Multiple Regulatory Elements Are Required to Direct Trophoblast Interferon Gene Expression in Choriocarcinoma Cells and Trophectoderm

Leaman, Douglas W.; Cross, James C.; Roberts, Michael R.

The Endocrine Society

http://hdl.handle.net/1880/48719
journal article

Downloaded from PRISM: https://prism.ucalgary.ca
Multiple Regulatory Elements Are Required to Direct Trophoblast Interferon Gene Expression in Choriocarcinoma Cells and Trophectoderm

Douglas W. Leaman*, James C. Cross†, and R. Michael Roberts

Departments of Animal Sciences and Biochemistry
University of Missouri
Columbia, Missouri 65211

Interferon-tau (IFNτ) is produced exclusively by the trophectoderm during the preimplantation stage of pregnancy in ruminant ungulate species. Human choriocarcinoma cells (Jar) stably transfected with 1.8 kilobases of promoter from a bovine IFNτ gene ahead of a human GH (hGH) reporter gene constitutively synthesize hGH, but expression is not increased further by exposure to Newcastle disease virus. This and earlier experiments suggest that the transcriptional cues regulating IFNτ expression are distinct from those operating on other type I IFN genes. Transient transfection experiments reveal that two distinct promoter regions are required for full constitutive expression: one proximal (to position −126), which directs basal expression, and a more distal promoter region (positions −280 to −400), which acts as an enhancer. Nuclear extracts prepared from ovine conceptuses during the period of IFNτ expression interact with the proximal promoter region (positions −34 to −126) to form several complexes of high electrophoretic mobility. Although nucleotide sequence motifs potentially capable of binding the transcription factor IRF-1 are present in this region, IRF-1 does not transactivate the IFNτ gene. The distal part of the promoter contains only one region (−322 to −358) that forms a complex with these conceptus nuclear extracts. Both proximal and distal gel shift patterns become dramatically different when IFNτ gene expression ceases, perhaps reflecting the appearance of transcriptional repressors. Together these experiments support the conclusion that the control of IFNτ gene expression is very different from that of other type I IFN genes and that trophoblast-specific expression depends upon distal as well as proximal promoter regulatory elements. (Molecular Endocrinology 8: 456-468, 1994)

INTRODUCTION

Interferon-tau (IFNτ), known previously as ovine and bovine trophoblast protein-1 (TP-1), is a type I IFN, related to but serologically distinct from the IFNω subtype (1), that is secreted in large quantities by cells of the trophectoderm. It has been implicated in the process of maternal recognition of pregnancy in ruminants (1, 2), where it acts on the uterus to suppress the pulsatile production of the luteolytic prostaglandin F₂, thereby allowing the corpus luteum of pregnancy to persist. IFNτ expression can first be detected around the time of blastocyst hatching (3), but becomes high only as the blastocyst expands from a spherical form to an elongated thread-like structure (around days 12-14 of pregnancy in sheep and days 15-17 in cattle) (4, 5). IFNτ expression declines markedly as implantation begins, and by day 22 of pregnancy in the sheep, when most of the trophoblast has become fully attached, expression is virtually undetectable (4-8).

Genes for IFNα, −β, and −ω are normally quiescent, but are rapidly induced in many cell types by exposure to virus (9). Only about 120 bases beyond the site of transcriptional initiation appear to be required for full virus induction of their genes (10-12). A variety of transcription factors have been implicated in directing both the induction and silencing of expression. These include the IFN regulatory factors (IRF), IRF-1 and IRF-2 (13-15), nuclear factor xB (NF-xB) (16), activating transcription factor (ATF)/cAMP response element-binding protein, and Oct-like factors (IFNβ only) (17) as well as several additional, less thoroughly characterized regulatory factors (12, 17, 18). The induction of expression depends upon many minienhancer sites that provide a flexible and graded response to virus and various modulating stimuli (17). To date, however, the precise roles of individual transcription factors in the regulation of IFNα or -β expression are uncertain. Control of the IFNω genes, which are most closely related structurally to the genes for IFNτ, remains even more enigmatic.

Genes for IFNτ have been cloned from several related...
Transcriptional Control of IFN-\(\gamma\) Expression

ngulate ruminant species, including sheep (19, 20), cattle (21, 22), goat (20), musk ox (20), and giraffe (Liu, D. W. Leaman, and R. M. Roberts, unpublished results). The promoter regions of these genes are highly conserved, even between species, through at least the first 400 bases up-stream of the transcription start site. Although the IFN-\(\gamma\) genes bear some sequence similarity to other type I IFN within the proximal regions of the promoters, most of the general features that apply to the transcriptional regulation of other type I IFN do not pertain to the IFN-\(\gamma\), which are exclusively expressed in cells of trophoblast origin. They are only poorly inducible by double stranded RNA in conceptuses (23) and by virus in leukocytes (24), and unlike other IFN, high level expression is sustained for a period of several days rather than just a few hours (6, 8). Thus, it is likely that expression of the IFN-\(\gamma\) is regulated in a manner distinct from that of the other type I IFN.

Study of transcriptional regulation of IFN-\(\gamma\) has been hampered because there are currently no well defined trophoblast cell lines available from cattle or sheep. Previous work had demonstrated that human choriocarcinoma cells, e.g. Jar, were competent to support constitutive IFN-\(\gamma\) promoter activity (24), despite the apparent absence of IFN-\(\gamma\) genes in humans (20). Although expression by these cells appears to be significantly lower than that observed in the developing ovine or bovine conceptus, they have provided a means to investigate the trophoblast-specific regulation of the IFN-\(\gamma\) genes. In the present study we have sought to define which regions of the gene promoter are involved in tissue-specific regulation of the IFN-\(\gamma\) in transfected human choriocarcinoma cells. We have also extended these studies to determine whether similar sequences might play a role in IFN-\(\gamma\) expression in the developing conceptus.

