Recent analyses of the upstream regulatory regions of the class I major histocompatibility complex genes in higher primates provided a generalized structural basis for the differential expression of A- and B-locus gene products in response to specific physiological stimulus. Among the regulatory sequences that differ between the loci is the interferon-responsive element (IRE). While the B-IRE is conserved, the A-IREs have species-specific sequence variation. We previously demonstrated that the B-IRE was an interferon (IFN)-inducible enhancer, whereas none of the A-IREs were functional. In the present study, we examined the biochemical basis for the enhancer activity of the conserved B-IRE and found that this may be attributed to a novel γ-IFN-inducible factor. This factor accumulated in nuclei of cells within minutes of exposure to γ-IFN. Its appearance was independent of de novo protein synthesis. However, it was not detected in nuclei of cells treated with herbimycin A, suggesting that its appearance depends on a protein kinase activation pathway. Supershift assays indicated that it was distinct from STAT1α, IFN regulatory factor-1, and p48, transcription factors known to bind IRE-like sequences found in regulatory regions of many non-major histocompatibility complex γ-IFN-responsive genes. Competition assays show that this novel factor bound B-IRE with relatively high affinity, about 100-fold more than that for the A-IRE sequence. This factor was also present in STAT1α and p48 somatic mutants that also exhibited B-IRE enhancer activity in reporter gene bioassays in a manner similar to those seen with wild type cells. These observations indicate the existence of a novel γ-IFN-dependent transcriptional activation pathway that correlates with the differential enhancer activity of the HLA-B IRE.

The gene products of the classical class I major histocompatibility complex (MHC), referred to as HLA-A, -B, and -C in humans or H-2 K, D, and L in the mouse, play a central role in immune responses. They participate in the development of the repertoire of T cells in the thymus and, in the periphery, serve as antigen presenting molecules to CD8+ T cells (1–4). In adults, class I proteins are ubiquitously expressed, but the levels of expression vary widely (5) and may be modulated by various stimuli such as cytokines, viruses, oncogenes, and tumor-associated factors (6–15). Additionally, the loss and/or the aberrant expression of the class I MHC glycoproteins have been implicated in various forms of immune dysfunction. For instance, the loss of expression of class I molecules in mice has been associated with marked susceptibility to infections due to the lack of CD8+ T cells (16–20). In human malignancies, tumorigenicity and metastatic growth have been associated with the generalized loss of expression of class I HLA proteins (21–23) or with locus-/gene-specific suppression (7, 9, 24). Similarly, infection with certain viruses may result in the generalized loss, locus-specific loss, or locus-specific up-regulation of class I glycoproteins (25–29). Clinical studies indicate that the relative importance of these molecules differs in allograft rejection (30, 31). These observations strongly indicate that the regulation of expression of MHC class I genes may determine their functional relevance in a particular physiological context.

In humans, the expression of class I HLA-A and -B glycoproteins is not tightly coordinated (5). Previous studies indicate that immature cortical thymocytes express very low levels of HLA-B (32). However, the in vitro treatment of isolated cortical thymocytes and thymocyte-derived cell lines with type I or type II interferon (IFN) results in the preferential up-regulation of HLA-B (32, 39). This preferential induction of HLA-B by IFN has been reported to occur in other cell types as well (34, 35). In contrast, HLA-A has been shown to be more responsive to inducers, like tumor necrosis factor α, that are potent activators of the Rel family of transcription factors (35, 36).

Consistent with the observed differences in the basal and inducible expression of the human HLA-A and -B genes, results of the analyses of the 5′ proximal regulatory regions of the A and B homologues in the higher primates reveal strong locus-specific properties of the two gene loci (37). In the course of 25 million years of evolution, the A-locus promoters have accumulated significant structural changes, whereas the B-locus promoters are generally more conserved. Interestingly, these accumulating changes in the A-promoters during phylogeny are correlated with fewer transcription factor binding motifs. These observations provide a phylogenetic basis for the differential regulation of expression of class I MHC genes.

