# Identification of a functional transcriptional factor AP-1 site in the sheep interferon τ gene that mediates a response to PMA in JEG3 cells

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To examine regulatory mechanisms of sheep interferon $\tau$ (oIFN$\tau$) gene expression, potential enhancer/silencer elements of the oIFN$\tau$ gene were examined using a transient transfection system with oIFN$\tau$ gene–chloramphenicol acetyltransferase (oIFN$\tau$–CAT) reporter constructs in human choriocarcinoma cells, JEG3. Experiments with 5′-deletion constructs revealed that the upstream regions from bases −654 to −607 and from bases −606 to −555 were essential for oIFN$\tau$ gene expression. In a heterologous transcriptional system in which the upstream regions of oIFN$\tau$ were inserted in front of simian virus 40 (SV40) promoter, the regions between bases −654 and −555 were determined as being the enhancer region required for oIFN$\tau$–SV40–CAT transactivation. A subsequent study with the oIFN$\tau$–CAT constructs lacking the upstream region between bases −542 and −124 revealed that, in addition to the further upstream region between bases −1000 and −654, the sequences from bases −543 to −452 seemed to act as silencer regions. The oIFN$\tau$–CAT constructs with site-specific mutagenesis revealed that multiple enhancer elements existed between bases −654 and −555 of the oIFN$\tau$ gene. On the basis of nucleotide sequence analysis, there are numerous sites between bases −654 and −555 to which potential transcriptional factors, AP-1, GATA and GATA-related proteins, could bind. Furthermore, gel mobility-shift assays revealed that AP-1 or other nuclear factors could bind to these elements. In co-transfection studies, the expression of c-Jun plus c-Fos enhanced the transactivation of oIFN$\tau$–CAT but the expression of GATA-1, GATA-2 or GATA-3 did not. Taken together, these results suggest that the upstream region between bases −654 and −555 could be considered as the enhancer region for oIFN$\tau$ gene transactivation.

Key words: CAT reporter gene, enhancer elements, interferon gene expression, transcription.

## INTRODUCTION

In ruminant ungulates, large amounts of an anti-luteolytic factor, interferon $\tau$ (IFN$\tau$), are produced by the peri-implantation conceptus [1–4]. IFN$\tau$ is initially detected in a very small quantity soon after the blastocyst hatches [5]. In sheep, the large increase in IFN$\tau$ production by the trophectoderm is detected on day 13 of pregnancy [2,5]. The quantity of sheep IFN$\tau$ (oIFN$\tau$) production is positively correlated with the degree of trophectodermal elongation: on day 16, when conceptus elongation subsides and attachment to the endometrium begins, the highest production of oIFN$\tau$ is observed [2,6]. oIFN$\tau$ production declines subsequently and is not detectable after day 21 [2]. IFN$\tau$ acts on the endometrium to attenuate prostaglandin $F_{2\alpha}$ synthesis and/or secretion; consequently, luteolysis is prevented and progesterone secretion is maintained [7]. These processes are known collectively as the maternal recognition of pregnancy and are essential for the establishment of gestation in ruminants [8].

IFN$\tau$ is structurally related to IFN$\alpha$, IFN$\beta$ and IFN$\omega$ and has antiviral and anti-proliferative activities [9,10]. Unlike IFN$\alpha$ or IFN$\beta$, however, IFN$\tau$ expression is not induced by virus or double-stranded RNA [11]. A transcription factor, interferon regulatory factor 1, IRF-1, which regulates the expression of the IFN$\alpha$ and IFN$\beta$ genes, is not involved in IFN$\tau$ gene transcription [12,13]. More importantly, the expression of IFN$\tau$ is sustained for five or more days, whereas other IFNs are produced for only a few hours after viral stimulation [4]. These results support the notion that transcription of the IFN$\tau$ gene is regulated by mechanisms other than common induction pathways for IFN$\alpha$ or IFN$\beta$. In tissue culture systems *in vitro*, oIFN$\tau$ production is stimulated by the addition of granulocyte/macrophage colony-stimulating factor, interleukin 3 or PMA [14,15]. The effect of glycosylated granulocyte/macrophage colony-stimulating factor or PMA on the production of oIFN$\tau$ is confirmed by transient transfection studies with the human choriocarcinoma cell lines JAR and JEG3 [16]. These cell lines have typically been used because of a lack of trophoblast cell lines from ruminant ungulates [12,17,18].

To understand better the mechanisms regulating IFN$\tau$ gene expression, enhancer or silencer elements of the IFN$\tau$ gene and possible transcription factors have been investigated. Leaman et al. [12] have suggested that the basal promoter region for the transactivation of the bovine IFN$\tau$ gene is located between bases −34 and −126 of its upstream sequence. These investigators also suggest that the upstream region between −280 and −400 might act as an enhancer. However, Guesdon et al. [18] show that silencer regions exist in the bovine IFN$\tau$ gene between bases −338 and −247 and between bases −150 and −71. These results are difficult to interpret because the degrees of transactivation detected by these investigators differ greatly from

Abbreviations used: CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; GMSA, gel mobility-shift assay; IFN$\tau$, interferon $\tau$; oIFN$\tau$, sheep interferon $\tau$; SV40, simian virus 40.