RESULTS

Control of Constitutive IFN-\(\gamma\) Expression in Jar Cells

The initial onset of IFN-\(\gamma\) expression appears to be a genetically programmed event that coincides temporally with the formation of a functional trophoderm (1, 3), if it is unclear whether maternally derived factors are required to enhance IFN-\(\gamma\) expression once it has been initiated. Therefore, a pBTP-1.8GH reporter plasmid (containing 1.8 kilobases of up-stream promoter sequence) was stably introduced into Jar cells, so that a number of factors known to work through second messenger systems or to stimulate type I IFN gene expression in other systems could be tested directly on \(\gamma\) expression. One selected clone (-1.8cl6) constitutively expressed human GH (hGH), but none of the treatments had a major influence on IFN-\(\gamma\) promoter activity (Table 1). Significantly, exposure to Newcastle disease virus had no effect, although endogenous IFN-\(\gamma\) were induced in these same cells, as assessed by increased antiviral activity released into the culture me-

\begin{table}[h]
\centering
\caption{Induction of IFN-\(\gamma\) Promoter Activity in Stably Transfected 1.8cl6 Cells}
\begin{tabular}{ll}
\hline
\textbf{Treatment} & \textbf{Response} \\
\hline
CAMP (8-Bromo-cAMP) & None \\
TPA (Phorbolester) & None \\
Newcastle Disease Virus & Slight decrease \\
bolIFN-\(\gamma\)1 & Slight increase \\
Retinoic Acid & None \\
\hline
\end{tabular}
\end{table}

Concentrations of reagents and conditions for treatment are described in Materials and Methods.

dium (300 U/dish; data not shown). This observation is consistent with earlier findings which suggested that IFN-\(\gamma\) was only marginally virus inducible in leukocytes (24) and indicates that IFN-\(\gamma\) may be one of the few IFN subtypes that is not virally responsive.

IFN-\(\gamma\) Promoter Regions Required for Transcription in Jar Cells

In earlier work we showed that regions of the bovine IFN-\(\gamma\) promoter that supported constitutive transcription in Jar cells were within 450 basepairs (bp) of the transcription start site and that deletion to position 126 significantly reduced promoter activity (24). To identify regions between 126 and 450 with enhancer activity, additional 5'-truncations of the 450-bp promoter segment were constructed. Deletion of the 450-bp bovine (b) IFN-\(\gamma\) promoter (pBTP-450GH) to -400 (pBTP-400GH) did not affect promoter activity, whereas deletion to -280 (pBTP-280GH) decreased promoter activity to a level comparable to the -126 promoter plasmid (pBTP-126GH; Fig. 1). These results implicated promoter sequences between -280 and -400 as possessing enhancer-like activity. To test this assumption, sequences between -126 and -230 were deleted from the pBTP-400GH plasmid. The resulting construct (pBTP-400.14104GH) retained full transcriptional activity (Fig. 1).

As an additional test of enhancer activity, sequences between -130 and -400 were placed up-stream of the -126 promoter in an antisense orientation (plasmid pBTP130/400ASGH). The transcriptional activity of this construct was indistinguishable from that of plasmid pBTP-400GH. Furthermore, when IFN-\(\gamma\) promoter sequences from -130 to -400 were fused to a -100 ovine (o) IFN-\(\omega\) gene promoter, the resulting promoter construct (pBTP/Om-100GH) displayed approximately 2-fold higher expression than the parental plasmid pOm-100GH in Jar cells (Fig. 1). This enhancement, presumably provided by the bIFN-\(\gamma\) sequences, was comparable to the enhancement conferred to the -126 bIFN-\(\gamma\) promoter by distal bIFN-\(\gamma\) promoter sequences (Fig. 1). In contrast, when distal IFN-\(\omega\) sequences were placed ahead of the minimal IFN-\(\omega\) promoter, transcriptional activity was reduced to the background levels observed with the promoterless hGH reporter construct (pOm-450GH). These results suggested that the IFN-\(\gamma\) gene promoter region between positions -130 and
Fig. 1. Activity of blFN-r Promoter Deletion Constructs in Jar Cells

Jar cells were transfected with equimolar amounts of blFNr promoter/hGH plasmids. Human GH levels in culture medium 24 h after transfection were determined by immunoassay. hGH values are the mean ± SEM of three to five independent experiments; each treatment was performed in duplicate for all experiments, and results are expressed as the amount of hGH measured relative to that obtained with the pBTP-450GH construct. A map showing the lengths of the respective promoter fragments is shown at the left. □, blFNr promoter sequences; ■, IFNω promoter sequences. Expression values that differ significantly (P < 0.05) are identified with different letters.

-400 (and more specifically, -280 to -400; see above) acted as a classical enhancer.

Characterization of Nuclear Factor Binding to the IFNr Promoter

To identify factors that bind to the proximal and distal promoter regions, overlapping blFNr gene promoter fragments were isolated for use as probes in gel mobility shift assays. Although Jar (Fig. 1) and JEG-3 cells (data not shown) were both permissive for IFNr gene expression, the predominance of nonexpressing cells in these cultures probably limited their usefulness as a source of nuclear extracts for gel shift experiments (see below). Therefore, either day 13/14 or day 15 ovine conceptus nuclear extracts were employed in gel mobility shift experiments, because this stage of development represents the peak in IFNr gene expression (1, 4). As summarized in Fig. 2, only two regions within the first 430 bp beyond the transcription start site were able to bind consensus nuclear factors. The first was localized between positions -34 and -126, and the second was between positions -280 and -430 (Fig. 2). Significantly, these were the same regions implicated in regulating IFNr transcriptional activity in transiently transfected Jar cells (Fig. 1).

Examination of the blFNr promoter sequence for consensus transcription factor-binding sites revealed that the proximal promoter region contained sequence motifs resembling the binding sites for IRF-1 and IRF-2.
region that, when deleted, had no effect on promoter activity in conceptuses (Fig. 1). Because the ovine and bovine gene sequences are virtually identical in these regions up-stream of the cap site (29), results obtained with bovine constructs in combination with ovine nuclear extracts are considered appropriate.