Among the A-locus regulatory elements that have changed over time is the interferon-responsive element (IRE), a member...
of a family of enhancers that determines induction of many genes by IFNs (38–40). In reporter gene bioassays, none of the A-IRE variants are functional, whereas the conserved B-IRE is a strong IFN-inducible enhancer (41). A single mutation, common among the primate A-IREs, is sufficient to inactivate the enhancer element. Additional mutations specific to different primate branches did not rescue enhancer activity. It is important to note that the conserved B-IRE motif in the higher primates is the identical sequence found among the classical class I MHC genes of phylogenetically lower mammals and that these orthologous genes are also responsive to γ-IFN (40, 42, 43). This suggests a common γ-IFN-inducible regulatory machinery for the mammalian class I MHC genes. Thus, the present study was carried out to examine the biochemical basis for the differential enhancer activity between the MHC A- and B-IRE in response to γ-IFN. Inasmuch as the pathways for the activation of non-MHC IFN-stimulated genes (ISGs) have already been established (44), experiments were performed to determine whether or not the IFN-inducible enhancer activity of the class I MHC B-locus IRE is attributable to similar or unique transcription factors. Although the up-regulation of many MHC class I genes by IFN is well documented (34, 35, 40), the biochemical basis for this observation remains to be elucidated. Here, evidence is presented for a novel γ-IFN-inducible factor that correlates with the strong enhancer activity of the MHC class I B-locus IRE.

MATERIALS AND METHODS

Cell Culture—HeLa and K562 cells were maintained in suspension cultures as described previously (41). The fibroblast cell lines 2FGTH, U2A(Dp48), and U3A (ASTAT1a) were cultured as monolayers in Dulbecco’s minimum essential medium (Sigma) supplemented with 10% fetal calf serum (Life Technologies, Inc.), 60 µg/ml penicillin, 135 µg/ml streptomycin sulfate, 80 µg/ml gentamycin, 300 µg/ml L-glutamine, 10−5 M 2-mercaptoethanol, and 250 µg/ml hygromycin B (Sigma). These cell lines were provided by Dr. George Stark (Cleveland Clinic Research Institute).

Cell Surface Expression of Class I HLA—Cell surface expression of class I HLA molecules in the three fibroblast cell lines was determined as described previously (41). Briefly, monolayers were washed with phosphate-buffered saline, treated with trypsin-EDTA for 1 min, and washed in complete medium. Aliquots of 2 × 10^7 cells were re suspended in complete medium and incubated for 18 h at 37 °C with or without γ-IFN (BioSource, Camarillo, CA) and/or βIFN (Sigma) at the indicated concentrations. Class I HLA expression was determined by flow cytometry using the monoclonal antibody (mAb) W6/32 (ATCC, Bethesda, MD).

Transient Transfection and Luciferase Assay—Transient transfections of luciferase reporter plasmids and the normalizing β-galactosidase reporter were carried out as described previously (41). Transfected cells were incubated at 37 °C in the presence or absence of 1000 units/ml γ-IFN, and β-galactosidase activity was measured after 18 h. Enzyme activities were assayed by chemiluminescence using a luciferase kit (Promega, Madison, WI) and a β-galactosidase assay kit (Galactolight, Tropix, Bedford, MA). Light emissions were measured using a luminometer (Lumat LB9501, Berthold Analytical, Nashua, NH).