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those detected *in vivo*. In either case, the upstream regions of the IFN$\tau$ gene that regulate the degree of production of IFN$\tau$ *in vivo* have not been identified.

Recently, Ezashi et al. [19] showed that there is an Ets-2-binding site in the upstream sequence of the bovine IFN$\tau$ gene and that, when co-transfected into the human choriocarcinoma cell line JAR, Ets-2 enhances the transcriptional activity of the bovine IFN$\tau$ gene. AP-1, the major factor activated by PMA [20,21], has been shown to enhance IFN$\tau$ gene transactivation [13]. However, because Ets-2 and AP-1 are quite common transcription factors, they might not sufficiently explain the specific expression of IFN$\tau$ seen during the peri-implantation period.

We have shown previously [13] that the bacterial chloramphenicol acetyltransferase (CAT) reporter gene under the control of $-654$ bases upstream of the oIFN$\tau$ gene had significant transactivation after treatment with 50 nM PMA: approx. 50% of the CAT activity of the CAT construct containing a $\beta$-actin-promoter. The specific aims of the present investigation were therefore: to identify the possible enhancer regions of the oIFN$\tau$ gene by using various deletion or mutation constructs in transient transfection experiments with JEG3 cells; to demonstrate by gel mobility-shift assays (GMSAs) that several nuclear factors including AP-1 could bind to these elements; and to evaluate whether various transcription factors could enhance the transactivation of the oIFN$\tau$–CAT construct in the co-transfection study.

## MATERIALS AND METHODS

### Plasmid constucts

Construction of the oIFN$\tau$–CAT ($-654$oIFN$\tau$–CAT) plasmid has been described previously [13]. To prepare 5′-deletion constructs, upstream regions of the oIFN$\tau$ gene (oIFN$\tau$ o10, accession number M88773 [22]), identical with those reported by Charlier et al. [23], were amplified by PCR and inserted into pCAT basic vector (Promega, Madison, WI, U.S.A.). Constructs lacking the middle portion of $-654$oIFN$\tau$–CAT and those with site-specific mutations were prepared by using an inverse PCR procedure [24] with Ex-Taq™ (TaKaRa, Tokyo, Japan). Nucleotide sequences confirmed by dideoxy sequencing indicated that all constructs had the exact sequences originally intended.

To construct a plasmid with the 5′ regions of oIFN$\tau$ fused to the simian virus 40 (SV40) promoter, the various segments of the 5′ regions of oIFN$\tau$ were amplified by PCR and inserted into the pCAT®3-promoter vector (Promega); the nucleotide sequences of these constructs were confirmed by DNA sequencing. Human GATA-1 and GATA-2 expression vectors are under the control of human elongation factor 1$\alpha$ promoter, which exhibits high transactivity in various cell types [25]. The human GATA-3 expression vector was obtained from Dr. J. D. Engel (Northwestern University, Evanston, IL, U.S.A.). The nucleotide structure of murine c-Jun and c-Fos expression vectors, pRVSV c-Jun and c-Fos respectively, have been described previously [13]. The expression of c-Jun and c-Fos in these plasmids is driven by the Rous sarcoma virus long terminal repeat. The Ets-2 expression vector was a pSG5-based construct containing the SV40 promoter/enhancer [26].

### Cell culture and transient transfection

Human choriocarcinoma cells, JEG3, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 40 i.u./ml penicillin and 40 $\mu$g/ml streptomycin. Cells were plated in six-well plastic microplates and grown to 50–60% confluence. Plasmid DNA was transfected into cells by using TransFast™ (cationic lipids; Promega). In brief, 4 $\mu$g of DNA construct and 15 $\mu$l of TransFast™ were mixed in 1 ml of DMEM. After 12 min, plated cells were overlaid with DNA–cationic lipids and incubated at 37 °C for 1 h. To each well, 5 ml of DMEM containing PMA (final concentration 50 nM) was added and cell incubation continued at 37 °C for 48 h under air/$CO_2$ (19:1). To assess background levels of CAT activity, pCAT basic plasmid without enhancer and/or promoter regions of the IFN$\tau$ o10 gene was transfected into JEG3 cells. For co-transfection studies, 3 $\mu$g of reporter plasmid and 1 $\mu$g of each expression vector or control plasmid were used for transfection.

