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Different Levels of Ovine Interferon-τ Gene Expressions Are Regulated Through the Short Promoter Region Including Ets-2 Binding Site

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ABSTRACT   Regulation of interferon-τ (IFNτ) production, a conceptus secretory protein implicated in the process of maternal recognition of pregnancy, has not been fully elucidated. Among more than 10 ovine IFNτ (oIFNτ) gene sequences characterized, approximately 75% of oIFNτ transcripts expressed in utero is derived from *oIFNτ-o10* gene and amounts of transcripts from other *oIFNτ* genes such as *oIFNτ-o8* or *oIFNτ-o2* are minimal. It was hypothesized that the variation in expression levels exhibited by *oIFNτ-o10* and *oIFNτ-o8/-o2* genes was due to differences in the proximal promoter regions of these *oIFNτ* genes. To test this hypothesis, transient transfection experiments with human choriocarcinoma JEG3 cells were executed with deleted and/or mutated 5′-upstream regions of these *oIFNτ* genes attached to the chloramphenicol acetyltransferase (*CAT*) reporter gene. Because only the Ets-2 binding site located in the *oIFNτ-o10* gene appeared to differentiate the expression levels of these constructs, the 6 base pair (bp) Ets-2 sequence from the *oIFNτ-o10* gene inserted into the *oIFNτ-o8/-o2* gene-reporter construct was examined. The insertion of this Ets-2 binding site into the oIFNτ-o8/o2-reporter construct failed to increase the degree of transactivation. Rather than this 6 bp sequence, a 22 bp sequence of the proximal promoter region, including the Ets-2 binding site, of the *oIFNτ-o10* gene was required for oIFNτ-o8/-o2-reporter transactivation. By electrophoretic mobility shift assay (EMSA), nuclear protein(s) bound to this 22 bp from the *oIFNτ-o10* and *oIFNτ-o8/o2* genes differed. These results suggest that the short promoter region including the Ets-2 binding site, not the Ets-2 binding region itself, may determine different levels of *oIFNτ* gene expressions seen in utero. *Mol. Reprod. Dev. 72: 7–15, 2005.*
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Key Words: ovine; IFNτ; promoter; Ets-2; AP-1; transcription

INTRODUCTION

Interferon-τ (IFNτ), produced by peri-implantation blastocysts, is a major protein implicated in the process of maternal recognition of pregnancy in ruminant ungulates (Godkin et al., 1982; Imakawa et al., 1987; Roberts et al., 1992). IFNτ acts on the uterine epithelium and attenuates secretion of a luteolysin, prostaglandin F2α (PGF2α), resulting in the maintenance of corpus luteum (CL) function (Vallet et al., 1988). Based on cDNA and amino acid sequences, this protein is classified into type I IFNs (Imakawa et al., 1987, 1989; Roberts et al., 1992). In addition to structural similarities, IFNτ polypeptides exhibit antiviral, antiproliferative, and immunomodulately activities like other IFNs (Pontzer et al., 1988, 1991; Roberts et al., 1989). The expression of IFNτ is quite different from that of other type I IFNs such as IFNα and IFNβ that are induced by viruses or double stranded RNA and maintained for only a few hours (Pestka, 1983; Farin et al., 1991). IFNτ exhibits temporal and spatial expression since its production is restricted to trophoblast cells during peri-implantation periods (Hansen et al., 1988; Farin et al., 1989; Guillomot et al., 1990; Demmers et al., 2001). In fact, ovine IFNτ (oIFNτ) production begins on day 8 of pregnancy (day 0 = first day of estrus). Its production increases as the conceptus elongates and reaches the highest production (up to 100 μg/conceptus/24 hr) on day 16, just before the attachment of the conceptus to the uterine epithelium (Godkin et al., 1982; Ashworth and Bazer, 1989; Imakawa et al., 1995). By day 22, when the placenta formation is initiated, oIFNτ is no longer detected (Godkin et al., 1982).

So far more than 10 *IFNτ* genes or cDNAs have been isolated and characterized for the ovine and bovine

species, which exhibit a high degree of similarity within and among ruminants (Leaman and Roberts, 1992; Nephew et al., 1993; Ryan and Womack, 1993; Imakawa et al., 1994; Ealy et al., 1998; Alexenko et al., 2000; Ealy et al., 2001). The majority of oIFNτ expressed in utero is derived from one gene, *oIFNτ-o10*, whose transcripts constitute more than 75% of oIFNτ mRNAs (Nephew et al., 1993). By transient transfection of reporter constructs with deleted or mutated 5′-upstream regions of the *oIFNτ-o10* gene in human choriocarcinoma JEG3 cells, the AP-1 binding site in the distal enhancer region was shown to be effective in oIFNτ-reporter transactivation (Yamaguchi et al., 1999, 2000). At the proximal promoter region, there exists another transactivation domain to which the transcription factor Ets-2 binds (Ezashi et al., 1998; Yamaguchi et al., 1999). Ezashi et al. (2001) have found that the action of Ets-2 on bovine IFNτ (bIFNτ) is inhibited while Oct-4 binds to Ets-2 protein. Once Oct-4 expression subsides, Ets-2 becomes effective in the activation of bIFNτ gene transcription. Recently, a transcription co-activator, cAMP-response element binding protein-binding protein (CBP), was shown to activate oIFNτ-o10 gene transcription (Xu et al., 2003). Both AP-1 and Ets-2 binding domains are located on the CBP polypeptide sequences, to which these transcription factors bound, possibly resulting in further activation of oIFNτ-o10 gene transcription.

