a promoterless hGH expression plasmid (pOGH). The activity of the resulting expression plasmid, pBTP-1.8GH, was compared in transient assays with that of pOGH and a plasmid bearing the murine metallothionein promoter (pXGH5). The bovine TP-1 promoter was active in JAR cells but was relatively inactive in non trophoblast cells (CHO and GBK-2) compared with the positive (pXGH5) and negative (pOGH) control plasmids (Fig. 4).

Culture medium from JAR cells transfected with pBTP-1.8GH contained 20–30 times more hGH than the promoterless controls. Truncation of the promoter to position −450 (pBTP-450GH) did not affect promoter activity, whereas deletion to position −126 decreased expression 4- to 5-fold (Table 2). Together these data indicate that the 5′ promoter region is sufficient to direct trophoblast-specific expression of the bovine TP-1 gene and that sequences 5′ to position −126 in the promoter may possess enhancer activity.

DISCUSSION

The data presented here imply that the genes for bovine TP-1 share certain common regulatory mechanisms with other type I IFNs. Although it seems unlikely that viruses resident within the female reproductive tract represent the normal inducer for TP-1 synthesis in the cow, bovine TP-1 mRNA synthesis is nonetheless virus-inducible in leukocytes. That TP-1 genes may be induced by viruses is supported by the observation that expression of TP-1 mRNA is increased 2.5-fold in day 11 ovine conceptuses after exposure to the synthetic double-stranded RNA, poly(I)-poly(C) (37). However, this induced level of expression does not approach that which is observed 2–3 days later when ovine TP-1 mRNA levels are maximal (21–23).

Coexpression of several type I IFN subtype mRNAs in conceptuses likely indicates that a common inducer and regulatory pathway interact with the genes for the IFN-α, IFN-ω, and TP-1 and that this pathway is active within trophoblast cells during early pregnancy. Potential nonviral IFN inducers include certain growth factors and cytokines (14). For example, colony-stimulating factor 1 may induce IFN-α synthesis in monocytes (38) and is also known to be produced within the murine uterus during early gestation (39).

The expression of IFN-α and -ω RNA is controlled largely at the level of transcription, and so it is likely that common transcription factors mediate the coexpression of all type I IFN genes. IFN-α promoters contain multiple IFN regulatory factor 1 (IRF-1) sites (40) and a novel viral responsive enhancer element of sequence GAAATG that binds a factor (TG protein) distinct from IRF-1 (19). TG protein binding sites are common to human (8, 19) and bovine (5, 8) IFN-α and -ω genes. TP-1 genes, however, lack a TG-like enhancer, but they do contain multiple potential IRF binding sites (5, 7). Although overexpression of IRF-1 is sufficient to induce modest expression from native IFN-α, -ω, and -β promoters (41), factors that induce IRF-1 transcription do not always result in associated transcription of IFN genes (42). It seems likely that activation of IFN promoters is achieved through an interaction of multiple transcription factors with their DNA recognition sequences (42–45). Therefore, the bovine TP-1 promoter, which contains only IRF-like enhancer elements, may be poorly activated in response to viral induction in the absence of other functional enhancer elements.

Although TP-1 is minimally virus-inducible and expressed in leukocytes, immunoneutralization assays clearly showed that it represented a negligible fraction of the total IFN produced. In contrast, TP-1 was the predominant type I IFN of day 18 bovine conceptuses, where its mRNA is localized to the trophoderm and absent in extraembryonic endoderm, yolk sac, and embryo proper (22). In addition, a transfected TP-1 gene was expressed in uninduced cells of trophoblast origin (JAR cells) but not in nontrophoblast cells. Together these data suggest that, unlike the related IFN-α genes, which are expressed by various cell types (14–16, 46), TP-1 IFN gene expression is largely restricted to cells of trophoblastic origin and probably dependent upon factors unique to such cells that bind to sequences within the 5′ flanking region of the TP-1 gene, including regions distal to −126. Importantly, expression of the TP-1 gene occurred under conditions in which JAR cells did not express endogenous IFN genes or transfected bovine IFN-ω gene. Differential expression of IFN species has been reported by others (46). In that study, various cell types produced different relative amounts of IFN-α, -β, and -γ and various -α subtypes in response to IFN inducers. However, the dramatic cell

Table 2. Expression from TP-1 promoter deletion constructs

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>GH secretion,* ng/24 h</th>
<th>Relative expression</th>
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<tbody>
<tr>
<td>pOGH</td>
<td>7.8 ± 3.6</td>
<td>1.0</td>
</tr>
<tr>
<td>pBTP-1.8GH</td>
<td>201.3 ± 23.4</td>
<td>25.8</td>
</tr>
<tr>
<td>pBTP-450GH</td>
<td>166.0 ± 9.2</td>
<td>21.3</td>
</tr>
<tr>
<td>pBTP-126GH</td>
<td>35.0 ± 7.0</td>
<td>4.5</td>
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*Mean ± SEM of duplicate plates.
specificity of TP-1 expression observed in the present work seems unique to the trophoblast IFN.

Unfortunately there are at present no well-characterized bovine trophoblast cell lines available for study. When bovine trophoblast cells are placed into primary culture they lose the ability to produce TP-1, and transfected TP-1 promoters are inactive in such cultured cells (unpublished observations).

The discovery that the bovine TP-1 promoter was active in JAR cells was surprising since these cells do not normally produce IFN unless induced by virus. IFN production by human placental tissues has been widely reported (1), and suggestions from a human placental-specific IFN have been made (24-45). Antiviral activity suggestive of IFN is associated with the conceptus and placenta in mice (50, 51), hamsters (52), and pigs (27). However, the overall amounts of IFN produced in these species are far lower than TP-1 production by early ruminant conceptuses, and we have been unable to detect TP-1-related mRNA in human, mouse, and pig conceptus tissues (ref. 51; C. E. Farin and R.M.R., unpublished observations). Moreover, TP-1-related genes may be restricted to ruminant ungulate species (53) even though the enhancer elements that mediate trophoblast-specific expression of bovine TP-1 presumably bind factors common to trophoblast cells across species. Deletion of the bovine TP-1 promoter from position −450 to −126 revealed that this intervening region is important for expression of the TP-1 gene in JAR cells, but we find no striking sequence similarity of this region with promoters of genes normally expressed in placenta such as chorionic gonadotropin subunits or placental lactogens. The fact that sequences distal to position −126 in the TP-1 genes possess enhancer function, at least in JAR cells, is also in contrast to other type 1 IFN genes where sequences distal to −126 appear dispensable for virus-inducible expression (20, 54).

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