Interferon-γ Induction of the Human Leukocyte Antigen-E Gene Is Mediated through Binding of a Complex Containing STAT1α to a Distinct Interferon-γ-responsive Element*

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Expression of the human major histocompatibility complex (MHC) class I genes has been shown previously to increase at the transcriptional level following exposure to interferon-γ (IFN-γ). In this report we have examined the molecular mechanisms involved in the IFN-γ-induced transcription of the human MHC class I gene, HLA-E. Functional analysis of CAT reporter gene constructs under the control of the HLA-E promoter transfected into U937 cells revealed the presence of a distinct IFN-γ-responsive element, termed the interferon response region (IRR), that was necessary and sufficient to mediate the response to IFN-γ. This cis-acting regulatory sequence contains an imperfect inverted repeat; the 5′-half of the IRR resembles the IFN-γ activation site (GAS), and the 3′-half of the IRR resembles the interferon-stimulated response element (ISRE). Gel mobility shift assays demonstrated that the IRR bound a single, specific, IFN-γ-induced complex (IRR-AC), which was formed rapidly following treatment with IFN-γ and independent of protein synthesis. Competition experiments with GAS and ISRE sequences from other IFN-inducible genes showed that GAS sequences competed for the IRR-AC, whereas ISRE sequences did not compete. Mutational analysis demonstrated that point mutations in either the 5′-half or 3′-half of the IRR prevented binding of the complex and abrogated or markedly reduced the IFN-γ responsiveness of reporter gene constructs. Supershift analysis revealed that the IRR-AC contains a factor that was recognized by antibodies specific for the protein STAT1α (signal transducer and activator of transcription). Taken together, these findings suggest that the mechanism of IFN-γ-induced transcription of the HLA-E gene is distinct from that of other MHC class I genes.

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1 The abbreviations used are: IFN, interferon; STAT, signal transducer and activator of transcription; ISRE, interferon-stimulated response element; GAS, IFN-γ activation site; IRF, interferon regulatory factor; GBP, guanylate binding protein; MHC, major histocompatibility complex; ICAM-1, intercellular adhesion molecule-1; GAF, IFN-γ activating factor; CTL, cytotoxic T lymphocyte; IRR, interferon response region; IRF-AC, IFN-γ activation complex; CHX, cycloheximide; bp, base pair(s); TPI, triose-phosphate isomerase; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; HLA, human leukocyte antigen; ISG, interferon-stimulated gene; GR, IFN-γ response region; SIF, cis-regulable factor; IRR, interferon-responsive element.
Dimerization of STAT1α is induced following phosphorylation of a specific tyrosine residue on the protein, which occurs with activation of the J AK-1 and J AK-2 tyrosine kinases in response to IFN-γ (14, 33–36). Recent studies by Quele et al. (37) demonstrate that tyrosine phosphorylation of purified STAT1 results in the appearance of a GAS-STAT1 complex, suggesting that other protein components are not required to form the DNA-protein complex, unlike the ISRE-ISGF3 complex, which requires p48 in addition to the STAT proteins.

The classical MHC class I genes are highly polymorphic and encode transmembrane glycoproteins, known as class I heavy chains, that associate noncovalently with β2-microglobulin (38). One of the primary functions of MHC class I molecules is to mediate the cellular immune response by presenting antigenic peptides to cytotoxic T lymphocytes (CTLs) (39), which results in CTL-mediated lysis of virus-infected cells and tumor cells. In contrast, expression of MHC class I molecules on target cells has been shown to have an inhibitory effect on natural killer cell lytic activity (40, 41). As noted above, MHC class I genes from both humans and mice are among those induced by both types of IFNs, with preferential induction by IFN-γ (2, 42). Previous studies have shown that the ISRE present in the 5′-flanking sequences of these genes is necessary for the induction of class I genes by IFN-γ, although in some cases the ISRE may not be sufficient to mediate the full response (22, 25, 43). The IRF-1 protein has been identified as an IFN-γ-induced factor that binds to the ISRE which may be involved in the IFN-γ-mediated induction of class I gene expression (44, 45). The binding of this factor requires ongoing protein synthesis, suggesting that induction of classical MHC class I genes by IFN-γ is not mediated by preexisting proteins (44).

In addition to the classical class I genes, the MHC class I region includes several nonclassical class I genes (reviewed in Refs. 46 and 47). The nonclassical class I molecules are generally characterized as having limited polymorphism, restricted tissue expression, and relatively low cell surface expression. The function of these molecules remains unclear; however, emerging evidence suggests that the nonclassical class I molecules may have a specialized role in the immune response by presenting a subset of antigenic peptides (47–49). Like classical MHC class I genes, the human nonclassical HLA-E (50) gene has been previously shown to be induced by IFN-γ, although the underlying mechanisms have not been elucidated (51). In the present studies, we have examined the mechanism of IFN-γ-induced expression of the HLA-E gene and present evidence suggesting that the regulation of the HLA-E gene differs from that previously described for other class I genes. Functional analysis of the HLA-E promoter using reporter gene constructs reveals the presence of a distinct IFN-γ-responsive regulatory element, which we have designated the interferon response region (IRR). The IRR contains an imperfect inverted repeat; the 5′-half of the IRR and 3′-half of the IRR act synergistically to mediate the full response to IFN-γ. Gel mobility shift assays reveal that the IRR binds a single, specific, IFN-γ-induced complex, termed the IRR-activation complex (IRR-AC), which is formed rapidly and independently of protein synthesis. Supershift analysis demonstrates that the IRR-AC contains a factor that is recognized by antibodies specific for the protein STAT1α.