In contrast to *oIFNτ-o10*, other *oIFNτ* genes like *oIFNτ-o8* and *-o2*, whose coding regions are >95% identical to that of *oIFNτ-o10*, are expressed at very low levels in vivo (Nephew et al., 1993). The homologies of their 5′-upstream regions (between −654 and −1 base pair, bp) are approximately 90%. The remaining 10% sequences and/or specific nucleotides may be responsible for the different degrees of expression observed for oIFNτ-o10 and oIFNτ-o8 or -o2. However, molecular mechanisms of these oIFNτ genes whose expressions are limited have not been characterized.

To understand the molecular mechanisms responsible for different degrees of oIFNτ gene transcriptions, three *oIFNτ* genes, *oIFNτ-o10*, *-o8*, and *-o2*, were examined using transient transfection analyses and electrophoretic mobility shift assays (EMSA).

MATERIALS AND METHODS
Plasmid Constructions

The upstream regions of *oIFNτ-o10*, *-o8*, and *-o2* (Nephew et al., 1993; GenBank accession numbers M88773, M88772, and M88770, respectively) between −654 and +51 bp were amplified through polymerase chain reaction (PCR) with specific primers (Yamaguchi et al., 1999). Each of these products was inserted into the *PstI* site of chloramphenicol acetyltransferase (CAT) basic vector (Promega, Madison, WI). For the preparation of chimeric enhancer (E)/promoter (P) sequences, the upstream regions from −654 to −452 bp of *oIFNτ-o8* and *-o2* genes were replaced with the same region of *oIFNτ-o10*, termed o10E + o8P and o10E + o2P, respec-

tively. The regions from −452 to +51 bp of *oIFNτ-o8* and *-o2* were replaced with the same region of *oIFNτ-o10*, termed o8E + o10P and o2E + o10P, respectively. AP-1 binding site, TGTGTCA, located at −594 to −588 bp of the *oIFNτ-o10* gene's upstream region was point mutated to TGTG<u>CA</u>A by inverse PCR procedure using the primers consisting of desired nucleotide changes (Yamaguchi et al., 1999). Using the same method, Ets-2 binding site located at −77 to −72 bp of *oIFNτ-o10* gene's upstream region was also mutated as CAGGAA to CA<u>TT</u>AA. Ets-2 binding site of oIFNτ-o10 (CAGGAA) and the sequence found at the equivalent region of oIFNτ-o8 (ATGAAA) were interchanged, resulting in oIFNτ-o10 with ATGAAA (o10-M6) and oIFNτ-o8 with CAGGAA (o8-M6) constructs, respectively. The 23 bp of *oIFNτ-o8* (GAAAACGCAAATGAAAGTGAGAG) was replaced with the 22 bp sequences of *oIFNτ-o10* (GAAAACAAA<u>CAGGAA</u>GTGAGGG; Ets-2 binding site is underlined), resulting in the construct *oIFNτ-o8-M22* (*o8-M22*). Expression vectors of murine c-Jun and c-Fos were driven by Rous Sarcoma Virus (RSV) E/P of pRVSV vector (Miyazawa et al., 1993). The Ets-2 expression vector was a pSG5-based construct and driven by the SV40 P/E (Wakiya et al., 1996).

Cell Culture and Transient Transfection

Human choriocarcinoma JEG3 cells (HTB36, American type culture collection) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Lois, MO) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich), 40 U/ml penicillin, and 40 μg/ml streptomysin under 5% CO_2 at 37°C. One day before transient transfection was performed, JEG3 cells were re-plated onto 6-well plastic culture plates and at 50%−60% confluency, they were transiently transfected with reporter constructs using TransFast (cationic lipid; Promega) according to the manufacturer's protocol (Yamaguchi et al., 1999). In each well of 6-well plates, 3 μg of oIFNτ-CAT reporter plasmids, 0.2 μg of β-gal plasmids, and 9 μg of TransFast mixed in 1 ml DMEM was overlaid on the cells. After 1 hr incubation at 37°C, 3 ml of DMEM was added to each well and transfected cells were cultured for 48 hr. The effects of nuclear factors on *oIFNτ* gene-reporter transactivation were determined by co-transfection analyses. One micrograms of expression vectors or pRVSV empty vector was co-transfected along with 3 μg of oIFNτ-CAT plasmids.