### CAT assay

At 48 h after transfection, the cells were washed twice with 1 ml of PBS. The transfected cells were lysed by freezing and thawing three times in 50 $\mu$l of 250 mM Tris/HCl, pH 7.8. Cell debris was pelleted by centrifugation at 10000 ***g*** for 10 min and 20 $\mu$l of the extract was used for the CAT assay. CAT activity was assayed by the solvent partition method [27]. In brief, a 240 $\mu$l reaction mixture containing 100 mM Tris/HCl, pH 7.8, 1 mM chloramphenicol, 3.7 kBq of [$^{14}$C]acetyl-CoA (NEN, Boston, MA, U.S.A.) and the cell extract was overlaid with 5 ml of scintillation fluid (Econofluor2; Packard, Meriden, CT, U.S.A.). Reactions were performed at 37 °C and the production of [$^{14}$C]acetyl-chloramphenicol was monitored by liquid-scintillation counting. For the determination of CAT activity due to oIFN$\tau$–CAT, background CAT activity was first determined from the lysate of JEG3 cells that had been transfected with pCAT basic vector without enhancer/promoter regions of oIFN$\tau$. Net radioactivity was calculated by subtracting background CAT radioactivity from that of the sample with oIFN$\tau$–CAT constructs. These were then expressed as values (fold activation) relative to an appropriate control within the experiment. Results are expressed as means $\pm$ S.E.M. for three separate experiments. Differences in CAT activity were tested by a one-way analysis of variance, followed by Scheffe's multiple comparison test with STATVIEW 4.0 (Abacus Concepts, Berkeley, CA, U.S.A.).

### Preparation of nuclear extracts

Nuclear extracts from JEG3 cells were prepared as described previously [28]. In brief, $(1–2) \times 10^6$ cells were collected and washed twice with PBS. These cells were resuspended in 400 $\mu$l of buffer A [10 mM Hepes (pH 7.9)/10 mM KCl/0.1 mM EDTA/0.1 mM EGTA/1 mM dithiothreitol/0.5 mM PMSF/2 $\mu$g/ml leupeptin/2 $\mu$g/ml aprotinin] and left to swell on ice for 15 min. Nonidet P40 solution (25 $\mu$l; 10%, v/v) was added and the tube was vortex-mixed vigorously for 10 s. After centrifugation, the nuclear pellet was resuspended in 50 $\mu$l of buffer C [20 mM Hepes (pH 7.9)/0.4 M NaCl/1 mM EDTA/1 mM EGTA/1 mM dithiothreitol/1 mM PMSF/2 $\mu$g/ml leupeptin/2 $\mu$g/ml aprotinin]. The tube was then shaken for 15 min at 4 °C on a shaking platform. The nuclear extracts were centrifuged for 5 min at 4 °C and the supernatant collected was frozen in aliquots at $-80$ °C.

### GMSA and gel supershift assay

Various upstream sequences (probes) representing oIFN$\tau$ enhancer regions used for GMSAs are shown below (see Figure 5). Oligonucleotide probes representing consensus AP-1, GATA, Sp-1 and GATA mutation sequences were 5′-CGCTTGA-TGAGTCAGCCGGAA-3′, 5′-CACTTGATAACAGAAAG-TGATAACTCT-3′, 5′-ATTCGATCGGGGCGGGGCGAGC-

3′and5′-CACTTCTTAACAGAAAGTCTTAACTCT-3′respectively, with the positions of the AP-1, GATA, Sp-1 and mutated GATA sites underlined. These oligonucleotide probes were end-labelled with [$\gamma$-$^{32}$P]ATP (NEN) and T4 polynucleotide kinase (Promega), which were then purified with NICK Columns® (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). For GMSA, 4 $\mu$g of nuclear extract was incubated in a binding buffer containing 50 $\mu$g/ml poly(dI-dC)·poly(dI-dC), 10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA and 4% (v/v) glycerol for 20 min at room temperature with or without a 100-fold molar excess of unlabelled competitor. After incubation, 0.175 pmol of $^{32}$P-labelled probe was added and the mixture was incubated for an additional 20 min at room temperature. Reaction mixtures were then applied to 4% (w/v) polyacrylamide gel in 0.5 × TBE (Tris/borate/EDTA) buffer. After electrophoresis, the gel was dried and autoradiographed. The supershift assay for AP-1 was performed with anti-(human c-Fos) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). In brief, 4 $\mu$g of nuclear extract was incubated in the binding buffer, as described above, for 20 min at room temperature. After incubation, 0.175 pmol of $^{32}$P-labelled probe was added to the mixture and incubated for an additional 20 min at room temperature. Subsequently, 2 $\mu$g of anti-(c-Fos) antibody or control IgG was added and the sample mixture was incubated for 15 min at 37 °C. Reaction mixtures were then applied to 4% (w/v) polyacrylamide gel in 0.5 × TBE buffer. After electrophoresis, the gel was dried and autoradiographed.