MATERIALS AND METHODS

Cell Culture—Pronocytic U937 cells (ATCC CRL 1593) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin (Life Technologies, Inc.). Cells were induced with IFN-γ (R & D Systems) at 250 units/ml for the times indicated. Cyclheximide (CHX, Sigma) was used at 50 µg/ml and added to the cultures 30 min prior to induction with IFN-γ where indicated.

For RNA isolation, cells were scraped off the plates into a mixture of 0.5 ml of TRIzol Reagent (GIBCOBRL) and 0.2 ml of proteinase K solution (10 mg/ml in 0.1 M Tris·HCl, pH 7.5). Cells were incubated for 1 h at 37°C, then a mixture of 0.2 part of chloroform and 1 part of isoamyl alcohol was added, and the samples were vortexed for 15 s followed by a 15-min incubation at room temperature. After centrifugation at 12,000 × g for 15 min at 4°C, the aqueous phase was transferred to a fresh tube, and 0.5 part of isopropanol was added. Samples were centrifuged at 12,000 × g for 15 min at 4°C, and the RNA pellet was washed once with 70% ethanol. After removing the supernatant, the RNA was resuspended in 0.2 ml of RNase-free water. RNA was quantified by measuring absorbance at 260 nm and washed with 70% ethanol. RNAs were stored at −80°C.

For Northern blotting, RNA (5 µg) from untreated or treated cells was electrophoresed in a 1% formaldehyde–agarose gel and transferred to a Hybond-N+ membrane (Amersham Corp.). The membrane was prehybridized at 42°C in 50% formamide, 5× Denhardt’s solution, 5× SSPE, 0.5% SDS, and 0.5 µg/ml salmon sperm DNA for 2 h. The membrane was then hybridized at 42°C in the same solution containing 10–20,000 cpm of a radiolabeled probe for HLA-E. Subsequently, the membrane was washed three times in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS for 15 min at room temperature. The membrane was then washed two times in 0.1× SSC and 0.1% SDS for 15 min at 65°C.

RNase Protection Assays—Cytoplasmic RNA was isolated using a rapid nuclear-cytosolic fractionation method described previously (52). For RNase protection assays, single-stranded, radiolabeled cDNA probes were synthesized using T7 RNA polymerase (GIBCOBRL). T7 polyadenylated RNA was detected by hybridizing the radiolabeled probe to a mixture of cytoplasmic RNA (5 µg) from untreated or treated cells and a molecular size marker (total size 100–1200 nucleotides) (53).

For RNase protection assays used to identify the transcription initiation site(s) of the HLA-E gene, the probe was synthesized as described above but with template DNA subcloned into pGem-3zf(−), which consisted of an HLA-E genomic fragment that included sequence from exon 1 and the 5′-flanking region (positions −1530 to −576 bp) (50), as predicted by alignment with other class I gene sequences. The samples were hybridized and processed as described above and then analyzed by electrophoresis on a 6% polyacrylamide, 7 M urea sequencing gel along with a set of size sequencing reactions using a 26-nucleotide primer (positions 742–767 bp, (Ref. 50)) and template DNA containing the HLA-E genomic fragment as size markers.

Preparation of CAT Reporter Gene Constructs—The pE CAT clone contains approximately 1800 bp of 5′-flanking sequence and was generated by sequentially subcloning HindII-PstI (−1800 to −174) and PstI-AlwNI (−174 to +2) promoter fragments from the HLA 6.2 genomic clone (kindly provided by Harry Orr (54)) into the HindII and PstI sites of the promoter and chloramphenicol acetyltransferase (CAT) reporter gene vector pCAT-Basic (Promega). The 5′-deletion mutants designated pE−386CAT, pE−174CAT, and pE−128CAT were generated by restriction enzyme digestion of larger clones and subsequent religation of the plasmid. The 5′-deletion mutant pE−193CAT was generated by polymerase chain reaction (PCR) using a primer corresponding to the HLA-E sequence −193 to −181 with the addition of a HindII site at the 5′-end and a primer corresponding to sequences from the pCAT-Basic plasmid together with pE−386CAT as the template DNA. Following PCR, the product was gel-isolated, digested with HindII and Sall to make cohesive ends, and ligated into the HindII and Sall sites of pCAT-Basic. The deletion mutant pE−193d60/170−129CAT contains an internal deletion of bases −170 to −129 and was generated by subcloning the −170 to −129 HindII fragment from the pE−193CAT upstream of pE−128CAT. The pTKCAT construct was generated by subcloning the sequences from −105 to +51 of the herpes simplex virus thymidine kinase (TK) promoter into the HindII and Xbal site of pCAT-Basic. The pE−193−146TKCAT construct was generated by subcloning the HLA-E fragment from −193 to −146 upstream of the TK promoter of pTKCAT. The 5′-half and 3′-half mutant constructs were prepared by cloning synthetic double-stranded oligonucleotides into the HindII and PstI sites of pE−174CAT. The 3′-half IRR point mutants were generated by using PCR overlap extension techniques (55) together with the pE−193CAT construct as template DNA. Following PCR, the product containing the point mutations was gel-isolated, digested with HindII and AscI to make cohesive ends, and ligated into the HindII and AscI sites of pCAT-Basic. pECAT clone was constructed by using the dideoxy chain termination method to verify the deletion end points and mutated sequences.

