Interferon-γ regulation of the human mimecan promoter

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Purpose: The human mimecan/osteoglycin promoter contains multiple interferon-stimulated response elements (ISRE) and interferon-γ-activation sites (GAS). ISRE and GAS motifs are present in a variety of interferon (IFN)-inducible genes. The purpose of this study was to investigate whether IFN-γ affects mimecan gene expression and, if so, to determine the cis-elements and transcription factors that mediate its action.

Methods: Electrophoretic mobility shift assay (EMSA) was used to investigate whether nuclear proteins from IFN-γ-treated cells bind to regions of the human mimecan promoter containing ISRE sites. Incubation of nuclear extracts with specific antibodies was used to identify transcription factors that bind to these sites. Transcriptional activity of the promoter was evaluated by transient transfections of human mimecan promoter/luciferase reporter constructs into corneal keratocytes and non-corneal cells. Co-transfection experiments were used to study the role of transcription factors that bind ISRE elements in the promoter and mediate the IFN-γ response. Expression of mRNAs was analyzed by reverse transcription-polymerase chain reaction.

Results: Using probes that correspond to two ISRE sites located in the first intron of the human mimecan gene, we detected specific DNA-protein complexes with nuclear extracts from IFN-γ-treated cells. Formation of DNA-protein complexes was abrogated by competition with unlabeled probe and one of the complexes was supershifted by the anti-interferon regulatory factor-1 (IRF-1) antibody. Interestingly, when probe that corresponds to a conserved E-box (CACATG) in the proximal promoter and nuclear extracts from IFN-γ-treated cells were used in EMSA, increased binding of upstream stimulatory factor-1 (USF-1) was observed. Co-transfection of a mimecan promoter construct that contained the entire first intron with IRF-1, or with both IRF-1 and USF-1 expression plasmids, suppressed luciferase activity of the promoter in corneal keratocytes and T-47D cells. In contrast, co-transfection experiments with IRF-2, or with both IRF-2 and USF-1, led to increased luciferase activity of the same promoter construct. RT-PCR analyses demonstrate that IFN-γ rapidly and transiently suppresses mimecan expression and induces IRF-1 and IRF-2 mRNAs in bovine corneal keratocytes.

Conclusions: IRF-1 binds to ISRE sites, located in the first intron of the human mimecan gene, and negatively regulates mimecan expression at the level of transcription. Consistent with these observations, an inverse correlation between the expression of mimecan and IRF-1 in bovine corneal keratocytes and non-corneal cells was demonstrated. IRF-2 positively regulates mimecan transcription in corneal keratocytes. Because the intact E-box in the proximal promoter was required for IRF-1 and IRF-2 effects on mimecan transcription, potential direct or indirect interactions between USF-1 and IRF-1 and IRF-2 are likely.

The IFNs are a family of secreted glycoproteins that play roles in regulation of antiviral, antiproliferative, and immunomodulatory responses in vertebrate cells. Two types of IFNs have been described, the type I IFNs (α, β, ω, τ, and γ) that are produced by leukocytes and fibroblasts, and the type II IFN (γ) that is produced by T-lymphocytes and natural killer cells in response to infections by viruses and bacteria, as well as in response to noninfectious agents [1,2]. Both types of IFNs exert their biological effects through the signal transduction pathways that initiate by binding of IFNs to their corresponding cell surface receptors; interferon α receptor (IFNAR) for all type I IFNs and interferon γ receptor (IFNGR) for type II IFN. IFN receptors have been detected in almost all mammalian cell types [3,4]. Binding of IFNs with their receptors leads to activation of the Janus kinases (Jaks), which in turn leads to tyrosine phosphorylation and activation of signal transducers and activators of transcription (Stat) proteins [5]. Activated Stat-1 proteins form dimers that translocate to the nucleus and selectively regulate the genes containing the GAS element in their promoters. Heterotrimers of Stat-1, Stat-2 and p48 (known as ISGF3γ and IRF9) also translocate to the nucleus and results in regulation of genes containing ISRE in their promoters [1,2]. The interferon regulatory factors also are induced by these pathways and form a group of secondary transcription factors that bind to ISRE sequences and affect transcription of different sets of genes, depending on the cell type and the nature of cellular stimuli [6]. In addition to the pathways described above, other signaling proteins and transcription factors have been shown to participate in the IFN-induced cellular responses. These include p38 mitogen-activated protein kinase (MAPK), transcription factors CBP/p300, USF1 and NF-κB, shown to act as co-activators, and the class II transactivator (CIITA) that can act as a suppressor of transcription [7-11]. IFNs are known to affect the expression of more than 50 cellular genes. Among IFN-regulatable genes are several members of the proteoglycan gene family, including aggrecan, perlecan and glypican, members of the small leucine-rich proteoglycan gene family, including decorin and...
biglycan, as well as other matrix proteins such as collagen α2(I) [12-14].