## RESULTS

### Analysis of 5′-deletion constructs

Of the oIFN$\tau$–CAT constructs representing 5′-sequential deletions, the −654oIFN$\tau$–CAT construct exhibited the highest CAT activity after treatment with PMA; other constructs demonstrated remarkably lower ($P < 0.01$) CAT activities (Figure 1). The −1000oIFN$\tau$–CAT construct exhibited a lower CAT activity than the −654oIFN$\tau$–CAT ($P < 0.01$). These results suggest that one or more enhancer regions might exist between bases −654 and −452, whereas a silencer lies between bases −1000 and −654 of the oIFN$\tau$ gene. To investigate the enhancer elements in detail, −606oIFN$\tau$–CAT, −555oIFN$\tau$–CAT, −503oIFN$\tau$–CAT and constructs lacking the portions between

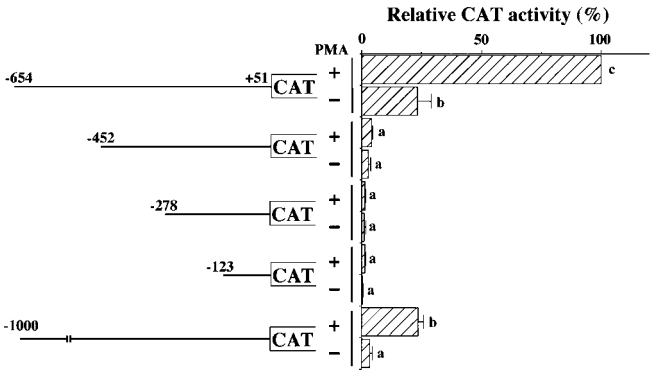


**Figure 1 Deletion analysis of enhancer/promoter regions of the oIFNτ gene**

CAT constructs with oIFN$\tau$ 5′-deletion mutants were transfected into JEG3 cells; the cells were then treated with 50 nM PMA. CAT activities are expressed relative to that of the control (−654oIFN$\tau$–CAT). Results with different letters differ at the $P < 0.01$ level.

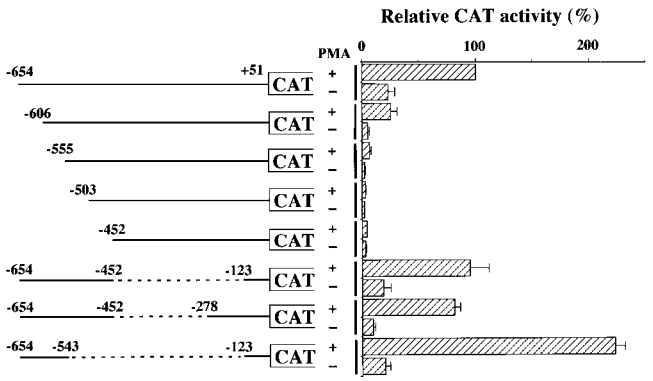


**Figure 2 Detailed deletion analysis of the upstream regions of the oIFNτ gene in transfected JEG3 cells**

Deletion analysis was further conducted in the region between bases −654 and 452 of the oIFN$\tau$ gene. CAT activities are expressed relative to that of the control (−654oIFN$\tau$–CAT).

potential enhancer and basal promoter regions were constructed and transfected into JEG3 cells that were then treated with 50 nM PMA (Figure 2). Relative CAT activities were: −654oIFN$\tau$–CAT > −606oIFN$\tau$–CAT > −555oIFN$\tau$–CAT, −503 or −452oIFN$\tau$–CAT ($P < 0.01$). The deletion mutants lacking the regions between bases −452 and −123 (Δ−452–123oIFN$\tau$–CAT) or bases −452 and −278 (Δ−452–278oIFN$\tau$–CAT) exhibited a high degree of CAT activity. In fact, the CAT activity was similar to that of the original construct, −654oIFN$\tau$–CAT, indicating that the sequence between bases −654 and −452, but not the region between bases −452 and −123, is essential for oIFN$\tau$ gene transactivation. However, the CAT activity of the deletion mutant lacking the sequence between bases −543 and −123 (Δ−543–278oIFN$\tau$–CAT) was approx. 2.2-fold higher than that of the −654oIFN$\tau$–CAT construct ($P < 0.01$). These results suggest that the enhancer sequence of the oIFN$\tau$ gene is located in the upstream region between bases −654 and −606 and bases −606 and −555, whereas the silencer sequence is between bases −543 and −452.