Identification of Factors Binding to the Proximal Promoter

A proximal promoter sequence probe (positions −34/−126) formed at least two high mobility complexes with day 13/14 conceptus extracts in gel shift experiments (Fig. 3A). These complexes were readily displaced by a 100-fold molar excess of unlabeled competitor DNA from the proximal promoter region, but not by a 100-fold molar excess of nonspecific DNA derived from distal IFN-α promoter sequences (−280/−430). When competitor DNA derived from the −100/45 region of the related oIFNω gene was used, only slight apparent displacement of label from the shifted bands was observed, even though the IFN-α and IFNω promoters share over 85% sequence identity within this region (20). Indeed, when this proximal IFNω promoter region was used as a probe in gel shift studies with ovine conceptus nuclear extracts, no specific binding was observed (data not shown). When the −34/−126 promoter IFNω probe was tested with day 21 ovine conceptus or Cos-1 cell nuclear extracts, the shifted pattern became considerably more complex, with several lower mobility complexes becoming evident (Fig. 3B). Complementary oligonucleotides representing IFNω-promoter sequences between −69 and −91 were then tested with day 15 nuclear extracts (Fig. 3C). At least two high mobility DNA-protein complexes similar to those observed with the −34/−126 probe were obtained, suggesting that this short stretch of the proximal promoter, whose sequence is shown in Fig. 3D, accounted for part but not all of the binding activity associated with the longer probe.

IRF-1 and IRF-2 Do Not Bind or Regulate the Proximal Promoter in Early Conceptuses

Among the factors implicated in the regulation of type I IFN gene expression are the IFN regulatory factors, IRF-1 (transcriptional activator) and IRF-2 (repressor) (14, 15). Included within the −34/−126 promoter region are motifs that resemble IRF-binding sites (1, 22) (Fig. 3D). IRF-1 and IRF-2 were, therefore, tested for their abilities to bind to an IFN-α promoter. Recombinant IRF-1 was able to associate with an IRF-binding tetrahexamer sequence ([AAGTGA]4; Fig. 4A) and also bind to the proximal region of the IFN-α promoter (Fig. 4B). These complexes were specific in that they were competed for by the tetrahexamer, but not by oligonucleotides containing unrelated sequences. In a separate experiment, IRF-1 and IRF-2 were synthesized by in vitro translation and tested for binding to the tetrahexamer probe (Fig. 4, C and D). In this system, the probe formed a complex with some unknown component in the reticulocyte lysates (all lanes). However, IRF-1- and IRF-2-binding activities were clearly evident in reticulocyte lysates that had been programmed with the appropriate IRF-1 or IRF-2 mRNA. IRF-1 binding to the probe was inhibited by both IFN-α and IFNω promoters, whereas IRF-2 binding was not (Fig. 4D). These experiments clearly showed that the proximal promoter region of the IFN-α gene was capable of binding IRF-1.

To determine whether IRF-1 was capable of transactivating a bovine IFNω promoter, murine IRF-1 was expressed in transfected cells. In cotransfection experiments in Jar and Chinese hamster ovary (CHO) cells, the activity of the pBTP-1.8GH plasmid was not stimulated by IRF-1 (Table 2). In fact, basal expression was reduced by 100-fold in CHO and 70% in Jar cells. In contrast, a bovine IFNω promoter was stimulated in both cell lines (data not shown). To eliminate the possibility that repressor elements up-stream of the proximal promoter might have inhibited the ability of IRF-1 to activate transcription, the cotransfection experiments were repeated with the pBTP-126GH plasmid. IRF-1 expression produced only a modest (<2-fold) stimulation of transcription from this truncated promoter in CHO cells and had no effect on expression in Jar cells (data not shown). These data indicate that although IRF-1 can bind the IFNω promoter, it cannot activate transcription. This situation contrasts with other type I IFN promoters, which are strongly transactivated by IRF-1 (14).

Northern analysis of bovine conceptus RNA and screening day 18 bovine conceptus and day 13 ovine conceptus cDNA libraries with a murine IRF-1 probe failed to detect IRF-1 transcripts (data not shown). To pursue the issue, primers based on conserved regions of mouse and human IRF-1 were used to amplify reverse transcribed RNA from bovine leukocytes and day 19 conceptuses by the polymerase chain reaction (PCR) (24). The mRNA for IRF-1 was virus inducible in leukocytes, but was expressed in barely measurable amounts in conceptuses, where the transcript could only be detected by hybridization to an IRF-1 probe and long term exposure of the gel to x-ray film (Fig. 5). The amplified IRF-1 transcript from leukocytes was sequenced and showed 93% identity to the human IRF-1 transcript (30) over the short region (406 bases) examined (data not shown).

As a final experiment to assess the presence of IRF-1 in conceptuses, gel mobility shift assays were performed with the tetrahexamer probe and nuclear extracts from bovine and ovine conceptuses. Complexes were formed, but were unaffected by inclusion of antiserum to IRF-1 or IRF-2 in the binding reaction (data not shown). Together, these data suggest that IRF-1 and IRF-2 are not expressed to any significant extent in bovine and ovine conceptuses at the time of maximal IFN-α expression and are not, therefore, likely to be among the factors involved in binding to the proximal IFN-α promoter region.
**Localization of Distal Binding Sequences**

The binding of nuclear proteins to the distal IFN-τ promoter region was examined in detail. A probe, representing positions -280 to -430, yielded a single complex in gel shift experiments with day 13/14 ovine conceptus nuclear extracts (data not shown). This distal complex was readily displaced by a 100-fold molar excess of unlabeled probe DNA, but not by competitor DNA derived from IFN-τ promoter sequences between positions -34 and -126. Day 18 bovine and day 14 or 15 ovine conceptus extracts yielded a complex with identical mobility (Fig. 6). However, when extracts from day 21 ovine conceptuses were tested, additional complexes of much slower electrophoretic mobility were observed. A similar binding pattern was obtained with Cos-1 nuclear extracts (Fig. 6). Whether the additional complexes of similar mobility in the poorly expressing Cos-1 cell or day 21 conceptus extracts represented the binding of transcriptional repressors remains to be determined. Surprisingly, similar gel shift patterns were also obtained with Jar cell extracts (data not shown). A 500-fold molar excess of specific competitor DNA was required to displace the complexes obtained with these day 21 extracts (Fig. 6), whereas the single complex obtained with the day 13/14 conceptus extracts could be dissociated by as little as a 10-fold molar excess (data not shown).