IFNs appear to play important roles in pathogenesis of many ocular diseases as evidenced by the following observations; (i) interferon α receptor 1-deficient mice are highly susceptible to viral infection [15], (ii) ectopic expression of IFN-γ in the lens of transgenic mice affects the growth of the whole eye, resulting in microphthalmia, microphakia, persistent hyperplastic primary vitreous, corneal vascularization, and arrest of retinal differentiation [16], (iii) IFN-γ contributes to corneal allograft rejection, as well as to development of herpetic stromal keratitis [17,18], and (iv) many interferon regulatory factors, including IFN consensus sequence-binding protein (ICSBP), which is thought to be expressed only in cells of macrophage and lymphocyte lineages, are constitutively expressed in the mouse lens [19,20]. Interferon regulatory factors not only affect the expression of IFN-regulated genes, but also play roles in chromatin remodeling, susceptibility to oncogeneic transformation, regulation of the cell cycle, and apoptosis [6]. Therefore, identifying genes that are regulated by IFNs and that are expressed in the eye may be of medical interest because it may suggest potential new therapeutic targets for therapy of diseases causing corneal opacity, corneal transplant rejections, aging and cancer.

Mimecan/osteoglycin is a small leucine-rich proteoglycan of the extracellular matrix that is transcribed and translated several-fold greater in the cornea than in other tissues. Importantly, in human and bovine corneas mimecan carries a long keratan sulfate chain attached to the protein core and thereby is converted to a keratan sulfate proteoglycan, whereas in other connective tissues mimecan is present as a non-sulfated matrix glycoprotein [21]. Similar to other members of the small leucine-rich proteoglycan gene family, mimecan plays a role in collagen fibrillogenesis, as illustrated by in vivo studies using mimecan-deficient mice [22,23]. Similar to other corneal keratan sulfate proteoglycans, mimecan may play roles in the acquisition and maintenance of corneal transparency [24-26]. It is also likely that corneal keratan sulfate proteoglycans contribute to the relative immunotolerance of the cornea [27]. Additional roles for mimecan in cellular growth control are likely, as judged by observations that; (i) the tumor suppressor protein p53 activates transcription of bovine and human mimecan genes [28,29], (ii) mimecan mRNA is absent or found in low levels in the majority of cancer cell lines and tumors [28], (iii) the level of mimecan mRNA is high in corneal keratocytes maintained in low serum or serum-free media, but rapidly decreases if these cells are grown in media containing serum [30], and (iv) growth factors, such as bFGF, modulate mimecan mRNA expression in corneal keratocytes and vascular smooth muscle cells [31,32].

The purpose of the present study was to investigate whether IFN-γ affects mimecan gene expression and, if so, to characterize the cis-elements mediating the IFN-γ action. The rationale for this study stems from; (i) the presence of multiple ISRE (consensus sequence, AGTTTCNNTTTCNY) and GAS (consensus sequence, TTNCNNNAA) binding motifs in the human mimecan promoter (Figure 1), (ii) the presence of similar sequences in the bovine mimecan promoter, and (iii) our previous work demonstrating that nuclear proteins from MG-63 cells bind to a region in the first intron of the human mimecan gene that spans ISRE-c [29]. In this report we show that IFN-γ transiently suppresses mimecan transcription in different cell types. We demonstrate that IRF-1 and IRF-2 are constitutively and inducibly expressed in bovine corneal keratocytes and that in these cells IRF-1 acts as a suppressor of mimecan transcription, whereas IRF-2 has the opposite effect. The intact E-box in the proximal promoter is required for IRF-1 and IRF-2 effects on mimecan transcription. Although interactions between USF-1 and IRF-1 and IRF-2 are not directly demonstrated in this study, potential direct or indirect interactions are likely.

METHODS

**Plasmids:** Expression plasmid pcDNA3.1/hIRF1, containing human IRF1 cDNA driven by the promoter of the immediately early gene of human cytomegalovirus (CMV), was obtained.

Figure 1. Schematic representation of the 5'-region of the mimecan gene. The positions of IFN-responsive regulatory elements, conserved E-box and p53 DNA-binding sites are indicated. The translation initiation start site (ATG), numbered with +1, is indicated in human and bovine exon 2. Alternative splicing sites within the second exon of human and bovine mimecan genes are indicated with white lines. The white rectangle within the arrow that marks the p53-binding site indicates the presence of additional half p53-binding sites in the bovine promoter.

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from Research Genetics (Huntsville, AL, catalog number H-K1000). Expression plasmid pCDM8/h-IRF-2, containing human IRF2 cDNA driven by the CMV promoter, was kindly provided by Dr. T. Taniguchi, Department of Immunology, Tokyo University, Tokyo, Japan. Expression plasmid pCX-USF1, containing USF-1 cDNA driven by the promoter of human cytomegalovirus (CMV), was kindly provided by Dr. Robert Roeder, Laboratory of Biochemistry and Molecular Biology, the Rockefeller University, New York, NY [33]. Expression plasmid A-USF, containing the USF dominant-negative mutant (A-USF) coding sequence, was kindly provided by Dr. Charles Vinson, Laboratory of Biochemistry, National Cancer Institute, Bethesda, MD [34,35]. The human mimecan promoter/luciferase reporter constructs, containing different lengths of the 5'-flanking region of the human mimecan gene including the first intron, have been described previously [29,36].