### Enhancer analysis with a heterologous promoter

To study further the enhancer activity of upstream regions of the oIFN$\tau$ gene shown in Figures 1 and 2, CAT constructs with the possible oIFN$\tau$ enhancer regions inserted in front of the SV40 promoter were transfected into JEG3 cells and treated with 0 or 50 nM PMA (Figure 3). The highest activity was observed when the region between bases −659 and −543 of the oIFN$\tau$ gene was used as an enhancer for SV40 promoter CAT (−659–543oIFN$\tau$–SV40–CAT). However, oIFN$\tau$–SV40–CAT containing the sequence between bases −659 and −602, the region determined as a possible enhancer element (Figure 2), did not exhibit enhancer activity. Enhancer activity was also detected when the sequence between bases −602 and −543 was examined, suggesting that two distinct sequences, bases −659 to −602 and bases −602 to −543, are required for the transactivation of oIFN$\tau$–SV40–CAT constructs. Constructs −659–444oIFN$\tau$–SV40–CAT and −602–444oIFN$\tau$–SV40–CAT exhibited a lower CAT activity than −659–543oIFN$\tau$–SV40–CAT or −602–543oIFN$\tau$–SV40–CAT ($P < 0.01$). These results suggest the presence of one or more silencer regions between bases −543 and −444, which is consistent with the results shown in Figure 2.

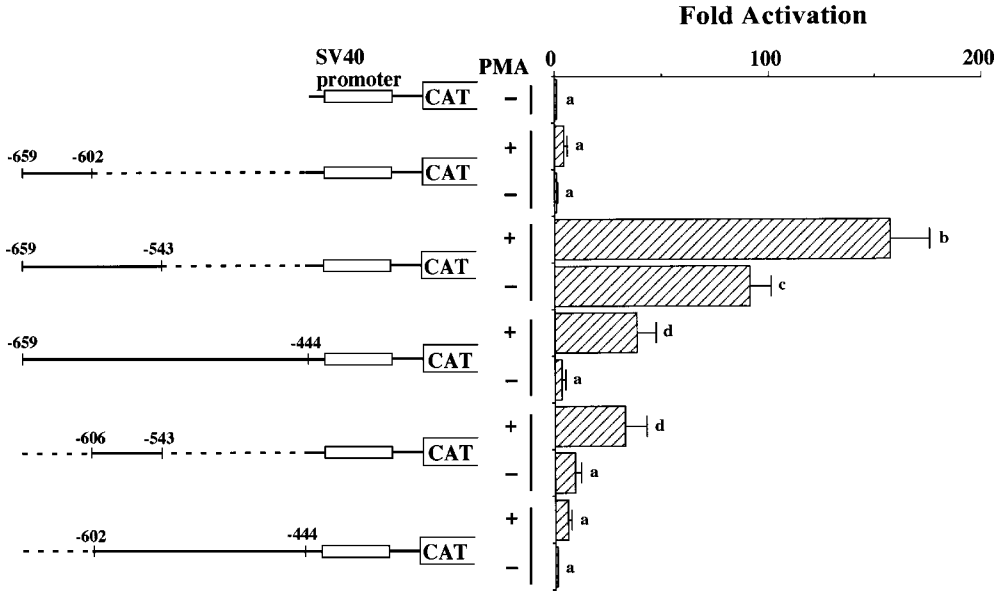


**Figure 3 Upstream region of the oIFNτ gene regulates and confers the PMA response with SV40 promoter on JEG3 cells**

Various fragments from the upstream region of the oIFN$\tau$ gene were inserted in front of the SV40 promoter and assayed for CAT activity in the absence or presence of PMA. Results are shown as fold activation relative to the control plasmid (SV40–CAT). Results with different letters differ at the $P < 0.05$ level.

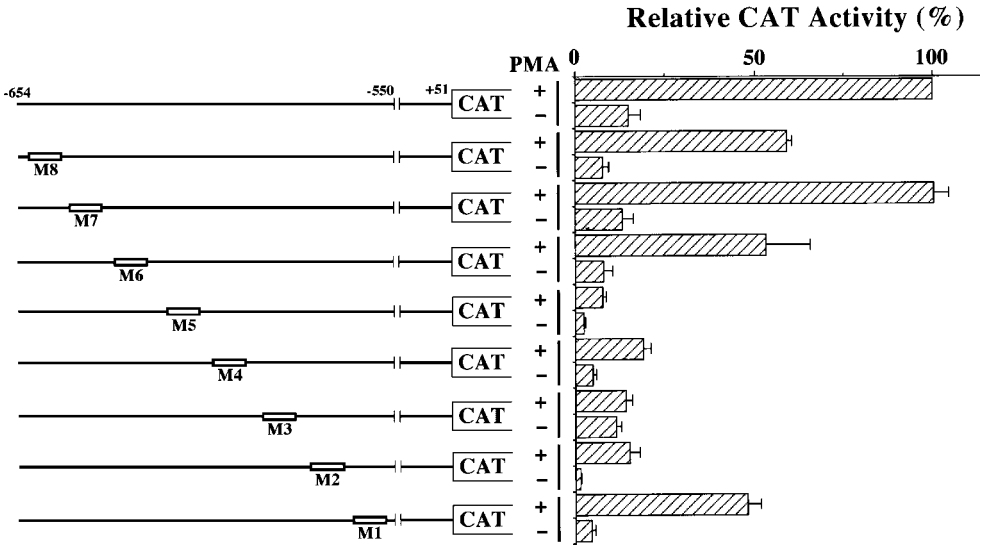


**Figure 4 Site-specific mutagenesis analysis of possible enhancer elements of the oIFNτ gene**

Ten bases each of the enhancer region replaced with the sequence of *Xho*I and *Bgl*II linker were linked to CAT reporter plasmid and tested in the absence or presence of PMA. CAT activities are expressed relative to that of the control (−654oIFN$\tau$–CAT).