Promoter sequences between positions -430 and -280 were subjected to bidirectional deletional analysis to localize the sequences involved in distal complex formation. Through this process, sequences between positions -322 and -358 were implicated in nuclear factor binding (Fig. 7A). Complementary oligonucleotides representing the sequence from -322 to -358 yielded a single complex similar in mobility to those
Transcriptional Control of IFN-\(\gamma\) Expression

Fig. 4. Analysis of IRF-Binding Activities in Vitro

Recombinant IRF-1 was produced in E. coli (A and B), and IRF-1 and IRF-2 were produced by in vitro translation (C and D). Proteins were tested for binding to a tetrahexamer oligonucleotide probe (A, C, and D) or an IFN-\(\gamma\) promoter probe (B). A, Binding of 5-fold increasing amounts of partially purified recombinant IRF-1 to the tetrahexamer (TH) probe in the presence (+) or absence (−) of a 100-fold excess of unlabeled oligonucleotide. B, Recombinant IRF-1 was tested for binding to the IFN-\(\gamma\) promoter probe in the presence or absence of unlabeled oligonucleotides representing the tetrahexamer probe, engrailed motif, or NF-\(\kappa\)B/Rel motif. C, Binding of 3-fold increasing amounts of reticulocyte lysate, programmed without (CON) or with IRF-1 and IRF-2 mRNA, to the tetrahexamer probe. D, Competition of IRF-1 and IRF-2 binding to the tetrahexamer probe by unlabeled tetrahexamer (TH) or plasmids containing the IFN-\(\gamma\) promoter (TP), IFN-\(\alpha\) promoter (Om), or nonspecific DNA from the HBV genome (NS).

Table 2. Effects of IRF-1 on IFN-\(\gamma\) Promoter Activity

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Reporter Plasmid</th>
<th>GH Production (ng/ml)</th>
<th>Fold Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>pOGH</td>
<td>3.9</td>
<td>−IRF-1 +IRF-1</td>
</tr>
<tr>
<td></td>
<td>pBTP-1.8GH</td>
<td>7.1</td>
<td>5.4</td>
</tr>
<tr>
<td>Jar</td>
<td>pOGH</td>
<td>0.3</td>
<td>−IRF-1 +IRF-1</td>
</tr>
<tr>
<td></td>
<td>pBTP-1.8GH</td>
<td>5.2</td>
<td>2.0</td>
</tr>
</tbody>
</table>

obtained with the slightly longer promoter fragments (Fig. 7B). Significantly, the −322 to −358 oligonucleotide competed for binding to the −280 to −430 promoter fragment in gel shift experiments with day 15 ovine conceptus extracts (Fig. 7C). The banding patterns obtained with both Jar and Cos-1 extracts were again more complex. Thus, the distal binding site was probably located between positions −322 and −358. Not shown, but also tested, were nuclear extracts from nonexpressing L929 cells and day 21 ovine conceptuses. The patterns obtained with these extracts were comparable to those shown for Jar and Cos-1 cells.

Figure 7D shows an alignment of sequences between positions −322 and −358 from a boIFN-\(\gamma\) gene and four oIFN-\(\gamma\) genes. This sequence is highly conserved across all genes.

The IFN-\(\gamma\) Promoter Is Active in Only a Subset of Jar Cells

Although we detected stage-specific factors that bind to the proximal and distal promoter elements in extracts from conceptuses, similar gel shift patterns were not
Fig. 5. IRF-1 mRNA Is Expressed at Very Low Levels in Bovine Conceptuses
PCR analysis of IRF-1 mRNA in control (CL) and Sendai virus-induced (VIL) bovine leukocytes and a day 19 bovine conceptus. Reverse transcriptase PCR was performed with primers representing highly conserved regions of murine and human IRF-1 cDNA. After amplification, PCR products were separated by electrophoresis, blotted to nylon membrane, and hybridized with a murine IRF-1 cDNA probe under conditions of moderate stringency. The blot on the left was exposed to x-ray film for 1 day; that on the right for 1 week.

observed when using extracts made from Jar or JEG-3 choriocarcinoma cells. Because only a small percentage of Jar cells constitutively express hCG α- or β-subunit (31, 32), it was of interest to determine whether expression from an IFNγ promoter was also confined to certain cell subpopulations.

To explore this possibility, cells from the clonal line -1.8cl6 that had been selected for high hGH production were tested in similar experiments. Transient transfection of these cells with pBTP-1.8βGal yielded similar numbers of βGal-positive cells as pSVβGal transfectants (3.5% vs. 5%; Fig. 8, D and E). These results suggested that only a subpopulation of cells within the normally heterogeneous population of Jar cells was able to support significant expression from the IFNγ promoter, and that a higher expressing phenotype could be clonally selected.

DISCUSSION

To confirm this hypothesis, cells from the clonal line -1.8cl6 that had been selected for high hGH production were tested in similar experiments. Transient transfection of these cells with pBTP-1.8βGal yielded similar numbers of βGal-positive cells as pSVβGal transfectants (3.5% vs. 5%; Fig. 8, D and E). These results suggested that only a subpopulation of cells within the normally heterogeneous population of Jar cells was able to support significant expression from the IFNγ promoter, and that a higher expressing phenotype could be clonally selected.

These experiments were designed to characterize the IFNγ promoter sequences that are required for IFNγ expression in trophoblasts. This pattern of expression is very unusual, in that it is massive, localized to the trophoblast, and confined to only a few days of the period immediately preceding implantation (1, 4–8). The experiments with stably transfected Jar cells, which are summarized in Table 1, confirmed yet another unique feature of IFNγ gene expression that had been suspected earlier (24, 33), namely that gene transcription was not readily inducible by virus, whereas other type I IFN were expressed after exposure to Newcastle disease virus. Unfortunately, these cell lines failed to provide much further information about what might regulate the transcription activity of IFNγ genes, except that the control systems operating were probably different from those implicated for other type I IFN.
Fig. 7. Localization of Distal Binding Sequences

A, Bovine IFN-α promoter sequences from positions –430 to –280 were subjected to bidirectional deletional analysis to identify sequences involved in distal complex formation. Resulting promoter fragments were used as probes in gel mobility shift assays with day 13/14 ovine conceptus nuclear extracts. Competitor DNA consisted of a 100-fold molar excess of unlabeled probe DNA.