Chloramphenicol Acetyltransferase (CAT) Assay

CAT activity was measured using CAT ELISA kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. After 48 hr incubation, JEG3 cells were washed with phosphate-buffered saline (PBS) and lysed by the addition of 600 μl Lysis buffer (Promega), and cell debris was then removed by centrifugation. Extracts (200 μl) were added to each well of anti-CAT-coated microtiter plate, which was then incubated at 37°C for 1 hr. The well was washed with the washing buffer (10 mM sodium phosphate, 150 mM

sodium chloride, and 0.1% Tween 20). To each well, 400 ng of anti-CAT-Digoxigenin were added and the plate was incubated at 37°C for 1 hr. After the washing step, 150 mU of anti-digoxigenin-peroxidase was added to each well and incubation continued at 37°C for 1 hr. The wells were washed again, 200 μl of peroxidase substrate was added and incubated at room temperature (22–25°C) for 30 min. CAT activities were measured by determining the absorbance of each sample at 405 nm. β-gal activities of each sample were also determined by using β-gal ELISA kit (Roche Diagnostics). CAT activity resulting from various oIFNτ-reporter constructs was normalized through the determination of transcriptional efficiency with β-gal activity. In the figures, CAT activities were expressed as fold activation relative to an appropriate control within the experiment.

Preparation of Nuclear Extracts and EMSA

Nuclear proteins were extracted from JEG3 cells following the protocol described by Angel et al. (1987). Cells were grown to 70%−80% confluency, then washed with PBS and lifted off the plate. The remaining procedures were described by Matsuda et al. (2004).

Bindings of nuclear proteins extracted from JEG3 cells to the 5′-upstream region of oIFNτ-o10 or -o8 were examined by EMSA. The three oligonucleotide probes examined were the 22 bp of *oIFNτ-o*10 gene's upstream region including Ets-2 binding site, 23 bp of *oIFNτ-o*8 (equivalent to the 22 bp of *oIFNτ-o*10), and 23 bp of *oIFNτ-o*8 sequences, to which Ets-2 binding site from the *oIFNτ-o*10 gene was inserted (o8-M6 probe, 5′-GAAAACGCAACAGGAAGT-GAGAG-3′; inserted sequence is underlined). These probes were end-labeled with [γ-^{32}P] ATP (6,000 Ci/mmol, NEN, Boston, MA) and T4 polynucleotide kinase (Takara, Shiga, Japan). End-labeled oligonucleotide probes were cleaned by ethanol precipitation, and annealed with non-labeled antisense

oligonucleotides by placing them into a 95°C water bath and slowly cooling to room temperature. The sequences of consensus Ets-2 and Sp-1 oligonucleotides were 5′-CTAGGACCAGGAAGTGGGAGT-3′ and 5′-ATTCGA-TCGGGGCGGGGCGAGC-3′, respectively (binding site of each is underlined), and competitors of oIFNτ-o10, oIFNτ-o8, Ets-2 consensus, and Sp-1 consensus were constructed by annealing non-labeled sense and antisense oligonucleotides. JEG3 nuclear proteins (10 μg) were incubated on ice for 15 min in the binding buffer (7 mM HEPES (pH 7.9), 0.5 mM $MgCl_2$, 35 mM KCl, 7% (v/v) glycerol, 70 nM EDTA, 0.3 mM DTT, 100 μg/ml poly(dI-dC), and 50 μg/ml BSA) with or without a 100-fold molar excess of unlabeled competitor. End-labeled probe (2–3 ng/probe, 3,000 cpm) was added and incubated at room temperature for 15 min. Reaction mixtures were loaded onto a 5% polyacrylamide gel and upon the completion of electrophoresis, the gel was dried and autoradiographed.

Statistical Analysis

The results of CAT assays were expressed as means ± SEM. Differences in fold activation were examined by one-way ANOVA followed by Tukey's multiple comparison tests.

RESULTS

Contribution of Proximal and Distal 5′-Upstream Regions on oIFNτ Gene Transcription

Reporter plasmids constructed with the wild-type 5′-upstream sequences of *oIFNτ-o*10, -o8 or -o2 genes were examined for the degrees of transactivation using a transient transfection method (Fig. 1). Similarly to its expression in vivo, the reporter plasmid with the upstream region of the *oIFNτ-o*10 gene exhibited high CAT activity whereas the one with *oIFNτ-o*8 or -o2 gene had very low activity, less than 20% of that expressed by
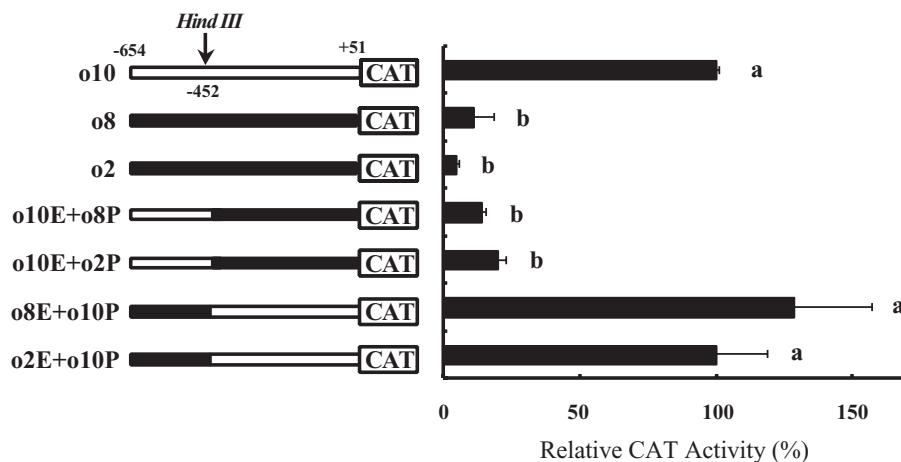