Nuclear Extracts and Mobility Shift Assay—Nuclear extracts were prepared using a high salt extraction protocol described previously (45). Briefly, 2 × 10^7 cells were incubated in complete medium containing 1000 units/ml γ-IFN for the indicated periods. Cells were then washed in cold phosphate-buffered saline and lysed in a HEPES hypotonic buffer. Nuclei were isolated by centrifugation and washed twice in 500 µl of wash buffer. Nuclear proteins were extracted in 75 µl of extraction buffer and cleared of nuclear debris by centrifugation, and the concentration of extracted proteins was determined using a protein assay kit (Bio-Rad). Samples were kept on ice during the entire procedure with the exception of the latter extraction steps that were done at 4 °C. All centrifugation steps were also carried out at 4 °C using a tabletop microcentrifuge. Nuclear extracts were aliquoted into smaller quantities, snap-frozen in liquid nitrogen, and stored at −70 °C.

To examine the role of protein synthesis on the activation of γ-IFN-inducible factors, nuclear extracts were prepared from HeLa cells exposed to γ-IFN in the presence of cycloheximide. Cells were pretreated with 50 µg/ml cycloheximide (Sigma) in serum-free media for 30 min, washed, and resuspended in the same medium containing 1000 units/ml γ-IFN. Fresh aliquots of cycloheximide at varying concentrations were also added and then cells were incubated for an additional 30–45 min. Nuclear extracts were prepared as described above.

The role of protein kinases in the activation of γ-IFN-inducible transcription factors was also examined. This was carried out in experiments using a kinase inhibitor herbimycin A (45). Cells were initially incubated in serum-free media containing a non-cytotoxic dose of 50 µM herbimycin A (LC Labs, Woburn, MA) or an equal volume of dimethyl sulfoxide, the drug carrier, for 4 h at 37 °C. Cells were washed, resuspended in the same culture medium containing the drug and 1000 units/ml γ-IFN, and incubated at 37 °C for the indicated period. Nuclear extracts were prepared as described above.

Electrophoretic mobility shift assays were carried out as described previously (45). About 20 µg of nuclear extract was combined with 15 µl of binding buffer, 3 µg of poly(dI-dC) (Sigma), and 3 µg of nonspecific oligonucleotide (Table I). The total volume of the reaction mixture was adjusted to 25 µl with the wash buffer and left on ice for 30 min. The radiolabeled specific double-stranded oligonucleotide probe (see below) was added to the reaction and incubated at room temperature for an additional 30 min. Protein-DNA complexes were resolved in 6% nondenaturing polyacrylamide gels and autoradiography. The relative intensities of the shifted bands were determined by optical imaging of multiple autoradiograms using the AMBIS 4000 System (Ambis Inc., San Diego, CA). The NF-κB probe (GGGATCCTGGGGATTCCCCA) was used in initial experiments to demonstrate the γ-IFN inducibility of the γ-IFN-inducible binding activity described here (see text). Results of the assays showed no detectable γ-IFN-induced NF-κB binding activity (data not shown).

In supershift assays, specific antibody was added before the addition of the oligonucleotide probe and incubated at 4 °C for 30 min. Antibodies used in the study included anti-STAT1 (Transduction Lab., Lexington, KY), rabbit antiserum to human p48, and IRF-1. About 1 µg of anti-STAT1 mAb or a 1:50 dilution of either anti-p48 or anti-IRF-1 was added to the reaction. The same amount of an irrelevant mouse IgG2b (Sigma) was added to control reactions. Antiser to p48 (46) and IRF-1 (47) were provided by Drs. David Levy (NYU Medical Center) and Richard Pine (Public Health Research Institute, NY), respectively.

Oligonucleotide probes used in this study are listed in Table I. Their binding specificity for known transcription factors are as indicated. With the exception of the nonspecific oligomer, the complementary strands of all oligonucleotides were synthesized (DNA synthesizer Model 301A, Applied Biosystems, Foster City, CA) and purified as described previously (48). Equimolar amounts of both strands of the oligomers were radiolabeled with [γ-32P]ATP (DuPont NEN) by the standard end-labeling reaction; the unincorporated radiolabel was removed by centrifugal column chromatography (QuickSpin, Boehringer Mannheim); and the eluates of the respective oligomer pairs were combined and annealed. The annealed probes were phenol/chloroform-extracted, ethanol-precipitated, lyophilized, and resuspended in sterile water to a final concentration of 40 fmol/µl. Probes were stored at −20 °C and used within 7 days.