**Cell culture, transfections, luciferase, and β-galactosidase assays:** Primary bovine keratocytes were isolated and cultured as described [21,32]. MG-63 cells (human osteosarcoma cell line), T-47D cells (breast ductal carcinoma cell line), and U-937 cells (histiocytic lymphoma cell line) were obtained from the American Type Culture Collection (Manassas, VA). Primary bovine keratocytes were isolated and cultured as described [21,32]. MG-63 cells (human osteosarcoma cell line), T-47D cells (breast ductal carcinoma cell line), and U-937 cells (histiocytic lymphoma cell line) were obtained from the American Type Culture Collection (Manassas, VA).

**Isolation of RNA and reverse transcription-polymerase chain reaction:** Total RNA was isolated from the cells at indicated intervals before and after IFN-γ treatment using the Totally RNA Total RNA Isolation Kit (Ambion, Inc., Austin, TX). RNA (1 μg) was reverse-transcribed using the anchor primer oligonucleotide (dT)18, and Superscript II Reverse Transcriptase (Life Technologies, Inc., Githersburg, MD) for 50 min at 42 °C in buffer supplied by the manufacturer. The single-stranded cDNA products (2 μl) were used as templates in a 50 μl PCR amplification reaction that contained 10 mM TRIS, pH 9, 50 mM KCl, 0.1% Triton X-100 (v/v), 1.5 mM MgCl₂, Taq polymerase (5 U), 0.2 mM dNTPs and 100 ng of each GSP, and carried out for 20-24 cycles (95 °C/45 s, 60 °C/1 min, 72 °C/2 min) to remain in the linear phase of the logarithmic growth during PCR amplification. Resulting PCR products were analyzed by electrophoresis on 2% agarose gels, with DNA visualized by ethidium bromide staining. Polaroid photographs of gels were scanned and the intensity of the bands was estimated by densitometry using the Scion Image 1.60 program. The human mimecan cDNA (AF112456) was amplified using the following primers; Hm+2, 5’-tct cat tca ccc tcc cac tct g-3’ and Hm-1265, 5’-taa tgc ctt gcc cag cag gag atg g-3’. For amplification of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), human control amplifier set from Clontech (Palo Alto, CA) was used (catalog number 5406-1). The human IRF-1 cDNA (NM_002198) was amplified using the following primers; hIRF1+187; 5’-cag aca caa tgc cca tca ctc gg-3’ and hIRF1-1180; 5’-tgc tac gtt gca cag gga atg g-3’. The human IRF-2 cDNA (NM_002199) was amplified using the following primers; hIRF2+290; 5’-gat gca tgc ggc tag aca tgg ggt g-3’ and hIRF2-837; 5’-ggt aga gct cgc tca tgc tga 3’. The human IRF-1 cDNA (NM_002198) was amplified using the following primers; Bm+411; 5’-ttg gat ctc gat cag ctc gtc cag g-3’. The bovine mimecan cDNA (M37974) was amplified using the following primers; Bmp+111; 5’-tgg gat ctc gat cag ctc gtc cag g-3’. The bovine IRF-2 cDNA (AJ490935) was amplified using the following primers; bovIRF1+11; 5’-aga tga att ccc aac aaa ttc cag g-3’ and bovIRF1-432; 5’-gga gct act gag tcc atg gta g-3’. The bovine IRF-1 cDNA (AJ490936) was amplified using the following primers; bovIRF2 +301; 5’-tga gcc atc ttt ggg ggt gat tag ctc ccg g-3’ and bovIRF2-730; 5’-gtt gat cgc aga aat cag ctc g-3’. The bovine GAPDH cDNA (U85042) was amplified using the following primers; Bgapdh+451; 5’-gtc atc cat cac ctt ggc atc gtt g-3’ and Bgapdh-800; 5’-ttg aag tcc cag gag aca acc tgg-3’. All primers were synthesized by Integrated DNA Technologies Inc. (Coralville, IA).
Preparation of nuclear extracts and electrophoretic mobility shift assay: Nuclear extracts were prepared from MG-63 cells before and after treatment with IFN-γ using NE-PER, Nuclear and Cytoplasmic Extraction Reagents, from Pierce (Rockford, IL), according to the manufacturer’s protocol. Nuclear extracts from U-937 and T-47D cells were obtained from Geneka Biotechnology Inc. (Montreal, Canada). Oligonucleotides for EMSA were synthesized by Integrated DNA Technologies, Inc., and annealed to generate double-stranded DNA probes. The following oligonucleotides were used in this study (sequence of the sense strand shown); ISRE-a, 5'-cac agt ttc aca ttc tgc a-3'; ISRE-b, 5'-ctc tgg ttt aag ttt cag tta cct c-3'; ISRE-c, 5'-act att ctt tca ctt ttc ctt c-3'; E-box, 5'-ctg gca aag atc tct acc acg tgg aaa actg-3'. Double-stranded DNA probes were 5' end-labeled with [\(\gamma\)^32P] ATP and T4 polynucleotide kinase. Nuclear extract (5 µg) was incubated with 25,000 cpm of labeled probe in a 20 µl volume of reaction buffer containing 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 10% (v/v) glycerol and 2 µg poly(dI-dC)-poly(dI-dC, Amersham Biosciences, Piscataway, NJ). Binding reactions were performed at room temperature for 25 min and resolved on a 4% non-denaturing polyacrylamide gel in 50 mM Tris-HCl, 0.38 M glycine, 2 mM EDTA, pH 8.5 for 2 h at 100 V. The gels were dried and autoradiographed. For the supershift assay, 1 µg of each antibody directed against USF-1 (sc-229), c-Myc (sc-232), IRF-1 (sc-497), IRF-2 (sc-498) or Statp84/p91 (sc-346) was added 20 min after the nuclear extract had been incubated with the probe. This mixture then was incubated for an additional 45 min at 4 °C. All antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA.