Fig. 1. Wild type and chimeric enhancer/promoter analyses of the 5′-upstream regions of oIFNτ-o10 and oIFNτ-o8 or -o2 reporter plasmid transfected into JEG3 cells. Distal and proximal upstream regions between −452 and +51 bp of *oIFNτ-o*10, -o8, and -o2 were interchanged at the *Hind III* restriction site (o10E + o8P, o10E + o2P, o8E + o10P, o2E + o10P) and transcriptional activities were compared to those of wild-type *oIFNτ-o*10, -o8, and -o2. Results are expressed as relative CAT activity to that of the oIFNτ-o10-reporter plasmid and values represent means ± SEM. Results with different letters differ at *P* < 0.01 (n = 4 each).

oIFNτ-o10. To determine the upstream region critical for high or low levels of oIFNτ gene transcriptions, distal and proximal regions of *oIFNτ-o*10, *-o*8 or *-o*2 were fused at the *Hind III* restriction site (−452 bp) and the importance of distal (E) and proximal (P) regions was evaluated (Fig. 1). Proximal region of *oIFNτ-o*8 or *-o*2 fused to the distal region of *oIFNτ-o*10 (o10E + o8P and o10E + o2P, respectively) exhibited the same level of transactivation as that of the wild-type oIFNτ-o8-reporter plasmid. However, the proximal region of *oIFNτ-o*10 fused to the distal region of *oIFNτ-o*8 or *-o*2 (o8E + o10P or o2E + o10P) exhibited a high degree of transactivation similar to that of the wild-type *oIFNτ-o*10.

Examination of Distal AP-1 and Proximal Ets-2 Binding Sites on oIFNτ Gene Transcription

Because AP-1 and Ets-2 binding sequences are located in the oIFNτ-o10's distal enhancer and proximal promoter regions, respectively, the effects of these sites on the transactivation of oIFNτ-reporter constructs were examined through point mutations to these binding sites (Fig. 2). Two-base mutation to the AP-1 binding site resulted in the reduction of CAT activity, approximately 50% of that of the wild-type oIFNτ-o10. Two-base mutation to the Ets-2 binding site also reduced the activity of oIFNτ-o10 to less than 40%. When both AP-1 and Ets-2 binding sites were point mutated, its CAT activity became approximately 25% of that of the wild-type oIFNτ-o10. In addition, co-transfection with AP-1 or Ets-2 expression plasmid failed to activate mutated constructs (Fig. 3A,B). These experiments show that it is a lack of binding of these factors to the mutated templates rather than the lack of these factors themselves.

Examination of Proximal Ets-2 Binding Site on oIFNτ Gene Transcription

The importance of the Ets-2 binding site of the oIFNτ-o10 promoter region was further examined by exchanging the Ets-2 binding site of oIFNτ-o10 (CAGGAA) with the sequences of oIFNτ-o8 (ATGAAA) located at the same region (Fig. 4A). Ets-2 site was originally described as a 10 bp sequence (Wasylyk et al., 1993). The 6 bp change made in this study was due to the fact that several nucleotides at both side of the 6 bp sequence were the same between *oIFNτ-o*10 and *-o*8 genes. When the 6 base sequence from oIFNτ-o8 was inserted into the equivalent site of oIFNτ-o10 (o10-M6), the CAT activity was reduced to 5% of the wild-type oIFNτ-o10 (Fig. 4B). The insertion of Ets-2 binding sequence to the equivalent region of the oIFNτ-o8-reporter construct (o8-M6), however, did not increase the degree of transactivation, which was at the same level as the wild-type oIFNτ-o8 and less than that of oIFNτ-o10 (Fig. 4B).

Examination of a Short Promoter Region of the *oIFNτ* Genes

The 22 bp sequence including the Ets-2 binding site of oIFNτ-o10 (GAAAAC-AAA<u>CAGGAA</u>GTGAGGG, Ets-2 binding site underlined) was inserted into the equivalent site of oIFNτ-o8's proximal promoter region (o8-M22; Fig. 5A). Its degree of transactivation was then compared with that of the wild-type oIFNτ-o10, wild-type oIFNτ-o8, and o8-M6 constructs. Differing from o8-M6, which had a low degree of transactivation similar to the wild-type oIFNτ-o8, o8-M22 construct had strong transactivation similar to that of the wild-type oIFNτ-o10 (Fig. 5B).

Examination of a Nuclear Protein Binding to the Short Promoter Region of *oIFNτ* Gene