RESULTS

A γ-IFN-inducible Transcription Factor Binds the MHC B-Locus IRE Sequence—Reporter gene bioassays have previously demonstrated that none of the MHC A-locus IRE variants were functional, whereas the conserved B-IRE exhibited IFN-inducible enhancer activity (41) comparable with a known γ-IFN-inducible enhancer, SIE (45). By virtue of the design of reporter gene constructs in which the 12-nucleotide core B-IRE sequence flanked by MHC A-locus sequences, the enhancer activity of the element was attributed to a single nucleotide in the middle of the sequence, i.e. an A → T change between the B-IRE and the A-IREs. In order to determine what transcription factor(s) may be responsible for this enhancer activity, similar IRE oligonucleotide probes were synthesized and used in gel shift experiments. As shown in Table I, the MHC IRE probes were 17-mers that differed from each other by one or two nucleotides.

Using nuclear extracts from K562 and HeLa cells, gel shift assays revealed the appearance of a B-IRE binding factor in the nuclei of cells within minutes of exposure to γ-IFN, i.e. between...
Novel γ-IFN-inducible Factor Binds the Class I MHC IRE

### Table 1

| Name            | Gene            | Sequence<sup>a</sup> | Transcription factor binding | Refs. |
|-----------------|-----------------|-----------------------|------------------------------|-------|
| B-locus         | Conserved MHC-B | CGCAGTTTTCATCTTCTC CC<sup>b</sup> | STAT1<sup>a</sup> | 41    |
| Human/Chimp-A   | HLA-A/Patr-A    | CGCAGTTTTCATCTTCTC CC<sup>b</sup> | IRF-1 | 41    |
| Gorilla-A       | Gogo-A          | CGCAGTTTTCATCTTCTC CC<sup>b</sup> | p48  | 41    |
| Orangutan-A     | Popy-A          | CGCAGTTTTCATCTTCTC CC<sup>b</sup> | Yes  | 41    |
| C13             | Synthetic oligo | TACGTTCACCTAACCTC TT | Yes  | 57, 58 |
| ISG15           | IFN-stimulated gene 15 | TGGTCAGTTTTCATCTTCTC CGCGGCCCGGGTTCGG | Yes  | 54, 56 |
| ISG54           | IFN-stimulated gene 54 | TCGAGTTTTCATCTTCTC TTGGTA | Yes  | 54, 56 |
| OAS             | Oligo-A synthetase | TTTTCGTTTTCCTCAG | Yes  | 59, 60 |
| SIE             | c-fos           | CGGATTTTTCGCTAAATC | Yes  | 45, 62 |
| 6/18            | Gene 6/18       | TGGAGCTCTATTTCCTGCT | Yes  | 39    |
| 9/27            | Gene 9/27       | GAGCTCTATTTCCTGCTC | Yes  | 39    |
| B-locus         | Conserved MHC-B | CGGCCATTTCCCGTAAATC | Yes  | 37    |
| Human-A         | HLA-A           | ACCTCCGAGCTCTATTTCCTCCTC CC<sup>c</sup> | Yes  | 37    |
| SP1             | Human AS promoter | GCTCCAGCGGGGCGCCGCCGGCTTCGG | Yes  | 63    |
| Nonspecific oligonucleotide | TCGAAGTACTAATGCTCGAGATGATCAGTCTAATCCGACTCC | | | |

<sup>a</sup> Except for the nonspecific oligonucleotide probe, sequences shown were synthesized along with their complementary strands. For gel shift assays, equimolar amount of both strands were radiolabeled, annealed, and used as binding probes.

<sup>b</sup> Sequences of these IRE probes are identical to previously described IRE-luciferase reporter plasmids (41); hence, they are referred to as the shorter 17-mer reporter versions of the IREs.