Transient Transfection of U937 Cells and Reporter Gene Assays—U937 cells were transfected by electroporation in RPMI 1640 medium at 210 V and 900 microfarads using a BTX Electro Cell Manipulator 600 (Biorheology & Experimental Research, Inc.). A total of 1.5 × 106 U937 cells were transfected with 40 µg of CAT reporter gene plasmid and 1 µg of the internal control plasmid consisting of the gene encoding β-galactosidase driven by the cytomegalovirus promoter. Cells from four replicate cuvettes were pooled and then divided among four flasks to ensure equal transfection efficiency between duplicate untreated and IFN-γ-treated samples. The cells were incubated for 2 h and then left untreated or treated with IFN-γ and incubated for an additional 8 h. Cell lysates were prepared and CAT assays were performed according to the CAT enzyme assay system protocol (Promega) with slight modifications. Briefly, cells were washed twice in phosphate-buffered saline, and the cell pellet was resuspended in 120 µl of 1 × reporter lysis buffer.
buffer. Following a 15-min incubation, the lysates were subjected to a single freeze-thaw cycle, vortexed, and centrifuged for 2 min at 4 °C to pellet cellular debris. The lysates were transferred to a new tube, and an aliquot was heated at 65°C for 10 min prior to determining CAT activity using n-butyl-2-oxo-3-en-4-one and (4%)[H]thymidine reporter assay system (Tropix, Inc.). CAT activity was normalized to the internal control β-galactosidase activity.

Preparation of Nuclear Extracts—Nuclear protein extracts from U937 cells were prepared according to the method of Dignam (56). Extracts were prepared in the presence of the protease inhibitors leupeptin (10 μg/ml), pepstatin (10 μg/ml), aprotinin (10 μg/ml), and phenylmethylsulfon fluoride (0.5 mM). Protein concentrations were determined by the Bradford protein assay (57) using bovine serum albumin as a standard.

Gel Mobility Shift Assays—DNA fragments were labeled by filling in the 5′-protruding ends with Klenow enzyme using [α-32P]dATP (Amer sham). Binding reactions (20 μl) contained 10 μg of nuclear extract, 50 fmol of double-stranded DNA probe, and 4 μg of poly(dI-dC) in a buffer consisting of 12 mHEPES (pH 7.9), 50 mM KCl, 5 mM MgCl₂, 0.12 mM EDTA, 0.06 mM EGTA, 10% (v/v) glycerol, 1 mM ATP, and 1 mM dithiothreitol. The reaction mixtures were incubated at room temperature for 30 min and then separated on 4% nondenaturing polyacrylamide gels in a buffer that consisted of 50 mM Tris (pH 7.9), 3.3 mM sodium acetate, and 1 mM EDTA. Gels were dried and subjected to autoradiography or exposure to a PhosphorImager (Molecular Dynam ics) for quantitation. In competition analysis, nuclear extracts were incubated with unlabeled double-stranded oligonucleotides prior to the addition of the radiolabeled probe. In supershift analysis, antibodies were added to the binding reactions and incubated on ice for 60 min prior to the addition of radiolabeled probe. The following rabbit polyclonal antibodies were used in supershift analysis and purchased from Santa Cruz Biotechnology: STAT1α (sc-345X), STAT3 (sc-482X), ISG54 (sc-259X), SP1 (sc-482X), IFN-γ (sc-218X), IL-4 (sc-226X), IL-10 (sc-236X), IL-13 (sc-237X), and IL-12 (sc-238X).

The following DNA fragments and double-stranded oligonucleotides were used as probes or competitors in gel mobility shift assays (only the sequence of the top strand is shown): IRR--193 to 175, 5′-GTCGGGAACTCTGCGATTCCTTGCTCCCTCACCTAAGCTG-3′; IRR--189 to 179, 5′-GTCGGGAACTCTGCGATTCCTTGCTCCCTCACCTAAGCTG-3′; IRR--193 to 175, 5′-GTCGGGAACTCTGCGATTCCTTGCTCCCTCACCTAAGCTG-3′; and IRR--193 to 175, 5′-GTCGGGAACTCTGCGATTCCTTGCTCCCTCACCTAAGCTG-3′. For the IRR probe (−193 to −146), the plasmid pE175 was digested with HindIII and EcoRI followed by gel purification of the fragment containing the insert and polyydol region, which was used as the probe. For the 2×-5′-half-IRR and 2×-3′-half-IRR probes, complementary oligonucleotides were annealed and ligated into the HindIII and XbaI sites of pGEM-3zf(−) plasmid, and the DNA fragment used as the promoter fragment was obtained by digestion of the plasmid with HindIII and EcoRI followed by gel purification. Oligonucleotides were purchased from National Biosciences or Life Technologies, Inc.

Methylation Interference Footprinting Analysis—The DNA fragments used as probes in these experiments contained the HLA-E genomic sequence from −193 to −146 and were prepared as follows. The PAC13 reverse primer (5′-GAGAAAAGCTTGGGATCC-3′) and the T7 promoter primer (5′-TATCTGCTAGACGCTACG-3′) were end-labeled by [γ-32P]ATP (Amer sham) with T4 polynucleotide kinase. This primer pair (one primer radiolabeled and one primer unlabeled) was used in PCR, using the plasmid pE175 (−32P-ends) as template DNA, to label the coding and noncoding strands, respectively. The radiolabeled PCR products were gel-purified and either partially methylated with dimethyl sulfate or subjected to the A+G sequencing reaction as described by Maxam and Gilbert (60).