The bindings of JEG3 nuclear protein to 22/23 bp of oIFNτ-o10/o8 promoter regions were examined by EMSA (Fig. 6). Protein bindings to oIFNτ-o10 and -o8 probes appeared specific since they disappeared when nonlabeled respective probes, competitors, were added whereas an unrelated competitor, Sp-1, did not inhibit the binding (Fig. 6). A second band appeared below the Ets-2 binding disappeared with the use of unrelated Sp-1 competitor, but the further characterization of this
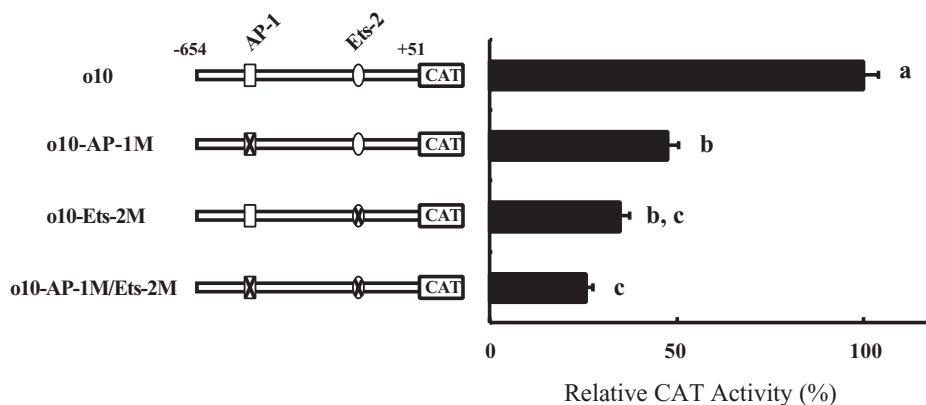

Fig. 2. Effects of point mutation to AP-1 or Ets-2 binding site of oIFNτ-o10. The wild-type oIFNτ-o10 reporter plasmid with mutated AP-1 and/or Ets-2 binding sites was transfected into JEG3 cells. Results are expressed as relative CAT activity to that of the oIFNτ-o10 reporter plasmid and values represent means ± SEM. Results with different letters differ at $P < 0.05$ (n = 4 each).

A

B

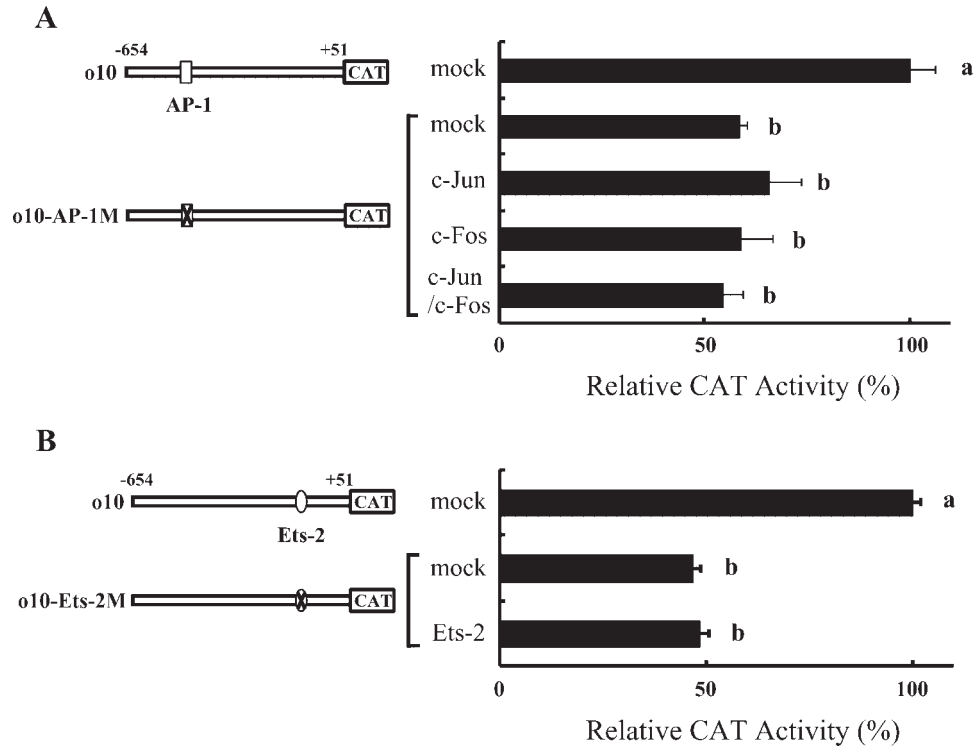**Fig. 3.** Co-transfection of wild type or mutated oIFNτ-o10 with AP-1 or Ets-2 expression plasmids. **A**: The wild-type oIFNτ-o10 reporter plasmid with mutated AP-1 site was transfected into JEG3 cells with empty vector (mock), c-Jun, c-Fos or c-Jun plus c-Fos expression vector. **B**: The wild-type oIFNτ-o10 reporter plasmid with mutated Ets-2 binding site was transfected into JEG3 cells with empty vector (mock) or Ets-2 expression vector. Results are expressed as relative CAT activity to that of the oIFNτ-o10-reporter plasmid and values represent means ± SEM. Results with different letters differ at $P < 0.05$ (n = 4 each).
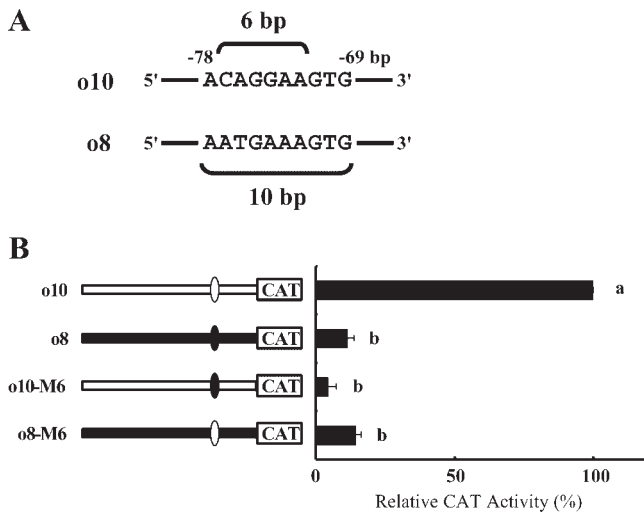
A