<sup>c</sup> These are the human A- and B-specific core IRE with their correct flanking locus-specific sequences (37) and are referred to here as the longer 28-mer in-context versions of the IREs.

![Figure 1](image-url)

**Fig. 1.** The MHC B-IRE sequence binds a γ-IFN-inducible factor. About 2 × 10<sup>6</sup> K562 (A) and HeLa cells (B and C) were incubated with 100 units/ml γ-IFN at 37°C. At the indicated times, nuclear extracts were prepared and used in gel shift assays using oligonucleotide probes corresponding to the MHC B-IRE (A and B) and the human/chimpanzee A-IRE (C). The sequences of the probes (Table I) were identical to those used previously in reporter gene constructs (41). Inset, quantitative representation of gel shifts. Relative intensities of the shifted bands were determined by optical imaging of autoradiograms using the AMBIS 4000 System. Values depicted are net signals after background subtraction for each lane of the autoradiogram shown.

10 to 45 min (Fig. 1, A and B). The appearance of this B-IRE binding gamma-IFN-activated factor, herein referred to as BIGAF, is fairly transient in that the observed protein binding activities of B-IRE dropped to base-line levels after 1–2 h of exposure to γ-IFN. The levels of induction of BIGAF varied considerably between extracts ranging from 5.6- to 171-fold in excess of the detectable base-line binding activity (Table II). The reason for this variability is not known, although it may be attributed to differences in endogenous BIGAF activity of untreated cells possibly caused by variations in the density of cells at harvesting. Treatment of cells with type I IFN had no effect on the presence of BIGAF (data not shown).

The inducible protein binding profile of the B-IRE sequence was not seen in similar gel shift assays using the human/chimpanzee A-IRE variant (Fig. 1C). We noted however, that A-IRE had a detectable but consistently low level of protein binding activity. This suggests that there may be two factors (or factor complexes) with the similar mobilities that were being detected in these assays, namely a γ-IFN-inducible factor that binds B-IRE (i.e. BIGAF) and a constitutive factor that binds the A-IRE variant.

The B-IRE Binding γ-IFN-activated Factor (BIGAF) Is Distinguishable from a Constitutive A-IRE Binding Factor—To determine whether the observed preferential binding of BIGAF to B-IRE and the low level of A-IRE binding activity reflected differences in the affinity of BIGAF for the two IRE sequences or whether there are two distinct IRE-binding factors, reciprocal competition assays were conducted using nuclear extracts from γ-IFN-stimulated and unstimulated cells. Consistent with previous observations (Fig. 1), gel shift assays using nuclear extracts from γ-IFN-stimulated cells showed vigorous B-IRE binding activity, whereas A-IRE binding activity was minimal (Fig. 2). In contrast, gel shift assays using extracts from unstimulated cells yielded the opposite results, i.e. a strong A-IRE, but not B-IRE, binding activity. Additionally, the B-IRE binding activity seen with extracts from γ-IFN-treated cells was not competed by A-IRE, and vice versa; the A-IRE binding activity seen with extracts from unstimulated cells was not
The binding activities of A- and B-IRE are distinguishable from each other. Nuclear extracts from HeLa cells either unstimulated or exposed to γ-IFN for 30 min were used in reciprocal competition gel shift assays using the indicated probes. Unlabeled competitor oligomers were added to binding reactions at the indicated concentrations in molar excess to the radiolabeled binding probe. Unlabeled arrows indicate the expected positions of the B- and A-IRE binding factors.

FIG. 2

competed by B-IRE. In either case, 300 M excess of unlabeled competitor did not cross-inhibit the IRE-binding activities, whereas the A- and B-IRE sequence-specific binding activities were blocked by 25 M excess of the competitor oligo of the identical sequence. This non-reciprocal nature of the relative competitiveness of the A- and B-IRE is therefore consistent with the hypothesis that a constitutive factor binding the A-IRE is different from the γ-IFN-induced factor, i.e. BIGAF, binding the B-IRE sequence.