RESULTS

Mimecan, IRF-1, and IRF-2 mRNA levels are inversely correlated following IFN-γ treatment: To evaluate whether IFN-γ treatment would affect mimecan expression, total RNA was isolated from cultured primary bovine corneal keratocytes, MG-63, U-937 and T-47D cells before and after treatment with IFN-γ. MG-63, U-937 and T-47D cells were included in these experiments because they are among the few cancer cell lines that express mimecan (high levels in MG-63 and detectable levels in U-937 and T-47D cells) and because they respond to

![Figure 2](http://www.molvis.org/molvis/v9/a39)

Figure 2. IFN-γ suppresses mimecan mRNA expression. A: Left: Semi-quantitative RT-PCR using primers for bovine mimecan, IRF-1, IRF-2 and GAPDH; Right: Quantitation of mimecan, IRF-1, IRF-2 and GAPDH mRNA pools by scanning densitometry. Data are presented as means with standard error bars (n=3); B: Expression of endogenous mimecan, IRF-1, IRF-2 and GAPDH mRNAs in three cell lines before and after treatment with IFN-γ. Left: Semi-quantitative RT-PCR; Right: Quantitation of mimecan, IRF-1, IRF-2 and GAPDH mRNA pools by scanning densitometry. Data are presented as means with standard error bars (n=3).
a variety of factors, including IFN-γ [37-39]. mRNA pools were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). The amplifications were carried out for 20-24 cycles to remain in the linear phase of the logarithmic growth during PCR, thereby allowing semiquantitative assessment of the mRNA pools. In addition to mimecan, the levels of IRF-1, IRF-2, and GAPDH mRNAs also were evaluated. As shown in Figure 2A, the level of mimecan mRNA was decreased within 3 h of exposure to IFN-γ. This effect was reversible and at 24 h of exposure to IFN-γ the level of mimecan mRNA was restored and slightly increased. An inverse correlation was observed between the expressions of mimecan and those of IRF-1 and IRF-2 in IFN-γ-treated cells. In addition, the results (Figure 2A, 0 h) demonstrated that IRF-1 and IRF-2 are constitutively expressed in corneal keratocytes. As expected, transient induction of IRF-1 and IRF-2 mRNAs was observed after 3 h of treatment with IFN-γ with IRF-1 displaying an approximately 2.5-fold induction and IRF-2 increasing approximately 2-fold.

Similar results were obtained with MG-63, U-937 and T-47D cells (Figure 2B). The level of mimecan mRNA was decreased in these three cell types 3 h after IFN-γ treatment. Under the same conditions, the levels of IRF-1 and IRF-2 mRNA were increased in all three cell lines, thus confirming the lack of cytotoxic effect of IFN-γ in our assay. These results demonstrate that IFN-γ suppresses mimecan gene expression in the cell types described above. The results also demonstrate that mimecan, IRF-1, and IRF-2 mRNA levels are inversely correlated following IFN-γ treatment.