### Analysis of possible enhancer elements by site-specific mutation

To understand the enhancer elements in detail, reporter plasmids with site-specific mutations were constructed: ten sequences between bases −654 and −550 of the oIFN$\tau$ gene were replaced with *Xho*I and *Bgl*II linker (CTCGAGATCT) (Figure 4). Mutation constructs of oIFN$\tau$–CAT (M2 to M5) showed a lower CAT activity, suggesting that the enhancer sequence is located in the upstream region between bases −616 and −568. A GATA-like sequence is present in M2 and an AP-1-like sequence is found in M3 (Figure 5).

### Examination of nuclear factors by GMSAs

To determine whether the positive enhancer elements between bases −616 and −568 (Figures 1–4) really function as nuclear-factor-binding sites, GMSAs were performed with nuclear

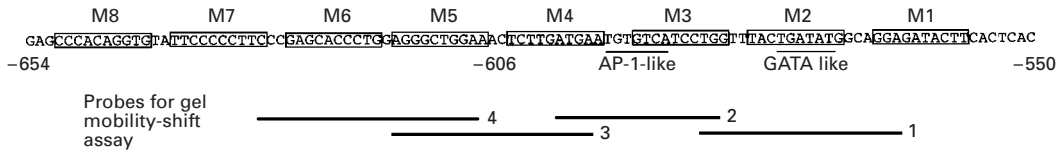


**Figure 5 Nucleotide sequences of possible enhancer elements of the oIFNτ gene**

Sites of specific mutations (see Figure 4) are boxed; AP-1-like and GATA-like sequences are underlined. Nucleotide sequences used for GMSA (see Figure 6) are indicated.