B

Fig. 4. Transcriptional activities of oIFNτ-o10 or -o8 after the 6 bp replacement at Ets-2 binding site. **A**: The core 6 bp Ets-2 binding sequence (-77 to -72 bp) in the oIFNτ-o10's promoter region and the equivalent 6 bp sequence of oIFNτ-o8 are shown. **B**: Transcriptional activities of wild-type oIFNτ-o10 and -o8, and those of o10-M6 and o8-M6. Results are expressed as relative CAT activity to that of the oIFNτ-o10-reporter plasmid and values represent means ± SEM. Results with different letters differ at $P < 0.01$ (n = 4 each).

protein was not made. Bands detected with oIFNτ-o10 and -o8 probes exhibited different sizes. The protein that bound to oIFNτ-o10 probe appeared Ets-2 since the band disappeared by the use of Ets-2 consensus sequence, which had been shown previously in the laboratory (Yamaguchi et al., 2000). However, a protein bound to oIFNτ-o8 probe, larger than the one with oIFNτ-o10, may not be Ets-2 as it was not competed off with the Ets-2 consensus competitor (Fig. 6).

Because the o8-M6 construct did not exhibit a high degree of transactivation (Fig. 4), protein binding to this probe was also examined. The 23 bp of o8-M6 probe showed specific binding, the same pattern as oIFNτ-o10 probe, though the binding appeared a little weaker than that of the oIFNτ-o10 probe (Fig. 7). This result indicated that Ets-2 protein could bind to the o8-M6 region, oIFNτ-o8 with the Ets-2 sequence.

DISCUSSION

This is the first report that investigates the molecular basis for differential transcription of *oIFNτ* genes in the ovine uterus. The results demonstrated that high and low expression levels from *oIFNτ-o*10 and *oIFNτ-o*8/*o*2 genes, respectively, are probably due to a short, contiguous region of the promoter that is unique to the *oIFNτ-o*10 gene. Because a ruminant trophoblast cell line proper for IFNτ analyses has not been established, human choriocarcinoma JEG3 or JAR cells have been
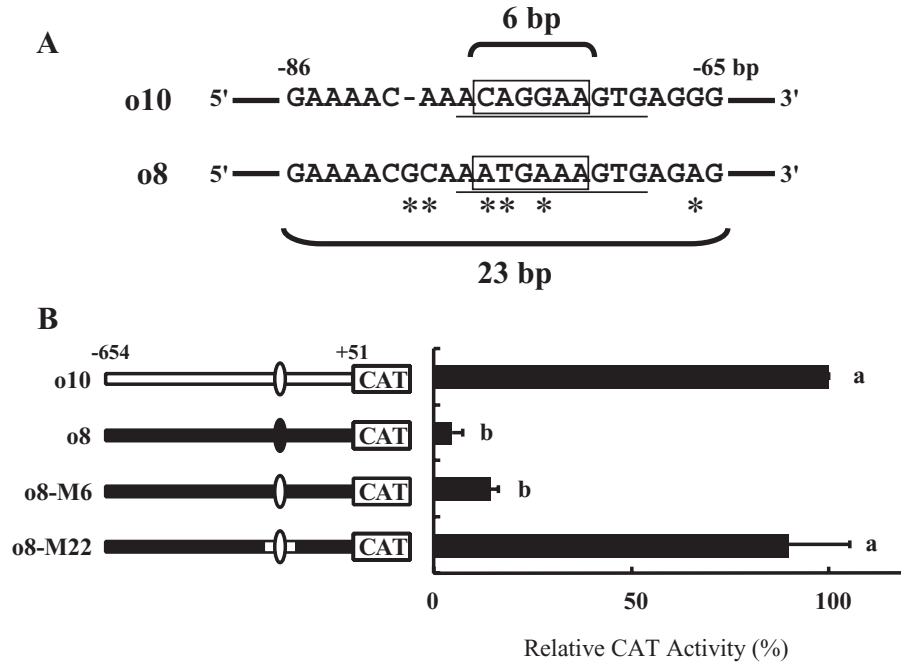
Fig. 5. Transcriptional activity of oIFNτ-o8-reporter construct after the short promoter region replacement. **A**: Comparison of the 22 bp sequences around Ets-2 binding site of oIFNτ-o10 (−86 to −65 bp) and the equivalent region of oIFNτ-o8. The different bases for oIFNτ-o10 and -o8 are indicated by asterisk. The core 6 bp shown in Figure 4A is indicated in boxes and a 10 bp Ets-2 sequence is underlined.