RESULTS

Induction of HLA-E mRNA by IFN-γ—Induction of HLA-E mRNA by IFN-γ was examined in promonocytic U937 cells, a human cell line previously utilized for the study of IFN-γ-responsive genes (30, 31). RNase protection assays were performed using RNA isolated from U937 cells that had been treated with IFN-γ for various times. Levels of steady state mRNA were induced 3–4-fold following treatment with IFN-γ for 4 h and remained elevated after treatment with IFN-γ for 16 h (Fig. 1A). To determine if the induction of HLA-E mRNA required ongoing protein synthesis, RNA was isolated from U937 cells that were treated with IFN-γ for 4 h in the presence or absence of the protein synthesis inhibitor cycloheximide. Induction of HLA-E mRNA by IFN-γ was not inhibited by cycloheximide, suggesting that the response to IFN-γ was likely mediated by posttranslational modification of preexisting factors (Fig. 1B). Interestingly, treatment with cyclohexi mide results in the appearance of a smaller protected fragment. This fragment may be the result of alternative splicing, but it has not been further characterized.

To identify the transcription initiation site(s) of the HLA-E gene, an RNase protection assay was performed using RNA isolated from untreated and IFN-γ–treated U937 cells. The analysis revealed one minor and two major initiation sites (Fig. 1C). The most 3′ major initiation site corresponded to a protected fragment 76 nucleotides in length, extending 13 nucleotides 5′ from the ATG codon, and was designated +1. The other two initiation sites were located upstream of the major site designated +1. The same protected fragments were obtained using RNA from U937 cells that were untreated (Fig. 1C, lane 2) or treated with IFN-γ (Fig. 1C, lane 3) but not when yeast RNA alone was hybridized to the probe (Fig. 1C, lane 1).

Functional Analysis of the HLA-E Promoter and Localization of the Interferon Response Region—To identify the regulatory sequences of the HLA-E promoter mediating the transcriptional activation by IFN-γ, a series of 5′ and internal deletion constructs were used, in which various portions of the 5′-flanking sequences of the HLA-E gene were subcloned into the promoterless chloramphenicol acetyltransferase reporter gene plasmid pCAT-Basic (Fig. 2, upper part). These constructs were transiently transfected into U937 cells that were treated with IFN-γ for 8 h or left untreated. The cells were harvested, and cell extracts were analyzed for CAT activity. As shown in Fig. 2, upper part, IFN-γ treatment of U937 cells transfected with the pCAT construct containing approximately 1800 bp of 5′-flanking sequence of the HLA-E gene resulted in an 8-fold induction of CAT activity compared with untreated cells. The pE−386CAT and pE−193CAT 5′ deletion constructs were induced 7.5- and 11.5-fold, respectively (Fig. 2, upper part). Although the differences in IFN-γ inducibility between these two deletion constructs suggested the possibility of a weak negative element between −386 and −193, both constructs remained strongly responsive to IFN-γ. However, further 5′ deletions of the HLA-E promoter producing the pE−174CAT and pE−128CAT constructs resulted in a complete loss of IFN-γ induced CAT activity. These results suggested that sequences in the region from −193 to −175 were necessary to mediate the IFN-γ response. To determine if the region from −193 to −175 was able to mediate responsiveness to IFN-γ, a DNA fragment containing this region was linked to the pE−128CAT construct. The CAT activity in extracts from cells transfected with the pE−193del−170/pE−128CAT internal deletion construct was in-
IFN-γ-induced HLA-E Gene Transcription

**Fig. 1.** Induction of HLA-E mRNA in response to IFN-γ and determination of the transcription initiation site(s) of the HLA-E gene by RNase protection analyses. A and B, the 32P-labeled antisense HLA-E RNA probe (upper part) and TPI RNA probe (lower part) (see “Materials and Methods”) were hybridized to cytoplasmic RNA isolated from U937 cells (30 and 5 μg respectively), digested with ribonucleases, and analyzed by denaturing polyacrylamide gel electrophoresis. A, time course of IFN-γ induction of HLA-E mRNA. U937 cells were untreated or treated with IFN-γ (250 units/ml) for the indicated times. B, effect of the protein synthesis inhibitor CHX on the induction of HLA-E mRNA by IFN-γ. U937 cells were untreated or treated with IFN-γ (250 units/ml) for 4 h. CHX (50 μg/ml) was added 30 min prior to the addition of IFN-γ where indicated. C, determination of the transcription initiation site(s) of the HLA-E gene. The 32P-labeled antisense HLA-E RNA probe derived from the first exon and 5' flanking region (see “Materials and Methods”) was hybridized with either yeast tRNA (15 μg, lane 1) or cytoplasmic RNA (5 μg) from U937 cells untreated (lane 2) or treated with IFN-γ (250 units/ml) for 48 h (lane 3). The most 3' major transcription initiation site is indicated by the arrow; upstream initiation sites are indicated by the asterisks. The sequence ladder, shown on the right, was used as a size marker (see “Materials and Methods”).