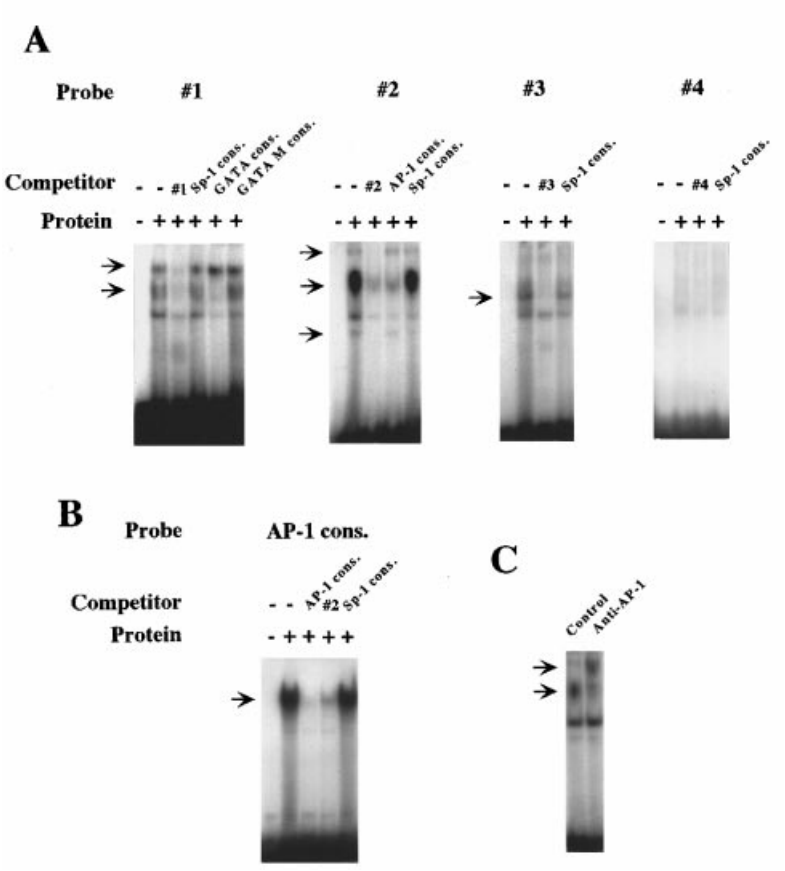


**Figure 6 GMSA and gel supershift assay**

(**A**) End-labelled oligonucleotide probes (1, 2, 3 and 4) shown in Figure 5 were incubated with nuclear extracts from JEG3 cells to demonstrate the formation of DNA–protein complexes. The specificity of each DNA–protein complex was assessed by competition assay with a 100-fold molar excess of unlabelled identical or heterologous (Sp-1 consensus sequence; Sp-1 cons.) competitor. In addition, specificity was further determined for probe 1 or 2 by competition with GATA consensus sequence (GATA cons.) and GATA mutation sequence (GATA M cons.) or AP-1 consensus sequence (AP-1 cons.) respectively. Arrows indicate the specific DNA–protein complex. (**B**) End-labelled AP-1 sequence was incubated with nuclear extracts of JEG3 cells. The arrow indicates the DNA–AP-1 protein complex that remained (Sp-1 cons.) or disappeared with the addition of a 100-fold molar excess of unlabelled identical competitor or probe 2. (**C**) Supershift analysis with end-labelled probe 2 and anti-(c-Fos) antibody. The control lane indicates probe 2 and AP-1 complex (lower arrow), and the anti-AP-1 lane indicates the supershift band (upper arrow).

extracts from JEG3 cells that had been treated with 50 nM PMA. On the basis of the results shown in Figure 4, four upstream regions (probes) were chosen for this experiment (Figure 5). Specific binding was observed with probes 1, 2 and 3, but not with probe 4. These bindings were competed for by the addition of a similar unlabelled competitor but were not affected by an unrelated competitor such as the Sp-1 consensus sequence (Figure 6A). When a GATA consensus probe was used as the competitor for the GATA-like sequence located in probe 1, a lower band disappeared. The addition of an unlabelled GATA mutation probe have no effect on binding, suggesting that probe 1 consists of the binding site for GATA family protein(s). The upper band was not affected by the addition of the GATA consensus probe, but was competed for by unlabelled probe 1. Taken together, these results indicate that GATA-related protein and another nuclear factor might recognize the sequence of probe 1. When labelled probe 2 was used, one distinct band and two weaker bands were observed. A putative AP-1 site exists within the sequence of probe 2; therefore an AP-1 consensus probe was used as competitor. Use of the unlabelled AP-1 consensus probe resulted in the disappearance of the distinct band, whereas the upper band was unaffected. A specific protein was able to bind

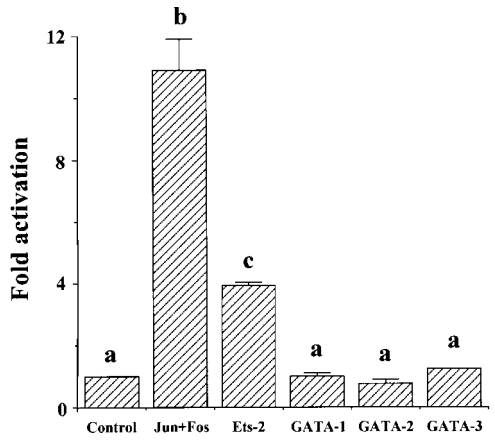


**Figure 7 Transactivation of −654oIFNτ–CAT by murine c-Jun plus c-Fos, human Ets-2 or human GATA-1, GATA-2 or GATA-3**

The CAT construct −654oIFN$\tau$–CAT and an expression plasmid (control, pRVSV or transcription factors) were co-transfected into JEG3 cells and CAT activity was measured. CAT activities are expressed relative to that of the control (−654oIFN$\tau$–CAT). Results with different letters differ at the $P < 0.01$ level.

to the labelled probe 3, which could be competed off with unlabelled probe 3. However, this nuclear protein could not be identified because there is no putative binding site in the nucleotide sequence represented by probe 3. Probe 4 did not seem to have any specific binding of any nuclear proteins. In GMSA with the labelled AP-1 consensus probe (Figure 6B), the band disappeared with the use of unlabelled probe 2. Furthermore, when a gel supershift assay with an anti-(c-Fos) antibody was performed, a supershift band was observed (Figure 6C). These results indicate that a putative AP-1 site located in the upstream region of the oIFN$\tau$ gene might in fact act as the AP-1 recognition site.

### Effects of several transcription factors

To determine the possible significance of GATA family transcription factors in the transactivation of oIFN$\tau$–CAT, co-transfection studies were performed with GATA-1, GATA-2 or GATA-3 expression plasmids (Figure 7). Co-transfection of −654oIFN$\tau$–CAT with positive controls, c-Jun plus c-Fos or Ets-2 expression plasmid, demonstrated enhanced transactivation of the oIFN$\tau$ gene. However, the expression of GATA-1, GATA-2 or GATA-3 had no significant effect on the transactivation of −654oIFN$\tau$–CAT.

## DISCUSSION

In the present investigation, the promoter/enhancer elements of the oIFN$\tau$ gene were analysed by using a transient transfection system with various oIFN$\tau$–CAT constructs in the human choriocarcinoma cell line JEG3. These experiments represent heterologous systems but the constructs (−654oIFN$\tau$–CAT) examined exhibited relatively high levels of transactivation, suggesting that sufficient transcription factors to regulate oIFN$\tau$–CAT constructs were activated by PMA in this cell line [13].