B: Transcriptional activities of wild-type oIFNτ-o10 and -o8, and those of o10-M6 and o8-M22 in which the 23 bp was exchanged with the 22 bp of oIFNτ-o10. Results are expressed as relative CAT activity to that of the oIFNτ-o10-reporter plasmid and values represent means ± SEM. Results with different letters differ at $P < 0.01$ (n = 4 each).
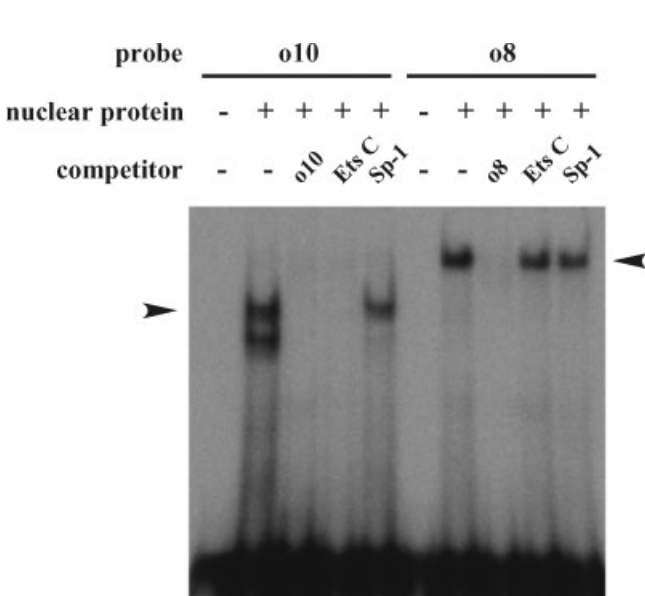

Fig. 6. Electrophoretic mobility shift assay (EMSA) analyses on 22 and 23 bp of oIFNτ-o10 and -o8 promoter regions. The sequences of oIFNτ-o10 and -o8 probes are shown in Figure 5A. Bindings of nuclear protein extracts from JEG3 cells to γ-^{32}P labeled oIFNτ-o10 or -o8 were examined. Specificities of the shifted bands were examined following addition of non-labeled oligonucleotide of the same sequences, Ets-2 consensus sequences (Ets C) and unrelated Sp-1 consensus sequence. A representative EMSA gel from three independent analyses is shown.
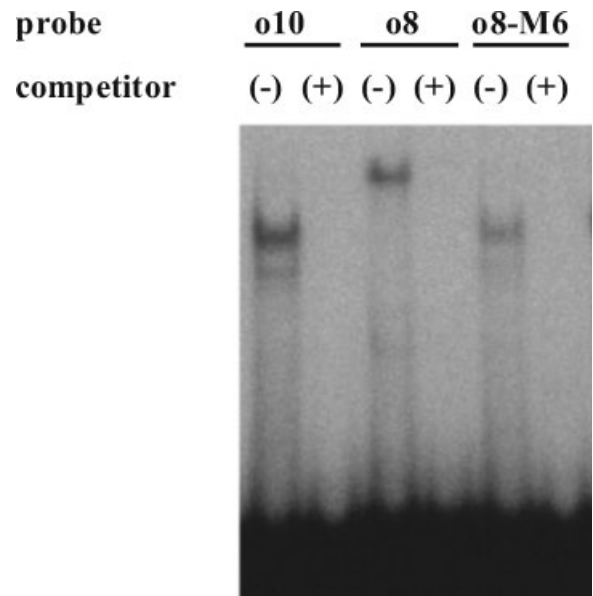
Fig. 7. EMSA analyses on o8-M6 probe. The oIFNτ-o8's 23 bp region inserted with the core 6 bp Ets-2 binding site from oIFNτ-o10 (5'-GAAAACGCAACAGGAAGTGAGAG-3', Ets-2 site inserted is underlined) was examined using nuclear extracts from JEG3 cells. Ovine IFNτ-o10 (22 bp) and -o8 (23 bp) probes were used as controls. Specificity of protein binding to IFNτ-o10 and -o8 probes was examined using non-labeled oligonucleotide of the same sequences (oIFNτ-o10, -o8, or o8-M6). A representative EMSA gel from three independent analyses is shown.

used to study regulatory mechanisms for *IFNτ* gene transcription. In fact, human trophoblast derived JEG3 and JAR cells have been shown to support the transcriptional activity of IFNτ-reporter constructs (Leaman et al., 1994; Yamaguchi et al., 1999). In this study, the CAT activity resulted from the upstream region of wild-type *oIFNτ-o*10, *-o*8 and *-o*2 genes transfected to JEG3 cells reflected the expression levels of these genes in utero (Nephew et al., 1993). These results indicate that the expressions of *oIFNτ* genes are regulated at transcriptional level and transient transfection analyses using JEG3 cells is one system for examining the regulatory mechanisms of *oIFNτ* gene expressions.