IFN-γ Rapidly Induces Binding of a Single Complex, IRR-AC, to the IRR—Inspection of the sequence of the IRR revealed the presence of an imperfect inverted repeat (Fig. 3A). The 5' half of the inverted repeat shares sequence homology with the GAS element, while the 3' half of the inverted repeat shares sequence homology with the ISRE (4). To identify IFN-γ-induced nuclear factors that bind to the IRR, gel mobility shift assays were performed using nuclear extracts from U937 cells that were untreated or treated with IFN-γ for various times together with a 32P-labeled DNA fragment containing the IRR (−193/−146) as the probe. Treatment with IFN-γ rapidly induced the formation of a single DNA-protein complex that appeared in extracts from cells treated for only 5 min and continued to be present at a comparable level 20 h after treatment with IFN-γ (Fig. 3B). This IFN-γ-induced complex was termed the IRR-AC. Formation of the IRR-AC was independent of ongoing protein synthesis, since nuclear extracts from U937 cells that had been treated with IFN-γ for 30 min in the presence of the protein synthesis inhibitor cycloheximide retained binding activity (Fig. 3B, lane 12). This result suggested that the IRR-AC was composed of preexisting factor(s) that required some posttranslational modification for DNA binding. Formation of the IRR-AC required an intact IRR, since DNA sequences containing either the 5' half or the 3' half alone...
failed to form an IFN-γ-induced complex that was stable under these electrophoretic conditions (data not shown).

The IFN-γ-induced IRR-AC Binds Specifically to the IRR and Is Competed by Other GAS Elements but Not by Other ISREs—

To determine if the binding of the IRR-AC to the IRR was specific, gel mobility shift assays were performed using nuclear extracts from untreated or IFN-γ-treated U937 cells in the presence of an excess of unlabeled double-stranded oligonucleotides. As shown in Fig. 3C, a 100-fold molar excess of the IRR (2193 to 2157) competed for the IRR-AC. This 37-bp sequence, which contains the imperfect inverted repeat, has thus far been determined to be the minimal sequence capable of binding the IRR-AC when used as a probe in gel mobility shift assays (data not shown). The 5'9-half of the IRR was able to compete as well as the intact IRR, whereas the 3'9-half of the IRR was only able to partially compete for the IRR-AC (Fig. 3C). In contrast, an unrelated sequence that binds the transcription factor SP1 did not compete for the IRR-AC (Fig. 3C).

Since the 5'9-half and the 3'9-half of the IRR share sequence homology with the GAS element and ISRE, respectively, GAS elements and ISREs from other IFN-inducible genes were also tested in gel mobility shift competition experiments. As shown in Fig. 3D, GAS elements from the IRF-1 and Ly-6E genes (28, 29) competed for the IRR-AC as efficiently as the intact IRR and 5'9-half-IRR. While the 3'9-half-IRR partially competed for the IRR-AC, the ISREs from the ISG54 and HLA-B7 genes (6, 58) did not compete for the complex (Fig. 3D). These results suggest that the IRR-AC contains factor(s) that are capable of binding to the GAS elements from other IFN-γ-inducible genes; lack of competition by the ISRE sequences suggests that ISRE-binding factors are not present in the IRR-AC.

Methylation Interference Analysis Shows the IRR-AC Binding Site Is Symmetrical—

To identify the potential contact points important for binding of the IRR-AC to the IRR, methylation interference analysis was performed using partially methylated end-labeled DNA fragments containing the sequence from −193 to −146 of the HLA-E promoter and extracts from U937 cells that had been treated with IFN-γ for 30 min. The methylation of several G residues located in both the 5'9-half and 3'9-half of the IRR interfered with formation of the protein-DNA complex (Fig. 4A). The interference pattern, summarized in Fig. 4B, was symmetrical, suggesting that the IRR-AC may consist of a dimeric complex. Interestingly, an interference pattern similar to that obtained for each half-site of the IRR was seen with methylation interference analysis of sis/platelet-derived growth factor-induced SIE binding to the SIE in the c-fos promoter (61, 62). The SIE shares sequence homology with GAS-like elements, and the platelet-derived growth factor-induced SIF-SIE complex has been previously shown to contain STAT1α by supershift analysis (63).

Point Mutation Analysis of the IRR Reveals a Correlation between the Binding of the IRR-AC and IFN-γ-induced Transcription—

To establish the functional importance of the IRR-AC in mediating the IFN-γ-induced activation of the HLA-E gene, point mutations were made in the 5'9-half and 3'9-half of the IRR and tested in both mobility shift assays and functional reporter gene assays (Fig. 5). As shown in Fig. 5A, two different oligonucleotides having mutations in the 5'9-half
of the IRR were used as competitors in mobility shift assays and compared with the wild type 5’-half of the IRR for their ability to compete for IRR-AC binding to the IRR. In the 5’-half-IRR-Tmut, two T residues were mutated to G residues. These point mutations have been previously shown to abolish the binding of IFN-induced factors to GAS elements from other genes (28, 30). In the 5’-half-IRR-Gmut, the three G residues mutated were those determined to be involved in IRR-AC binding to the IRR by the methylation interference analysis shown in Fig. 4. Competition experiments showed that these base changes either abolished or greatly reduced the ability of these sequences to compete for the IRR-AC (Fig. 5A). Similarly, the
the mutation analysis showed that binding of the IRR-AC to the IRR in vitro in mobility shift assays correlated with the IFN-γ inducibility of the HLA-E promoter in reporter gene constructs.