**IRF-1 binds ISRE-b and ISRE-c in the human mimecan promoter:** Using DNase I footprinting and EMSA, we previously demonstrated that nuclear proteins from untreated MG-63, but not from U-937 cells, as well as in vitro synthesized IRF-1, bind to a segment of the first intron of human mimecan gene that contains ISRE-c (Figure 3) [29]. In the present study, we examined whether IFN-γ could induce the binding of nuclear proteins to ISRE-a, -b, -c, and GAS sequences in the human mimecan gene. Nuclear extracts were prepared from MG-63, U-937 and T-47D cells treated with 200 U/ml IFN-γ for 2 or 4 h and EMSA was performed with labeled oligonucleotides spanning the sequences above. Nuclear extracts from these three cell lines formed complex bands with the probe (Figure 3A, lanes 1, 6, and 11). However, addition of non-specific competitor poly (dI-dC)-poly (dI-dC) reduced the density of the complex bands, thus demonstrating that binding of nuclear proteins to ISRE-a, -b, -c, and GAS sequences in the human mimecan gene. Nuclear extracts were prepared from untreated and IFN-γ-treated cells did not show protected segments within this region (data not shown). In contrast, when EMSA was performed with labeled ISRE-b probe, specific complex bands were formed in all three nuclear extract samples (Figure 3A, lanes 2, 7, and 12), thus demonstrating that binding of nuclear proteins to this ISRE is non-specific. Addition of anti-IRF-1, anti-IRF-2 and anti-Stat-1 antibodies did not lead to a supershift of the complexes (Figure 3A, lanes 3-5, 8-10, and 13-15) indicating that these complexes did not contain IRF-1, IRF-2 or Stat-1 proteins. DNase I footprinting using a probe that spans the GAS-b and -c elements and nuclear extracts from untreated and IFN-γ-treated cells did not show protected segments within this region (data not shown). In contrast, when EMSA was performed with labeled ISRE-b probe, specific complex bands were formed in all three nuclear extract samples (Figure 3B, top, lanes 1, 6, and 11). The addition of 50 times molar excess of the non-labeled ISRE-b probe reduced the density of the complex bands, thus demonstrating specificity of the binding proteins induced in these cells by IFN-γ (Figure 3B, top, lanes 2, 7, and 12). Addition of anti-

![Figure 3. EMSA analyses using labeled ISRE oligonucleotides and nuclear extracts from. A: EMSA analyses using labeled ISRE-a oligonucleotide and nuclear extracts from MG-63, U-937 and T-47D cells treated with IFN-γ for 2 h. Arrows indicate DNA-protein complexes formed with the probe; B: Top: EMSA analyses using labeled ISRE-b oligonucleotide and nuclear extracts from MG-63, U-937 and T-47D treated with IFN-γ for 2 h. Arrows indicate specific DNA-protein complexes formed with the probe. Red arrow indicates the complex that contains IRF-1 and green arrow indicates the position of supershifted complex; B: Bottom: EMSA using labeled ISRE-c and nuclear extracts from untreated and IFN-γ-treated U-937 and T-47D cells. Arrows indicate specific DNA-protein complexes formed with the probe. Red arrow indicates the complex that contains IRF-1 and green arrow indicates the position of supershifted complex.](image-url)
IRF-1, anti IRF-2, and anti-Stat-1 antibodies did not lead to supershift of the complexes formed with MG-63 and U-937 extracts (Figure 3B, top, lanes 3-5 and 8-10). However, addition of anti-IRF-1 antibody led to supershift of one of the complexes formed with T-47D extract (Figure 3B, top, lane 11, the complex marked with a red arrow contains IRF-1; lane 13, supershifted complex is marked with a green arrow). These results demonstrate that the binding proteins induced in T-47D cells by IFN-γ contained IRF-1. Similar results were obtained with the ISRE-c probe. Nuclear extracts from U-937 and T-47D cells treated with IFN-γ formed specific complexes with a labeled ISRE-c probe (Figure 3B, bottom, lane 1 versus 6 and lane 11 versus 16). Addition of anti-IRF-1, -2, and anti-Stat-1 antibodies did not supershift the specific complex formed in U-937 extract (Figure 3B, bottom, lanes 8-10, red arrow). However, similar to EMSA results with ISRE-b probe, addition of anti-IRF-1 antibody led to supershift of one of the complexes formed with T-47D extract (Figure 3B, bottom, compare lanes 16 and 18). These results demonstrate that the binding proteins induced in T-47D cells by IFN-γ interact with ISRE-b and ISRE-c sequences and that at least one of the interacting proteins is IRF-1.