The upstream regions at −452 bp of *oIFNτ-o*10, *-o*8, and *-o*2 genes were digested with *Hind III*, resulting in the separation of distal enhancer (−654 to −452 bp) and proximal promoter (−451 to +51 bp) regions. When the enhancer and promoter regions were interchanged heterologously and examined for their transactivation, the promoter of *oIFNτ-o*10 gene's proximal region was shown to possess strong activation (Fig. 1). As shown in Figure 4A, only the *oIFNτ-o*10 gene contains the Ets-2 binding sequence at −77 to −72 bp of the promoter region and this difference between *oIFNτ-o*10 and *oIFNτ-o*8/*o*2 genes was thought to determine the transcriptional expression levels of these genes. This hypothesis was confirmed by point mutation analyses of Ets-2 binding site, and by the 6 bp insertion of the oIFNτ-o8 sequence into the core 6 bp Ets-2 binding sequence of oIFNτ-o10 (Figs. 2–4). Similar to our previous observation (Yamaguchi et al., 2000), the binding of Ets-2 to the oIFNτ-o10's short promoter region was demonstrated by the EMSA analyses (Fig. 6). This result, the requirement of Ets-2 for oIFNτ-reporter transactivation, agrees with previous observation made by Ezashi et al. (1998).

It appeared that a protein binding to the Ets-2 site dictated the oIFNτ gene transcription, however, insertion of the core 6 bp sequence of Ets-2 binding site (CAGGAA) failed to increase the transactivation of oIFNτ-o8-reporter construct (Fig. 4). As shown in Figure 5A, it should be noted that because nucleotide sequences at both sides of this 6 bp were the same between oIFNτ-o10 and -o8, the Ets-2 site examined was the same as the 10 bp consensus Ets-2 site (Wasylyk et al., 1993). Thus, a lack of transactivation with the 6-bp insertion was not due to insufficient Ets-2 binding site. When the 22 bp sequence from the *oIFNτ-o*10 gene promoter, including the Ets-2 binding site (GAAAAC-AA<u>ACAGGAAGTGA</u>GGG; 10 bp Ets-2 site underlined), was inserted into the equivalent region of oIFNτ-o8-reporter plasmid, this mutation construct, o8-M22, increased the CAT activity to the level exhibited by the oIFNτ-o10 construct (Fig. 5B). From these results, it was suspected that Ets-2 protein did not bind to the core 6 bp Ets-2 binding sequence that had been inserted into the oIFNτ-o8's promoter region, o8-M6. Therefore, a different binding pattern was expected in EMSA with the o8-M6 probe, but the binding pattern was the same as the oIFNτ-o10 probe except that the binding signal of o8-M6 appeared to be weaker (Fig. 7). It

is possible that the flanking sequence around Ets-2 site may position the Ets-2 site such that it can act with other proteins, and that the sequence per se may not be relevant but the position of the Ets binding site is critical. It is likely that in addition to the 10 bp Ets-2 binding site, the 22 bp sequence may possess another transcription binding site(s), which increases oIFNτ-o10 gene transcription.

By searching for a possible transcription factor that binds to this oIFNτ-o10 short promoter region, but does not bind to the equivalent region of *oIFNτ-o*8/*o*2 gene, a candidate protein, hepatocyte nuclear factor-3 beta (HNF-3β), was found. Co-transfection analyses with expression vector of HNF-3β (Dr. R.H. Costa, University of Illinois) and point mutation analyses to the HNF-3β binding site of the oIFNτ-o10 short promoter region were performed, however, our hypothesis was not verified with these experiments (unpublished observations). Within the 23 bp region of the *oIFNτ-o*8/*o*2 gene, a nuclear factor Oct-4 binding site resides at the same location as the Ets-2 site exists in the *oIFNτ-o*10 gene. In the porcine and bovine species, however, the expression of Oct-4 is not restricted to ICM (Kirchhof et al., 2000), and has been demonstrated to inhibit bIFNτ gene transcription by binding to Ets-2 protein (Ezashi et al., 2001). For these reasons, co-transfection of the oIFNτ-o10-reporter plasmid with Oct-4 expression plasmid (Dr. C. Meno, Osaka University, Japan) was also performed, resulting in the reduction of oIFNτ-o10-reporter transactivation (data not shown). Furthermore, identity of a higher molecular weight protein, which was found in the EMSA experiment with the oIFNτ-o8/o2 probe, was not determined in this study. It is possible, however, that instead of Ets-2 site, the binding of Oct-4 itself or together with other transcription factor(s) to this 23 bp region may repress the expression of *oIFNτ-o*8/*o*2 gene in vivo.

In summary, using the 5′-upstream region of *oIFNτ-o*10, *-o*8, and *-o*2, the 22 bp with Ets-2 binding sequence of oIFNτ-o10 was found to be the short promoter region required for the full activation. From this and the previous observations on *IFNτ* genes' transcriptional regulation, Ets-2 should be considered an essential factor for the expression of *oIFNτ-o*10 gene (Yamaguchi et al., 2000; Ezashi et al., 2001). While Ets-2 was the factor critical for the oIFNτ-reporter transactivation, point mutation to the AP-1 binding site decreased the degree of oIFNτ-reporter transactivation (Fig. 2 and 3). Considering this observation with the results from Yamaguchi et al. (1999, 2000), AP-1 might also be the transactivation molecule of oIFNτ genes acting in concert with Ets-2 for higher degree of transcription (Gutman and Wasylyk, 1990; McCarthy et al., 1997). Since AP-1 and Ets-2 are relatively common transcription factors detected in many cell types, another factor, which by itself determines spatial and/or temporal expression of *oIFNτ* genes, is required. A factor that dictates AP-1 and Ets-2 to be spatial and/or temporal expression may also exist in the regulation of *IFNτ* gene expression.

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