STAT1α Is Involved in the Formation of the IRR Activation Complex—Previous studies have demonstrated that the IFN-γ-induced complex identified as GAF contains a homodimer of the factor STAT1α (33). GAF has been shown to bind to the GAS or GAS-like elements present in several IFN-γ-inducible genes (29, 31, 32, 64–66). Given the rapid appearance and binding specificity of the IRR-AC, we reasoned that the IRR-AC would likely contain the IFN-γ-induced factor STAT1α. To determine if the STAT1α protein or other known IFN-inducible factors were present in the IRR-AC, we tested several antibodies specific for proteins that have been shown to bind to the GAS or ISRE IFN-inducible elements for their ability to cross-react with the IRR-AC. Polyclonal antibodies were incubated with nuclear extracts from IFN-γ-treated U937 cells and tested in mobility shift assays to determine if the formation or mobility of the IRR-AC was affected by antibody binding. As shown in Fig. 6, incubation with the antibody against STAT1α resulted in a supershifted complex (designated by the asterisk). In contrast, antibodies against the proteins STAT3, ISGF3γ, p48, IRF-1, and IRF-2 had no effect on the formation or mobility of the IRR-AC, similar to the rabbit IgG control. From these results, we conclude that STAT1α, or an antigenically related protein, is involved in the formation of the IRR-AC. The inability to detect the presence of the STAT3, ISGF3γ, p48, IRF-1, and IRF-2 proteins by antibody reactivity in the supershift analysis suggests that these proteins are not present in the IRR-AC. The immunoreactivity of these antibodies was verified by supershift analysis using previously reported conditions and nuclear extracts from cytokine-induced U937 cells with the ISG54 ISRE or IRF-1 GAS element as probes (Refs. 6 and 31, data not shown).

The IRR Contains High and Low Affinity Binding Sites—Results from the methylation interference footprinting analysis shown in Fig. 4 demonstrated that the pattern of residues involved in the binding of the IRR-AC to the IRR was symmetrical, suggesting the possibility that the IRR-AC was composed of a dimeric complex. If the same proteins were binding to both the 5'-half and 3'-half of the imperfect inverted repeat in the IRR, one would predict that a sequence having a duplication of the 5'-half or the 3'-half of the inverted repeat would bind a complex that exhibited the same mobility as that of the IRR-AC when tested in gel mobility shift assays. However, if different proteins were binding to the 5'-half and 3'-half of the imperfect inverted repeat in the IRR, duplication of the half-sites may result in a sequence that would not support the formation of an IFN-induced complex or one that would bind a complex that migrated with a different mobility than the IRR-AC. To test this, DNA fragments containing either the wild type IRR or a duplication of the 5'-half or 3'-half of the imperfect inverted repeat of the IRR were used as probes in mobility shift assays with nuclear extracts from U937 cells. As shown in Fig. 7A, the 2×5'-half-IRR and 2×3'-half-IRR probes were able to bind specific IFN-γ-induced complexes that exhibited the same mobility as that of the IRR-AC bound to the wild type IRR; however, the efficiency with which these probes bound the complex differed. The 2×5'-half-IRR sequence bound the complex about 2.5 times more efficiently than did the wild type IRR, whereas the 2×3'-half-IRR sequence bound the complex 10 times less efficiently than did the wild type IRR as determined by gel mobility shift assays using radiolabeled sequences and competition experiments with unlabeled double-stranded oligonucleotides (Fig. 7, A and B). The results shown here are consist-
ent with the methylation interference analysis shown in Fig. 4, suggesting the IRR-AC may be composed of a dimeric complex, and further suggest that the 5'-half and 3'-half of the imperfect inverted repeat present in the IRR may act as high affinity and low affinity binding sites, respectively. These results are also consistent with the results from the competition experiments presented in Fig. 3D demonstrating the differential ability of the 5'-half and 3'-half of the IRR to compete for the binding of the IRR-AC to the IRR in gel mobility shift assays.

The supershift analysis described above showed that the IRR-AC contains STAT1α or an antigenically related protein. To further demonstrate that the IFN-γ-induced complexes bound to the 2×5'-half-IRR and 2×3'-half-IRR probes were the same as the IRR-AC, supershift analysis was performed to determine if the complexes that bound to these sites also contained the STAT1α protein. As shown in Fig. 7C, incubation with the anti-STAT1α antibody resulted in a supershift of the IFN-γ-induced complexes bound to the 2×5'-half-IRR and 2×3'-half-IRR probes (designated by the asterisk). Similar to the supershift analysis shown in Fig. 6 for the wild type IRR,
the other antibodies to GAS- and ISRE-binding proteins did not alter the formation or mobility of the complexes binding to the 2×5'-half-IRR and 2×3'-half-IRR probes (data not shown).

**DISCUSSION**

Previous studies have shown that expression from the non-classical MHC class I HLA-E gene can be induced by IFN-γ (51); however, to our knowledge, work aimed at defining the molecular mechanisms of IFN-γ-induced HLA-E gene expression has not been reported. In this report, we have examined the mechanism of IFN-γ induction of the HLA-E gene, and the major findings of these studies are summarized as follows. In U937 cells, IFN-γ induced the expression of HLA-E in the absence of ongoing protein synthesis. Functional analysis of the HLA-E promoter using CAT reporter gene constructs demonstrated that the region from −193 to −146, which we have termed the IRR, is necessary and sufficient to mediate the full response to IFN-γ. The IRR contains an imperfect inverted repeat; the 5'-half of the IRR mediates a partial response to IFN-γ, whereas the 3'-half of the IRR is functionally nonresponsive to IFN-γ. The IFN-γ inducibility of constructs containing the intact IRR demonstrate that 5'-half and the 3'-half of the IRR act synergistically to mediate the full response to IFN-γ. Gel mobility shift assays revealed that the IRR binds a single, specific IFN-γ-induced complex, the IRR-AC, that is formed rapidly and in the absence of ongoing protein synthesis. Methylation interference analysis of the IRR and gel mobility shift assays using the 2×5'-half-IRR and 2×3'-half-IRR probes with duplicated half-sites suggested that the IRR-AC may be composed of a dimeric complex and that the 5'-half and the 3'-half of the inverted repeat within the IRR function as high and low affinity binding sites, respectively. By supershift analyses we have demonstrated that the IRR-AC contains STAT1α or an antigenically related protein.