Transcription factors from IFN-γ-treated cells bind the E-box in the human mimecan promoter: We demonstrated previously that the E-box in the proximal promoter is important for transcription and is an element required for transcriptional activation of the human mimecan gene by p53 [36]. The E-box (CACRTG) is a DNA binding site for several transcription factors including the basic-helix-loop-helix-leucine zipper transcription factors, USF-1, USF-2, and their differentially spliced isoforms, the Myc/Max/Mad family of transcription factors, the Mitf (microphthalmia associated transcription factor), and Myo-D, myogenin [40-44]. The USF-1 was identified as a protein that binds to the E-box and supports transcription of mimecan in MG-63 cells [29]. In this study we sought to examine whether treatment of MG-63, U-937 and T-47D cells with IFN-γ could influence the binding of nuclear proteins to this E-box in the human mimecan promoter. Gel-shift analysis was performed using a double-stranded probe spanning the E-box motif (Figure 4). Consistent with our previous observations, nuclear extract from untreated MG-63 cells formed a complex band with labeled probe and this complex was supershifted by addition of anti-USF-1 antibody, but not by addition of anti-c-myc antibody (Figure 4, lanes 1-3). Nuclear extract from MG-63 cells, treated with IFN-γ for 2 h, formed a complex band with much higher density as compared to the band from untreated MG-63 cells. This complex band was competed by unlabeled probe, but could not be supershifted by addition of anti-USF-1 or anti-c-myc antibodies (Figure 4, lanes 4-8). Treatment with IFN-γ for 4 h led to reduced density of the complex (Figure 4, compare lanes 7 and 8 with lanes 9 and 10). These results demonstrate that a different complex is formed in IFN-γ-treated MG-63 cells. One possible explanation for these results is that additional proteins that bind to USF-1 may be contained in the dense band and may block access of USF-1 antibody to its target. Nuclear extracts from U-937 cells, treated with IFN-γ for 2 h, also formed a complex band that was competed by unlabeled probe but not by non-specific competitor poly (di-dC)-poly (di-dC, Figure 4, lanes 14-16). In contrast to results with MG-63, the complex formed with U-937 extract could be supershifted by addition of anti-USF-1 (Figure 4, lane 17). In addition, the density of supershifted complex was stronger in IFN-γ-treated cells (Figure 4, compare lanes 12 and 17, blue arrows)indi-
cating that IFN-γ-treatment of U-937 cells led to increased binding of USF-1 to the E-box. Similar experiments performed with untreated and IFN-γ-treated T-47D cells demonstrated that IFN-γ also induced the binding of nuclear proteins to E-box and that one of the complexes induced by IFN-γ contained USF-1 in its molecule (Figure 4, lanes 19-25).

Together, the results presented so far demonstrate that; (i) nuclear proteins from all three cell lines bind to ISRE-b and ISRE-c elements, both of which are located in the first intron of the human mimecan gene, (ii) the proteins induced in T-47D cells by IFN-γ that bind to ISRE-b and ISRE-c sequences contain IRF-1, (iii) the proteins induced in U-937 and T-47D cells by IFN-γ that bind to the E-box contain USF-1, and (iv) treatment with IFN-γ appears to abolish binding of USF-1 to the E-box in MG-63 cells.

**IRF-1 suppresses mimecan transcription in corneal keratocytes:** The transcriptional repression of mimecan by IFN-γ was further investigated by transient cell transfection assay utilizing mimecan promoter/luciferase reporter gene constructs. Varying lengths of the mimecan promoter region linked to the luciferase gene were transfected into bovine corneal keratocytes and T-47D cells (Figure 5). The p-3694/-1146 construct contained 2.5 kb of the human mimecan promoter, including GAS-a, GAS-b, GAS-c, and ISRE-a sequences, as well as the E-box and the three Initiator elements located at the 5'-end of the first exon (Figure 1). The p-2316/-1146 construct contained 1.1 kb of the human mimecan promoter in which upstream GAS and ISRE sequences were deleted. The p-1314/-1146 construct was the minimal promoter region (168 bp) that was shown before to be functional, and contained only the E-box and the three Initiator elements [36]. The p-1341/-153 construct contained the entire first intron, in addition to the E-box and the three Initiator elements. Following transfection (24 h), to allow reporter expression, the cells were incubated in the presence or absence of IFN-γ for 12 h. In several independent experiments, only the p-1314/-153 construct showed transcriptional repression in the presence of IFN-γ in both cell types (Figure 5). These data are consistent with the results from EMSA (Figure 2) and demonstrate that the first intron of the human mimecan gene contains IFN-γ responsive elements.

To further investigate the potential role of IRF-1 as a regulator of mimecan expression, we cotransfected the IRF-1 expression plasmid together with p-1314/-1146 or p-1314/-153 into corneal keratocytes and T-47D cells. As shown in Figure 6, IRF-1 expression markedly diminished luciferase activity of the p-1314/-153 construct, but had little effect on luciferase activity of the p-1314/-1146 construct (in which the first intron is missing). The negative effect of IRF-1 on mimecan transcription was dose-dependent, since increased amounts of IRF-1 led to increased suppression of mimecan transcription. Interestingly, cotransfections with the IRF-2 expression plasmid had an opposite effect on the mimecan promoter and led to increased luciferase activity of the p-1314/-153 construct, although the results from EMSA did not show binding of IRF-2 to intronic ISRE-b and ISRE-c sequences. These results can be explained in two ways. First, IRF-2 also may bind to intronic ISRE sequences, but this binding was not detected in our system. Second, binding of IRF-2 to ISRE sequences may not be required for its effect on mimecan transcription. In support of the second conclusion, the luciferase activity of p-1314/-1146 cotransfected with IRF-2 was also increased, although not as dramatically as with the p-1314/-153 construct. Consistent with our previous observations, co-transfection of USF-1 led to activation of the human mimecan promoter [36]. To test for potential functional interactions between IRF-1 and IRF-2 with USF-1, cotransfections of human mimecan promoter constructs, together with expression vectors encoding the USF-1, or the dominant-negative mutant of USF, A-USF, were performed. In the A-USF, the basic HLH domain of USF-1 is replaced with an acidic sequence, which stabilizes the heterodimers between A-USF and USF, resulting in inhibition of USF DNA binding [34,35]. As shown in Figure 6, cotransfections of p-1314/-153 with both USF-1 (50 ng) and IRF-1 (1 μg) led to diminished luciferase activity of this promoter construct. Compared to luciferase activity of p-1314/-153 cotransfected with IRF-1 alone, these results indicate that USF-1 and IRF-1 may have opposite effects on mimecan transcription. Cotransfections of p-1314/-153 with both USF-1 (50 ng) and IRF-2 (1 μg) led to slightly increased luciferase activ-