To further address the notion that there is a constitutive A-IRE binding factor distinct from that of BIGAF, gel shift assays were carried out using a different set of A- and B-IRE probes. Unlike the 17-mer IRE probes used in the previous experiments that contained the reporter A- or B-IRE sequences (41; refer also to Table I), this set of probes was 28-mer containing the core A- or B-IRE in the context of their correct flanking locus-specific sequences (37).

Results showed that there is no significant difference in the binding activities between the 17-mer and the 28-mer forms of the B-IRE probe (Fig. 3A). Using nuclear extracts from cells treated with γ-IFN, protein binding activities of both probes were equivalent, and these probes equally competed each other. Consistent with previous observations (Fig. 2), both forms of the B-IRE showed low binding activity with nuclear extracts from unstimulated cells (Fig. 3B). In contrast, the 28-mer form of A-IRE showed a greater binding activity with nuclear extracts from unstimulated cells compared with that seen with the shorter form or those seen with either forms of B-IRE. As seen previously, the A-IREs showed weak binding activities, if any, with nuclear extracts from γ-IFN-treated cells.

While the binding activities of the A-IRE and B-IRE sequences were clearly distinguishable from each other (Figs. 2 and 3), we noted binding activities similar to that seen with B- and A-IRE sequences when other related IRE-like sequences were used as binding probes (data not shown), e.g. ISG54, C13, oligo-adenylate synthetase, 6/16, and 9/27 (refer to Table I). Each of these sequences can compete B-IRE binding activity when they were added to the binding reactions at 300 M excess indicating that they are capable of binding BIGAF (Fig. 4). Furthermore, their respective binding activities were also effectively competed by A-IRE at 25–300 M excess (data not shown). These observations indicate that the fine specificities of the IRE-like probes were intermediate between that of B-IRE and A-IRE. The fact that A-IRE can cross-compete the IRE-like sequences, but not the B-IRE sequence (refer to Figs. 1–3), indicates that the γ-IFN-inducible factor described here has a strong binding preference for the B-IRE sequence.

BIGAF Is Antigenically Distinct from STAT1α, IRF-1, and p-48—Transcriptional activation of many ISGs by γ-IFN has
been previously attributed to STAT1α homodimers (also known as p91 or ISGF3α) and GAF, both of which specifically bind to the GAS regulatory element (44, 49–53). Our analysis of the structure of class I MHC upstream regulatory regions (37), however, did not reveal any sequence motif that resembled GAS. As demonstrated previously, the sequence we refer here-to as IRE is the element that controls responsiveness of MHC class I genes to γ-IFN as well as to α/β IFNs (34, 40, 41).

It is important to note that the MHC class I IRE sequence is structurally similar to the so-called ISRE sequence that accounts for the responsiveness of many non-MHC ISGs to α IFN (44, 54–61). Along these lines, two other transcription factors, namely IRF-1 and p48, have been previously found to bind ISRE sequences and are inducible with γ-IFN, respectively (54–61). In the present study, we examined whether or not BIGAF is one of the previously described transcription factors known to bind GAS (e.g. SIE) or ISRE (e.g. ISG54, 6/16, 9/27) sequences.

In order to distinguish BIGAF from STAT1α, IRF-1, and p48, gel shift experiments were performed using extracts from cells exposed to γ-IFN for longer periods. Results showed that the protein binding profile of B-IRE oligo probes was distinct from that of SIE (Fig. 5), a known STAT1α-binding sequence (45, 62). As seen previously, the peak of B-IRE binding activity of BIGAF occurred within 45 min of exposure to γ-IFN and then it dropped to base-line levels after 1.5 h. This protein binding profile of B-IRE was clearly distinguishable from that of SIE. As the results showed, SIE bound a nuclear factor with a slower mobility than that of BIGAF, and this SIE-bound factor was supershifted by anti-STAT1α mAb (Fig. 6). BIGAF bound to B-IRE was not recognized by anti-STAT1α mAb.