B, Complementary oligonucleotides, representing boIFNα promoter sequences from positions –322 to –358 were annealed and used as a probe in gel mobility shift experiments with day 15 ovine conceptus, Jar, or Cos-1 nuclear extracts. A 100-fold molar excess of unlabeled oligonucleotides was used as competitor.

C, Conceptus nuclear factor binding to the –280/–430 boIFNα promoter probe was competed for using a 100-fold molar excess of unlabeled probe DNA or a 100-fold (lane 3) or 500-fold (lane 4) molar excess of annealed oligonucleotides representing promoter sequences between –322 and –358.

D, Alignment of promoter sequences from positions –322 to –358 from a boIFNα and four oIFNα genes. Identical bases are represented by dots. The boIFNα gene sequence is from Hansen et al. (22), oTP-p7 and oTP-p9 sequences are from Leaman and Roberts (20), and oTP57 and oTP19 sequences are from Charlier et al. (19). A nearly perfect palindrome (from positions –352 to –338) is underlined.
Therefore, two additional approaches, cell transfection and gel mobility shift assays, were employed to dissect the up-stream promoter regions of the IFN-\(\gamma\) genes. Again, however, there were difficulties. Transfections have not been successful in primary trophoblast cell cultures (24), and Jar cells do not constitute an ideal alternative because the human genome does not appear to possess IFN-\(\gamma\) genes (20), and the cells, when cultured normally, do not display constitutive expression of any other type I IFN. Jar cells are, however, capable of expressing a transfected IFN-\(\gamma\) gene (24), presumably because they contain an appropriate complement of transcription factors.

It was somewhat surprising that the gel shift pattern obtained with the Jar cell extracts and both proximal and distal promoter region gel shift probes were very similar if not identical to those obtained with extracts from nonexpressing cell lines. However, as shown in Fig. 8, we found that only a small proportion of Jar cells within a heterogeneous Jar cell population was competent to support expression from an IFN-\(\gamma\) gene promoter. Others have indicated that Jar cells display a considerable breadth of phenotype and are exceedingly heterogeneous with regard to expression of CG subunits (31, 32). Therefore, even though the Jar line can be regarded as permissive for IFN-\(\gamma\) promoter activity, only a relatively small fraction of the cells in the population may actually be so, and this fraction may vary from culture to culture. The banding pattern is likely, therefore, to reflect the majority of nonpermissive cells. Clearly, the lack of a consistent phenotype in Jar cells makes them less than a desirable system for use in gene promoter analysis and probably leads to major shifts in cell responsiveness over time. However, Jar and related human choriocarcinoma cells have provided the only useful models to date for studying IFN-\(\gamma\) pro-

---

**Fig. 8. Histochemical Staining for \(\beta\)Gal Expression within Transfected Jar Cell Populations**

Wild-type Jar cells (A–C) or cells from the high expressing stable line –1.8c6 (D–F) were transiently transfected with plasmids pSV\(\beta\)Gal (A and D), pBTP-1.8\(\beta\)Gal (B and E), or a promoterless \(\beta\)Gal plasmid (C and F). Cells were cultured for 36 h after transfection, then fixed and stained for \(\beta\)Gal activity. Magnification, x100.
moter activity. Experiments with cells derived from rat trophoblast (34) have yet to be completed.

Despite these obvious difficulties, the promoter detection analyses performed in Jar cells and the gel shift experiments carried out with conceptus nuclear extracts showed remarkable agreement. Both implicated a proximal region (to position −126) and a more distal region between −280 and −400 which are required for full constitutive expression of the IFN- genes.

The first 126 bp of IFN- promoter sequence beyond the transcription start site provided an almost 10-fold increase in transcriptional activity relative to a promoterless reporter gene when transfected into Jar cells. Interestingly, a −100 oIFN promoter was found to exhibit low, but detectable, transcriptional activity in Jar cells, whereas no transcriptional activity was detected when longer oIFN promoter fragments were tested (Fig. 1). This result contrasted with what had been observed with the IFN- promoter constructs, where longer promoter fragments were found to display higher transcriptional activity than the shorter ones (24) (Fig. 1). Therefore, the evolution of the trophoblast-specific expression by IFN- genes probably involved the introduction of distal regulatory elements necessary to direct full gene expression in the developing trophoblast, perhaps with a concomitant loss of repressor elements that inhibits constitutive activity of other type I IFN genes in these same cells. Additional subtle changes within the proximal promoter region may also have contributed to this unique pattern of expression and perhaps to the loss of virus inducibility of the IFN- genes.

Our analysis of nuclear factor binding to the proximal promoter region indicated that this region formed several complexes when extracts from day 13–16 ovine conceptuses were used. The slower migrating band arose from a complex involving DNA sequences present between positions −69 and −91 (Fig. 4C). The binding pattern was altered when later stage (day 21) conceptus extracts or nuclear extracts from various cell lines were used. The changes in the shifted pattern may well have reflected the introduction of novel regulatory molecules, possibly transcriptional repressors in the day 21 extracts. Because binding of conceptus nuclear factors to the proximal IFN- promoter region was not affected by competition with a 100-fold molar excess of DNA derived from a comparable region of an oIFN- gene promoter (Fig. 3A), it seems likely that the factors that interacted with the IFN- promoter were distinct from those that formed complexes with the IFN- promoter. Although putative IRF-1- and IRF-2-binding sites (13) can be observed within the proximal promoter region and may play a role in the transactivation of other type I genes, including IFN- (14), they do not appear to be involved in IFN- gene expression. Recombinant IRF-1 was able to bind the promoter, but did so rather weakly (Fig. 4), and IRF-1 was incapable of transactivating IFN- transcription. More importantly, IRF-1 and IRF-2 were probably very poorly expressed in ovine or bovine conceptuses, as determined by both gel shift assays and analysis of conceptus RNA (Fig. 5).