The results presented here clearly demonstrate that the mechanism of IFN-γ-induced transcription of the HLA-E gene is distinct from those previously described for other MHC class I genes. The ISRE present in the 5'-flanking sequence of several classical MHC class I genes has been shown to be both necessary and sufficient for IFN-γ inducibility, although in some cases additional sequences are required to mediate the full response to IFNs (22, 25, 43). The IRF-1 protein has been identified as one factor that binds to the ISRE and is involved in the IFN-γ-induced expression of MHC class I genes (44, 45). Although the 3'-half of the IRR of the the HLA-E gene shares sequence homology with the ISRE (Ref. 4, Fig. 3A), reporter gene constructs containing the 3'-half of the IRR alone are not responsive to IFN-γ, and this sequence does not support the formation of an IFN-γ-induced complex in gel mobility shift assays. The sequence of the intact IRR, which contains the imperfect inverted repeat and is required to mediate the full response to IFN-γ, does not appear to be present in the 5'-flanking sequence of other class I genes. The binding characteristics of the IFN-γ-induced IRR-AC differ from the complex reported to bind to the ISRE of class I genes with respect to the time course of DNA-binding activity and the requirement for ongoing protein synthesis (44). Furthermore, our results demonstrate that the ISREs from the HLA-B7 and ISG54 genes are not able to compete for the IRR-AC, nor are known ISRE-binding proteins present in the complex based on the supershift analysis. These differences in the mechanism of IFN-γ induction of the HLA-E gene further distinguish this nonclassical class I gene from the classical class I genes. The significance of this locus-specific transcriptional regulation as it relates to the possible function(s) of the HLA-E molecule in the immune response remains to be determined.

Our findings suggest that the mechanism of IFN-γ-induced transcription of the HLA-E gene is, in some respects, similar to the IFN-γ induction of several immediate early genes that are mediated through the binding of STAT1α homodimers to GAS-like elements (29, 31–33, 64–66). The sequence of the IRR contains an extended imperfect inverted repeat in which the 5'-half of the IRR shares sequence homology to GAS-like elements (Ref. 4, Fig. 3A). Competition experiments demonstrated that the GAS elements from the IRR-1 and Ly-6E genes were able to compete for the IFN-γ-induced IRR-AC that binds to the IRR, suggesting a common factor binds to these sequences. Furthermore, gel mobility shift assays revealed that the IRR-AC contains STAT1α or an antigenically related protein.

Despite these similarities, several lines of evidence suggest the mechanism of IFN-γ-induced HLA-E gene expression is distinct from the IFN-γ induction of genes mediated through the binding of a STAT1α homodimer to a GAS-like element. The functional analysis of the HLA-E promoter demonstrates that the intact IRR is necessary and sufficient to mediate the full response to IFN-γ; the 5'-half and the 3'-half of the IRR together act synergistically to mediate the response. Consistent with these findings, mutations made in the 5'-half or 3'-half of the IRR abolish or markedly reduce the IFN-γ-induced activity. In gel mobility shift assays, the intact IRR is required for the formation of the IFN-γ-induced IRR-AC; neither the 5'-half nor 3'-half of the IRR alone binds an IFN-γ-induced complex when used as probe. The IRR-AC contacts DNA residues located in both the 5'-half and 3'-half of the IRR as determined by methylation interference analysis, demonstrating that the binding site extends beyond the sequence homologous to GAS-like elements.

Characterization of IRR-AC binding to the IRR by methyla-
tion interference analysis and gel mobility shift assays using the 2×5′-half-IRR and 2×3′-half-IRR probes with duplicated half-sites suggests that the IRR-AC may be composed of a dimeric complex. The complexes that bind to the 2×5′-half-IRR and 2×3′-half-IRR probes have the same relative mobility as the IRR-AC bound to the IRR and also contain the STAT1α.