![Figure 5](http://www.molvis.org/molvis/v9/a39)

**Figure 5.** Reporter analysis of the human mimecan promoter. Human mimecan reporter constructs were transfected into corneal keratocytes and T-47D cells. The day following transfections, cells were incubated with IFN-γ for 12 h and the luciferase activities were determined. The results are presented as the means with standard error bars from three separate transfections performed in duplicate.
ity, when compared to that of p-1314/-153 cotransfected only with USF-1, and decreased luciferase activity, when compared to that of p-1314/-153 cotransfected with IRF-2 (1 µg). Because co-transfections of USF-1 alone or IRF-2 alone led to increased promoter activities, these results can be explained with the involvement of an additional protein(s) that may bind both, USF-1 and IRF-2, and that may cause transcriptional repression of the mimecan promoter. Co-transfections of A-USF with either IRF-1 or IRF-2 abolished the effects of these factors when co-transfected alone, thereby supporting our conclusion that interactions between USF-1 and the two IRFs are likely.

To further confirm the roles of IRF-1 and IRF-2 on transcriptional regulation of human mimecan, bovine corneal keratocytes were transiently transfected with IRF-1 or IRF-2 expression plasmids. Transient introduction of IRF-1 in these cells, which had not been treated with IFN-γ, caused a moderate decrease in mimecan transcripts, whereas transient introduction of IRF-2 in these cells had the opposite effect (Figure 7). The modest effect is likely a result of the fact that transient transfection can introduce the transfected gene in only a portion of the cells and that transfection efficiency of primary cells is known to be low.

DISCUSSION

In this study we investigated the expression of mimecan in bovine corneal keratocytes and other cells types in response to IFN-γ. We demonstrated that IRF-1 and IRF-2 are constitutively expressed in bovine corneal keratocytes and that after IFN-γ treatment the levels of both IRF-1 and IRF-2 mRNAs are upregulated by IFN-γ, with greater increases seen in the levels of IRF-1 mRNA. Notably, the levels of IRF-1 and IRF-2 mRNAs were inversely correlated with those of mimecan mRNA in all cell types used in this study.

Herein, we identify the cis-acting elements (two ISRE sites located in the first intron of the human mimecan gene) and transcription factors involved in this response. Using EMSA, with oligonucleotides spanning intronic ISRE-b and ISRE-c sequences, we identify specific DNA-protein complexes formed with nuclear extracts from IFN-γ-treated cell lines. As indicated by supershift experiments, IRF-1 was contained in one of the complexes formed with nuclear extract

Figure 6. IRF-1 suppresses, whereas IRF-2 enhances transcription of the mimecan promoter. Bovine corneal keratocytes and T-47D cells were co-transfected with wild-type construct (p-1314/-153 or p-1314/-1146) and either IRF-1, IRF-2, USF-1 and A-USF expression plasmids alone or in combinations. Cell lysates were analysed for luciferase and β-galactosidase activities 24 h after transfection. The results are presented as the means with standard error bars from three separate transfections performed in duplicate.

Figure 7. Introduction of IRF-1 reduces the expression of endogenous mimecan whereas IRF-2 has the opposite effect. Left: Total RNA was isolated from cells transiently transfected with IRF-1 or IRF-2 48 h after transfection. RNAs were subjected to RT-PCR with mimecan-specific primers. GAPDH was used as a control. The PCR products were resolved on agarose gels and visualized by ethidium bromide staining. Right: Quantitation of mimecan and GAPDH mRNA pools by scanning densitometry. The results are presented as the means with standard error bars (n=3).
IRF-1 binds to ISRE-c in the human mimecan promoter. The also binds to ISRE-c [29]. Therefore, at least in two cell types, IRF-1 binds to ISRE-c in the human mimecan promoter. The results obtained with nuclear extract from IFN-γ-treated U-937 cells can be explained either that proteins other than IRF-1 directly bind to ISRE-b and ISRE-c in the human mimecan promoter, or that additional proteins that bind IRF-1 are contained in the complexes so that they block access of anti-IRF-1, anti-IRF-2 and anti-Stat antibodies to their targets. Supportive of these explanations are the results from EMSA with T-47D extracts, showing that multiple DNA-protein complexes were formed.