Deletional analysis of the 450-bp IFN- promoter in conjunction with transient transfection experiments in Jar cells has indicated that at least one enhancer region is located between positions −280 and −400. This region displayed full activity in either orientation and conferred enhancement to a heterologous promoter (Fig. 1). Whether this region consists of a single enhancer element or several distinct regulatory elements remains to be determined. It is conceivable (and perhaps likely) that negative regulatory elements also exist within this distal region, because certain cell lines, e.g. L6 myoblasts, permit a higher level of transcriptional activity from a 126-bp promoter than from a 450-bp promoter (data not shown). Further truncations of the −280/−400 promoter region in conjunction with transfection experiments into nonpermissive cell types will be necessary to determine where these putative negative regulatory factors might bind and whether overlapping positive and negative regulatory elements, such as those observed in other type I IFN genes (12, 17), are involved in regulating IFN- gene expression.

The single complex formed in association with the −322 and −358 region of the promoter appeared to be specific to the time of peak IFN- gene expression. Examination of the sequences within this stretch of DNA (Fig. 7D) revealed a nearly perfect palindrome between positions −352 and −338, and a heptanucleotide (−336 to −330) that resembles a motif (TCCAG/AAT) implicated in the trophoblast expression of the mouse placental lactogen-1 gene (34). Whether the ovine and bovine factors involved in distal IFN- promoter binding represent related proteins remains to be determined.

As with the proximal region, the marked change in the band shift pattern noted on day 21, when expression was in sharp decline (1) were again possibly indicative of the binding of repressor molecules. However, it should be emphasized that sheep conceptuses do not represent a single cell type. Trophoblast, comprised of IFN- expressing trophoblast cells as well as nonexpressing extraembryonic endoderm, makes up the major mass of tissue on days 13–14 when the embryonic disc is very small, but the embryo proper, yolk sac, and emerging allantois begin to contribute more heavily as development proceeds (35). Although attempts were made to dissect away non trophoblast tissues on day 21 before nuclear extracts were prepared, the band shift patterns observed may not entirely reflect a switch in gene expression within trophoblast.

Although the complexes obtained with the proximal and distal probes on the earlier stage conceptus nuclear extracts were predominantly of high electrophoretic mobility, there are several reasons to believe that these complexes were neither artifacts nor the result of proteolytic degradation. The procedure used to isolate conceptus nuclear extracts was identical across all stages of pregnancy, yet the day 21 nuclear extracts consistently yielded both low and high mobility com-
plexes (Figs. 3B and 6). Furthermore, similar gel shift patterns were observed with day 13–16 ovine conceptus extracts from at least seven different preparations, isolated by two different individuals (Cross, J. C., and D. W. Leaman) over a 3-yr period. It is also important to emphasize that gel shift complexes of similar high mobility have been described previously by others (36–38) and do not necessarily reflect proteins of low mol wt. Such proteins may share some common features that contribute to their high electrophoretic mobility under the nondenaturing electrophoretic conditions employed in the gel shift assays.

In conclusion, these studies have indicated that IFN-1 promoter regions that are critical for promoter activity in choriocarcinoma cells are also capable of binding conceptus nuclear factors isolated from ovine or bovine conceptuses during the times of peak IFN-1 gene expression. In particular, it has been shown that 1) both proximal and distal promoter sequences are required for full constitutive IFN-1 expression in Jar cells; 2) conceptus nuclear factors bind these regions; and 3) the conceptus protein/DNA binding pattern changes dramatically as IFN-1 gene expression ceases, perhaps reflecting the appearance of novel transcriptional repressors. These data provide valuable information about the promoter sequences that may be involved in regulating the cell-restricted and stage-specific expression of the IFN-1 genes. They indicate that the transcriptional controls operating may be quite different from those involved in trophoblast-specific expression of CG (39) and placental lactogen (40) in the human.

MATERIALS AND METHODS

Reporter Genes and Transfection Experiments

Plasmids pBTP-1.8GH, pBTP-450GH, pBTP-126GH, and pOGH have been described previously (24). The pBTP-450GH and pBTP-280GH plasmids were generated by nested deletions of the pBTP-450GH plasmid by exonuclease III and mung bean nuclease digestions (41). The lengths of the resulting promoter fragments were confirmed by sequence analysis. For plasmid pBTP-400A.104GH, biFN-1 promoter sequences from position –230 to –400 were PCR amplified from a cloned biFN-1 gene and then subcloned ahead of the –126 biFN-1 promoter in plasmid pBTP-126GH. The pOm-100GH plasmid was prepared by Ncol/HindIII digestion of a cloned ovine IFN-1 gene to liberate gene sequences from positions –100 to 45 (20). These sequences were blunt end ligated into the promoterless pOGH expression plasmid (Nichols Diagnostic, San Juan Capistrano, CA) at a unique SalI restriction site. For plasmid pBTP/Om-100GH, the biFN-1 promoter region from –130 to –400 was prepared by PCR amplification, and then subcloned up-stream of the –100 OIFN-1 promoter in plasmid pOm-100GH. The plasmid pOm-450GH was prepared by subcloning the Ncol-XbaI fragment of the bovine IFN-1 gene (42) into pOGH. The plasmid pCMWRF-1 was prepared by first cloning the murine IRF-1 cDNA (13) into the EcoRI site of the expression plasmid pCEC (43). The cDNA and down-stream SV40 polyadenylation signal (gained from pECE) were then liberated and cloned down-stream of the cytomegalovirus promoter-enhancer in the plasmid pEC (provided by Dr. Keith Marotti, Upjohn Co., Kalamazoo, MI).