Fig. 7. The IRR contains high and low affinity binding sites for the IRR-AC. A, gel mobility shift assays were performed with nuclear extracts (10 μg) from U937 cells untreated or treated with IFN-γ (250 units/ml) for 30 min and the double-stranded, 32P-labeled IRR, 2×5′-half-IRR, and 2×3′-half-IRR sequences as probes. Probes were 32P-labeled to the same specific activity. For the 2×5′-half-IRR probe, positions −158 through −162 were mutated to result in a palindromic sequence containing a duplication of base pairs −175 to −188. For the 2×3′-half-IRR probe, positions −183 through −187 were mutated to result in a palindromic sequence containing a duplication of base pairs −157 to −170. Where indicated, 100-fold molar excess of the unlabeled, double-stranded oligonucleotide IRR (−193/−157) was included in the binding reaction as competitor. B, quantitative analysis of gel mobility shift competition assays. Gel mobility shift assays were performed using the double-stranded, 32P-labeled IRR probe and analyzed as described in Fig. 3D to determine the percentage of IRR-AC that remained bound (% Complex Bound on the y axis). The IRR (−193/−157) and 2×5′-half-IRR unlabeled double-stranded competitor oligonucleotides were used at 2-, 5-, and 20-fold molar excess; the 2×3′-half-IRR unlabeled double-stranded competitor oligonucleotides was used at 2-, 5-, 20-, and 50-fold molar excess. C, antibody specific for STAT1α recognizes the IFN-γ-induced IRR-AC bound to the 2×5′-half-IRR and 2×3′-half-IRR sequences. Supershift analysis using the anti-STAT1α antibody was performed as described in Fig. 6. The asterisk indicates the position of the supershifted complexes obtained with the antibody specific for STAT1α.
protein as determined by supershift analysis. The IFN-γ-activated STAT1α protein has been shown previously to bind to the GAS element as a homodimer, although it also appears to heterodimerize with other STAT proteins, such as STAT3, and bind to GAS or GAS-like elements when activated by other cytokines (33, 67, 68). Given these findings, our studies suggest that the IRR-AC may be composed of dimers containing the STAT1α protein, which bind to both the 5′-half and 3′-half of the IRR. However, from these results it cannot be determined whether the STAT1α protein is binding to these sites as homodimers or possibly heterodimers with another STAT protein or other unknown factor.

The results of the gel mobility shift competition experiments and those utilizing the 2×-5′-half-IRR and 2×-3′-half-IRR sequences as probes suggest that the 5′-half and the 3′-half of the IRR function as high and low affinity binding sites, respectively. The 3′-half of the IRR appears to bind STAT1α, albeit with much lower affinity relative to the 5′-half of the IRR. In addition to the binding activity demonstrated using the 2×-3′-half-IRR sequence, functional studies show that the CAT activity of a construct containing the 2×-3′-half-IRR sequence driving the TK promoter is induced 4-fold by IFN-γ (data not shown). GAS-like elements that bind STAT1α have been identified in several IFN-γ-inducible genes, and a comparison of their sequences reveals the 9-bp palindromic motif TT(C/A)(C/T)N(G/A)(G/T)AA where the half-sites (either TTCC, TTAC, or TCTT) are separated by 1 bp (66, 69). The 5′-half of the IRR shares sequence homology with the GAS element and contains the sequence TTTCCNGGA, in which the half-sites are separated by 2 bp. In contrast, the sequence of the 3′-half of the IRR, which more closely resembles an ISRE, contains a direct repeat of two TTCC half-sites (TTCCNNTTCC) separated by 2 bp. While recent studies by Bluyssen et al. (70) have demonstrated that IFN-γ induction may be mediated through the ISRE by a complex composed of p48 and STAT1α, binding of STAT1α to the ISRE in the absence of p48 could not be detected. The results of our experiments suggest that STAT1α binds to the 3′-half of the IRR in the absence of p48, as determined by the inability of antibodies directed against p48 to affect the formation of the IRR-AC (data not shown). Taken together, these studies suggest that the IRR is a distinct STAT1α-binding element that contains the higher affinity 5′-half, which differs slightly from the GAS consensus sequence, and the lower affinity 3′-half, which contains several mismatches at conserved base pair positions. Based on the results of mutational analyses of several GAS-like elements, which demonstrate the importance of the palindromic sequence and the spacing between the half-sites with respect to STAT1α binding (28, 30, 69), it is surprising that the 3′-half of the IRR acts as a binding site for STAT1α. However, given the synergistic effect between the 5′-half and 3′-half of the IRR in both DNA binding and functional assays, it seems likely that the interaction of STAT1α with the 3′-half of the IRR is stabilized through protein-protein interactions with STAT1α that binds at the 5′-half of the IRR or other proteins binding to the IRR.

Preliminary results of gel mobility shift assays using minor groove-binding drugs and DNA oligonucleotide competitors suggest that the IRR-AC contains an additional protein, besides STAT1α, that may be critical to the observed synergism between the 5′-half and 3′-half of the IRR. It is interesting to speculate that the minor groove-binding protein high mobility group IY, which has recently been shown to play a role in the transcriptional regulation of several genes by facilitating the binding of transcription factors to their cognate DNA binding sites (71–75), might mediate this effect. Studies are under way to address this possibility.

Examples of other IFN-γ-responsive elements have been reported in which interactions between adjacent protein binding sites are required for full induction by IFN-γ. Pearse et al. (30) demonstrated that the GRR of the FcγRI gene contains both a GAS element and an adjacent 5′ protein binding site. While the GAS element alone functions to mediate 25% of the IFN-γ response, the intact GRR is required for maximal induction by IFN-γ. Furthermore, the GAS element of the GRR alone is able to bind STAT1α in mobility shift assays; however, the formation of an additional IFN-γ-induced complex required the intact GRR. In addition, Wong et al. (76) recently described another IFN-γ-responsive element, the γRE-1, as a unique palindromic element that mediates the IFN-γ induction of the mig gene. The γRE-1 contains an extended imperfect inverted repeat in which both half-sites share sequence homology with the palindromic GAS element. The intact γRE-1 is required for the full functional response to IFN-γ. The γRE-1 binds a complex (γRF-1) that cross-reacts with anti-STAT1α antibodies and consists of at least two proteins of approximately 95 and 130 kDa (76, 77). While the sequence of the IRR of the HLA-E gene is distinct from these other IFN-γ-responsive elements, the extent to which protein components of the IRR-AC differ from those binding other IFN-γ-responsive elements remains to be determined.

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