Our results also indicate involvement of the E-box in IFN-γ-induced suppression of human mimecan transcription. By EMSA, binding of USF-1 to E-box oligonucleotide was detected in untreated MG-63, T-47D and U-937 cells. However, increased binding of USF-1 after IFN-γ treatment was detected in two of these cell types, T-47D and U-937. In contrast, IFN-γ treatment of MG-63 cells appeared to abolish the binding of USF-1 to its target. Instead, increased density of the complex formed with E-box oligonucleotide in these cells was observed. Antibody against c-myc, one of many other E-box-binding proteins, also had no effect on the DNA-protein complex in MG-63 cells. Although in this study we did not attempt to identify all known E-box-binding proteins, one explanation for these results could be that proteins other than USF-1 bind to the E-box in IFN-γ-treated MG-63 cells. Alternatively, proteins that bind to USF-1 may block access of anti-USF-1 antibody to its target. Involvement of the E-box and intronic ISRE sites in transcriptional regulation of human mimecan by IFN-γ was further corroborated by transient transfection experiments. The expression of three constructs that contained only the 5′-upstream region of the human mimecan promoter was not affected by IFN-γ, demonstrating that the ISRE-a and GAS-a, -b, and -c elements play little role in mediating mimecan response to IFN-γ. In contrast, expression of the construct that contained the E-box plus the entire first intron was suppressed by IFN-γ in two cell types, corneal keratocytes and T-47D cells, demonstrating roles for the intronic ISRE and E-box elements. The effect of USF-1 and IRFs on mimecan gene transcription was evaluated by overexpression of each one of these transcription factors alone or in combination. We show here that overexpression of IRF-1 suppresses, but overexpression of IRF-2 activates, mimecan transcription. Importantly, cotransfection of A-USF abrogates the effects of IRF-1 and IRF-2. Based on these data, it can be concluded that mimecan transcription in response to IFN-γ is controlled, in part, by the presence and concentrations of USF-1 and IRFs and that functional interactions between these transcription factors appears to take place in the human mimecan promoter. In this view it is of interest to note that IRF-1 protein has been demonstrated to be very unstable (half-life about 30 min), whereas IRF-2 protein has been demonstrated to be stable (half-life about 8 h) [6]. The data presented in this study are consistent with these observations; the initial down-regulation of mimecan mRNA after treatment with IFN-gamma, mediated by IRF-1, is followed by up-regulation of mimecan mRNA, mediated by IRF-2. In addition, binding of IRF-2 to intronic ISRE sites may not be required for its effect on mimecan expression. USFs are ubiquitously expressed proteins known to interact with DNA as dimers, with USF-1-USF-2 heterodimer being the major one present in most cell types, less abundant USF-1 homodimers and very little USF-2 homodimers [45]. Interactions between USF-1 and other proteins have been described, including interactions between USF-1, IRF-1 and Stat1α in the type IV class II transactivator (CIITA) promoter in astrocytes, although the positions of E-box, ISRE and GAS regulatory elements in the CIITA promoter are different than those in the mimecan promoter [46,47]. Similar to mimecan, binding of IRF-1 and IRF-2 to an ISRE located in the first intron of the c-myb gene has been reported [48]. Furthermore, similar to mimecan, IRF-1 has been demonstrated to suppress transcription of the c-myb gene. Although in this report we do not address the exact molecular mechanism by which the described transcription factors may regulate mimecan transcription in response to IFN-γ, one possible explanation for the results presented in this study could be that binding of IRF-1 to intronic ISRE sites may cause pausing of RNA polymerase II and temporarily block transcription elongation of mimecan mRNA. As an attempt to overcome such a potential block in transcription, cells may respond with increased binding of USF-1 (and other transcription factors) to the E-box in the human mimecan promoter. A similar mode of transcriptional regulation has been shown to control expression of other genes, including c-myc, c-myc, c-fos, and adenosine deaminase [48-51]. Recently, recruitment of multiple interferon regulatory factors and histone acetyltransferase to the transcriptionally active IFN-γ promoters has been demonstrated [52]. Chromatin remodeling at the CIITA locus by IFN-γ also has been reported [53]. Although not directly addressed in this study, formation of chromatin-remodeling complexes at the mimecan promoter also may account for transcriptional regulation of this gene by IFN-γ. The roles of IRF-1 and IRF-2 on transcriptional regulation of human mimecan were further confirmed by transient transfections of bovine corneal keratocytes with IRF-1 or IRF-2 expression plasmids.

In conclusion, our study has several novel findings. First, it shows that mimecan expression in the cornea is suppressed by IFN-γ. Given the important role of corneal keratan sulfate proteoglycans in the maintenance of corneal transparency, as well as increased recognition of extracellular matrix proteins as important elements in lymphocyte (cells producing IFN-γ) positioning and effector functions in alloreactive responses, the results from these studies provide new information and may suggest new molecules as potential targets for immune intervention in corneal and organ transplantation. Second, our study identifies cis-regulatory elements and transcription factors that mediate the suppressive effect of IFN-γ on the human mimecan promoter in corneal keratocytes and other cell types. Third, this study shows that IRF-1 and IRF-2 are constitutively expressed in corneal keratocytes and therefore may have other, non-immunity-related functions in the cornea.
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