All plasmids were purified on cesium chloride density gra-
dients (41). Transient transfections were performed by the calcium phosphate technique (44) with 10 μg reporter plasmid. Relative promoter activities were assessed by measuring hGH content in culture medium 24 h after transfection using a commercially available RIA kit (Nichols Diagnostics). All reported transfection results are the average values obtained from two to five experiments, with treatments tested in duplicate or triplicate for each experiment. Differences in promoter activities were examined for significance by analysis of variance using the General Linear Models procedure of SAS (45) before conversion to relative values.

The pBTP-1.8/βGal reporter plasmid was prepared by subcloning a biFN-1 promoter (positions –1800/45) into the EcoRI site of the promoterless βGal expression plasmid pNASS (Clontech, Palo Alto, CA). The pSV/βGal plasmid was prepared by subcloning the SV40 promoter from plasmid pECE (42) into the EcoRI site of plasmid pNASS. Analysis of βGal expression was performed according to previously reported procedures (46). Cells were stained with X-gal for 12 h before visual analysis.

Stable Transfectants

Stable Jar transfectants were obtained by cotransfection of plasmid pBTP-1.8GH with the neomycin resistance (neo) plasmid, pSVNEO, at a ratio (wt/wt) of 10:1 (pBTP-1.8GH:pSVNEO). The pSVNEO expression plasmid was prepared by subcloning a neomycin resistance gene, derived from plasmid pNEO (Pharmacia, Piscataway, NJ), into the multiple cloning site of plasmid pECE (43). Selection of stable transfectants was conducted in Dulbecco’s Modified Eagle’s Medium containing 10% fetal bovine serum and 400 mg/liter G418 sulfate (Gibco, Grand Island, NY). After 3 weeks, surviving colonies were ring cloned and clonally propagated in 5% fetal bovine serum-Dulbecco’s Modified Eagle’s Medium containing 200 mg/liter G418. Independent isolates testing positively for hGH expression were analyzed for gene integration by PCR amplification of isolated genomic DNA with sense primer BTP-126 G6 (5′-AGCGAATTCGACTACATTTCCTAGGTC-3′) and antisense primer hGhspec.as (5′-AGGCAATGGTTGCTCTCTTCGTAAGAAGCACTGTC-3′).

Stably transfected cells were treated with the CAMP analog 8-bromo-cAMP (Sigma, St. Louis, MO) for 24 h at concentrations ranging from 50 μM to 2 mM and with TPA (phorbol 12-myristate 13-acetate; Sigma) for 24–48 h at concentrations ranging from 1 x 10^-9 to 1 x 10^-6 M. Cells were also treated with retinoic acid at concentrations ranging from 1 x 10^-8 to 1 x 10^-6 M for 2 days, followed by an additional 24-h culture period in retinoic acid-free medium before expression analysis. For virus induction experiments, cells were exposed to 5 hemagglutination units/ml Newcastle’s disease virus for 6 h, and then cultured in virus-free medium for an additional 6 h before expression analysis (24). Medium from IFN-stimulated cells was assayed for antiviral activity using a cytopathic effect reduction assay employing GBK-2 cells infected with vesicular stomatitis virus (47). IFN titers were expressed in units of activity in which 1 U was equivalent to that amount of IFN that protected 50% of the GBK-2 cells from lysis by vesicular stomatitis virus. Recombinant biFN-1 (Ciba-Geigy, Basel, Switzerland), used as a standard, was added to culture medium at concentrations ranging from 12.5–50 ng/ml.

Polymerase Chain Reaction Analysis of IRF-1 mRNA

RNA was isolated from cells, reverse transcribed, and analyzed by PCR, as described previously (24). Primers for amplification of IRF-1 cDNA were synthesized based on conserved regions of the murine (13) and human (30) sequences. Southern blots were probed with a murine cDNA for IRF-1 under conditions of moderate stringency [30% (vol/vol) formaldehyde and 5 x saline-sodium citrate (41) at 42 C] and washed with 0.1 x saline-sodium citrate at 42 C (41). The reverse
transcription experiments were analyzed in parallel for IFN\(\omega\) and IFN\(\gamma\) (24).

### Nuclear Extracts, Recombinant IRF-1/2, and Gel Mobility Shift Analysis

Nuclear extracts from Jar, JEG-3, COS-1, and L929 cells were prepared by standard procedures (48). Nuclear extracts from ovine and bovine conceptuses were prepared in the same manner, except that protease inhibitors (5 g/ml aprotonin, 30 g/ml leupeptin, and 0.7 g/ml pepstatin) were added to all extraction and dialysis buffers immediately before use. Day 13−16 ovine conceptuses were recovered from ewes by surgical flushing (8), and bovine conceptuses were recovered from uteri after slaughter (24). Day 21 ovine conceptuses were recovered from sheep uteri after hysterectomy, and extraembryonic membranes were dissected from the embryo proper before nuclear extract isolation. Ovine conceptus extracts were used in the majority of studies because of the expense of obtaining bovine material.

Partially purified recombinant murine IRF-1 was prepared in Escherichia coli (BL21 DE3 strain) by using the expression vector pET-IRF-1 (15). Synthetic mRNAs for murine IRF-1 and IRF-2 were transcribed from the vectors pET-IRF-1 and pET-IRF-2, respectively (15), and translated in reticulocyte lysates (Promega, Madison, WI).

DNA probes were incubated with nuclear proteins or recombinant proteins for 30 min at 30 C in DNA binding buffer containing 0.4 g/\(\mu\)l poly(dI-dC), 15% (vol/vol) glycerol, 13 mM HEPES (pH 7.9), 66 mM KCl, 0.1 mM EDTA, 0.25 mM dithiothreitol, and 1 mM MgCl\(_2\) (46). Competitor DNA, when included, was added to the reactions just before incubation. Reactions were run over 4% or 6% low ionic strength Tris-borate (0.025 M Tris, 0.025 M boric acid, and 0.00125 M EDTA) polyacrylamide gels (49), and complexes were visualized by autoradiography.