In all previous experiments studying the enhancer and silencer regions of the IFN$\tau$ gene with the use of stable or transient transfection systems, the upstream region representing bases −450 and +32 was used [12,17]. The use of this upstream region is based on the fact that a *Hin*dIII site is located at −450 bases from the gene and that the regulation of the IFN$\alpha$ and IFN$\beta$ genes were studied with short upstream regions, typically at −150 bases [29]. The results obtained here support that the region up to −123 bases from the IFN$\tau$ gene is used for basal transcription, whereas the sequences between bases −654 and −555 are the enhancer region. Furthermore, two silencer sites might exist in the upstream sequence spanning bases −1000 to −654 and between bases −543 and −452. The results presented in Figure 2 indicate that a deletion from bases −654 to −606 in the oIFN$\tau$–CAT construct decreased CAT activity to nearly one-quarter and further deletion of the upstream region to −555 bases caused a decrease in CAT activity to less than one-tenth of the control (−654oIFN$\tau$–CAT). These results from sequential deletion studies were supported by the observation that the heterologous SV40 promoter fused to upstream regions of IFN$\tau$ exhibited similar results.

It should be noted, however, that the sequence between bases −654 and −602 did not exhibit an enhancer activity in the heterologous system (Figure 3). One possible explanation is that some factors that by themselves do not display enhancer activity could regulate transcription of the oIFN$\tau$ gene co-operatively with other factors bound to one or more proximal regions. Another possibility is that some factors that bind to the edge of the sequence at or around −602 bases from the oIFN$\tau$ gene could not bind to the region, resulting in a lose of transactivation of the oIFN$\tau$–SV40–CAT construct. The results obtained from GMSAs (Figure 6) suggest that a nuclear factor binds to the region upstream of M4 and M5 (probe 3). In the site-specific mutation studies, the mutations represented by M4 and M5 demonstrated a remarkable decrease in CAT activities. However, this sequence exhibited little or no similarity to any other putative transcription factor binding sites, suggesting that one or more unidentified factors possibly bind to this region and regulate oIFN$\tau$ gene transcription in a co-operative and/or independent manner.

The site-specific mutations introduced into the region between M2 and M5 (Figure 4) decreased CAT activities compared with that of the non-mutated control, suggesting that the sequences between bases −616 and −568 constitute the core of enhancer elements in the transactivation of the oIFN$\tau$ gene. Other mutations, M1, M6 and M8, had slightly decreased CAT activities, suggesting that these regions might also be enhancer regions. It is possible, however, that these elements affect oIFN$\tau$–CAT transactivation indirectly through secondary or tertiary DNA structure. The GMSA studies (Figure 6) indicated that several transcriptional factors can bind to the regions between M2 and M5 (Figures 4–6). We had previously shown that AP-1 enhances the transactivation of the −654oIFN$\tau$–CAT construct in transient transfection studies [13]. In the present study with the use of deletions, mutations, heterologous enhancer and GMSA, the putative AP-1 site in the −654oIFN$\tau$–CAT construct is most probably an AP-1 recognition site, suggesting that AP-1 binds to this region and up-regulates the expression of IFN$\tau$. This is confirmed by the observation that the oIFN$\tau$–CAT M3, which lacks the AP-1 site, did not exhibit CAT activity after treatment with PMA. In addition, the involvement of AP-1 in the transactivation of −654oIFN$\tau$–CAT was also demonstrated by a co-transfection study (Figure 7).

In these experiments, PMA had an effect on all constructs containing an AP-1-like sequence between bases −593 and −587. However, the degree of transactivation by PMA treatment varied. This could be because AP-1 might associate with one or more other transcription factors for full transactivation. We also showed that GATA family proteins bound to the M2 site *in vitro*

but the expression vectors consisting of GATA-1, GATA-2 or GATA-3 did not affect the transactivation of oIFN$\tau$–CAT in co-transfection experiments. These results suggest that GATA binds to the upstream region of the oIFN$\tau$ gene *in vitro* but might not be involved in IFN$\tau$ transcription *in vivo*. It is also possible that unless combined with one or more other transcription factors, GATA alone might not transactivate the IFN$\tau$ gene. The presence and participation of nuclear factors other than AP-1 and GATA that bind to putative enhancer elements and/or associate with AP-1 or GATA that regulate oIFN$\tau$ transcription remain to be determined.

Here we have identified novel enhancer regions to which several nuclear factors including AP-1 could bind. However, because these experiments were performed in heterologous systems, it is not clear whether these transcription factors are actively involved in oIFN$\tau$ transcription *in vivo*. Therefore experiments to identify the nuclear proteins in the conceptus that influence oIFN$\tau$ gene transactivation *in vitro* and *in vivo* are in progress.

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