### Gel Mobility Shift Probes

Bovine IFN\(\gamma\) promoter fragments used as probes in gel mobility shift studies were obtained initially by PCR amplification between specific primer sets (see Fig. 3A for relative locations). The oligonucleotide primers used for amplification were bTP-34as (GGGAAGACGTTACTTTAGTCC), bTP-126as (AGGGATTACCTCGACCTTTCCCTAGTGTC), bTP-130as (CAATCTAATGGTC), bTP-220as (TTTCAATTAAATTCAATTAAA), bTP-280as (TTTCAATTGAATTTATGCA), and bTP-430as (TTTCAATTATTTAATGTAG). The -130/-280 and -280/-430 fragments were obtained by restricting the -130/-430 fragment with Rsal. Distal gel shift probes were obtained through nested deletions of the -280/-430 promoter fragment by exonuclease-III and mung bean nuclease digestion to yield the probes described in Fig. 8.

For IRF-1 binding studies, two oligonucleotides were synthesized which, when annealed, created a tetrahexamer probe containing four tandem repeats of the AAGTGA hexamer (IRF-2 like binding site). A fragment of the bovine IFN\(\gamma\) promoter (20, 22) from positions 65 to -450 was prepared by restriction endonuclease digestion to yield the probes described in Fig. 8.

### Acknowledgments

The authors would like to thank Neil Cosby and Limin Liu for helpful discussions and assistance with some of the reported transfection experiments. Our gratitude is also extended to Kyle Kramer, Bill Trout, Sancal Xie, Harriet Francis, and Bob Nagel for assistance with surgeries, and to Gal Foristal for preparing this manuscript. We are grateful to Dr. Tadatsugu Taniguchi (Osaka, Japan) for the IRF-1 and IRF-2 expression vectors and antisera, Dr. Bill Rutter (San Francisco, CA) for the pECE plasmid, and Dr. Keith Marotti (Kalamazoo, MI) for the pECL plasmid.

Received November 16, 1993. Revision received January 4, 1994. Accepted January 5, 1994.

Address requests for reprints to: Dr. R. Michael Roberts, 158 Animal Sciences Center, University of Missouri, Columbia, Missouri 65211.

This is publication 11,920 of the University of Missouri Agricultural Experiment Station.

† Present address: Molecular Biology Department, Research Institute, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44195.

‡ Present address: Laboratory of Radiobiology and Environmental Health, University of California, San Francisco, California 94143-0750.

### REFERENCES

31. Martell RE, Ruddon RW 1990 Patterns of human choric gonadotropin expression in untreated and B-bromodeox-
inosine-treated JAR choriocarcinoma cells. Endocrinology 126:2757–2764
32. Hochberg A, Rachmilewitz J, Eldar-Geva T, Salant T, Schneider T, de Groot N 1992 Differentiation of chori-
ocarcinoma cell line (Ja). Cancer Res 52:3713–3717
33. Stewart HJ, Guesdon FMJ, Payne JH, Charleston B, Vallet JL, Flint APF 1992 Trophoblast interferons in early preg-
nancy of domestic ruminants. J Reprod Fertil (Suppl) 45:59–68
34. Shida MH, N-g Y-K, Soares MJ, Linzer DCH 1993 Troph-
oblast-specific transcription from the mouse placental lac-
togen I gene promoter. Mol Endocrinol 7:181–188
35. Bazer FW, Giepert RD, Zavy WT 1987 Fertilization, cleavage
and implantation. In: Hafez ESE (ed) Reproduction in Farm
Animals. Lea and Febiger, Philadelphia, pp 210–228
Interferon-induced nuclear factors that bind a shared pro-
tector element correlate with positive and negative tran-
scriptional control. Genes Dev 2:383–393
Activation of transcription by IFN-γ: tyrosine phosphory-
ation of a 91-kD DNA binding protein. Science 258:1808–
1812
binding of transcription factor PII-1 to the growth hormone
promoter. Proc Natl Acad Sci USA 89:11451–11455
39. Nilson JH, Bokar JA, Clay CM, Farmerie TA, Fenster-
maker RA, Hamernik DL, Keri RA 1991 Different combi-
nations of regulatory elements may explain why placent-
specific expression of the glycoprotein hormone α-subunit
gene occurs only in primates and horses. Biol Reprod 44:231–237
40. Roberts RM, Anthony RV 1994 Molecular biology of tropho
Academic Press, San Diego, pp 385–439
41. Sambrook J, Fritsch EF, Maniatis T (eds) 1989 Molecular
Laboratory, Cold Spring Harbor
42. Capon DJ, Shepard HM, Goeddel DV 1985 Two distinct
families of human and bovine interferon-genes are coordi-
natedly expressed and encode functional polypeptides.
Mol Cell Biol 5:765–779
43. Ellis L, Clauser E, Morgan DO, Edery M, Roth RA, Rutter
WJ 1986 Replacement of insulin receptor tyrosine resi-
dues 1162 and 1163 compromises insulin-stimulated ki-
nase activity and uptake of 2-deoxyglucose. Cell 45:721–
732
44. Kingston RE 1987 Transfection of DNA into eukaryotic
cells. In: Ausubel FA, Brent R, Kingston RE, Moore DD,
in Molecular Biology. Greene and Wiley Interscience, New
York, pp 911–914
45. SAS 1985 Statistical Analysis Systems, ed 5. SAS Insti-
tute, Cary
46. MacGregor GR, Mogg AE, Burke JF, Casky CT 1987
Histochemoical staining of clonal mammalian cell lines ex-
pressing E. coli β-galactosidase indicates heterogeneous
47. Roberts RM, Imakawa K, Niwano Y, Kazemi M, Malitoph
PV, Hansen TR, Glass AA, Kronenberg LH 1989 Inter-
feron production by the preimplantation sheep embryo. J
Interferon Res 9:175–187
48. Dignam JD, Lebowitz RM, Roeder RG 1983 Accurate
transcription initiation by RNA polymerase II in a soluble
extract from isolated mammalian nuclei. Nucleic Acids
Res 11:1475–1489
49. Chodosh LA 1988 Mobility shift DNA-binding assay using
RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds)
Current Protocols in Molecular Biology. Greene and Wiley
Interscience, New York, pp 1221–12210