Additionally, BIGAF binding to B-IRE is also distinguishable from the protein profiles of two IRF-1/p48 binding oligo probes (Fig. 5), namely C13 (57, 58) and ISG54 (54, 56). The latter probes bound two proteins (or protein complexes) that had mobilities faster than that of BIGAF. One band appeared to be a nonspecific binding activity as it was observed even with nuclear extracts from unstimulated cells. The other band was detected in nuclear extracts after 1–1.5 h of exposure to γ-IFN, and the peak of binding activity was observed in 9 h. It must be noted that this latter faster migrating factor also binds B-IRE, but the intensity of the signal of shifted bands varied between experiments. In supershift assays, the binding of this latter factor was inhibited by anti-human IRF-1 antisera (Fig. 6).

Neither anti-p48 nor anti-human IRF-1 antisera affected the binding of BIGAF to B-IRE.

Activation of BIGAF Is Inhibited by Herbimycin A, but Not by Cycloheximide—The activation of γ-IFN-inducible transcription factors, like STAT1α, has been previously demonstrated to involve protein kinases resulting in the phosphorylation of critical tyrosine residues in their DNA binding domains (44).
For STAT1α, this phosphorylation could be detected by supershift assays using anti-phosphotyrosine mAbs, and DNA binding can be blocked by protein kinase inhibitors (45). Similar anti-phosphotyrosine supershift experiments involving BIGAF binding to B-IRE, however, did not show any detectable changes in its mobility (data not shown). On the other hand, treatment of cells with herbimycin A, a potent protein tyrosine kinase inhibitor, blocked the binding of BIGAF to the B-IRE oligo probes (Fig. 7). As seen previously (45), herbimycin A inhibited the binding of STAT1α to SIE oligo probes. The inhibition of BIGAF and STAT1α binding activities was not due to a generalized drug-induced inactivation of transcription factors. Results of gel shift assays showed that exposure of cells to either herbimycin A or γ-IFN did not affect SP1 (63) binding activities as expected. Previous studies indicated that activation of SP1 does not involve a protein tyrosine kinase but a DNA-dependent kinase activity resulting in the phosphorylation of serine and threonine residues (64).

However, the induction of BIGAF by γ-IFN was unaffected by the treatment of cells with cycloheximide (Fig. 8), suggesting that it does not require de novo protein synthesis. The regimens of cycloheximide treatment employed in these experiments were conditions found to significantly reduce levels of trichloroacetic acid-precipitable radioactivity of biosynthetically labeled cells (data not shown).

**Somatic Mutants of IFN Signaling Exhibit B-IRE Enhancer Activity**—The critical roles of STAT1α and p48 in the induction of ISGs by γ-IFN and αIFN, respectively, have been previously confirmed with the generation of somatic mutants for these two transcription factors (44). Immunofluorescence staining for surface class I HLA showed that these somatic mutants expressed high levels of surface class I molecules comparable with that seen in the wild type cells (Fig. 9) and as previously observed with HeLa cells (41). This suggested that the constitutive pathway for class I HLA gene expression was reasonably functional in these somatic mutants.

However, there were differences in the patterns of inducible expression of class I HLA molecules between the two mutants. In Δp48 cells, βIFN did not elicit a significant increase in the level of surface class I expression unlike those seen with cells exposed to γ-IFN or to a combination of βIFN and γ-IFN, suggesting a role for p48 in class I gene induction. Although induction of class I genes by βIFN is well documented (40), whether or not this is associated with the p48-containing ISGF3 (or an ISGF3-like) transcription factor complex (44, 46, 59–61, 65, 66) remains to be examined. Since ISGF3 is an αIFN-inducible factor complex, it is not known whether the same complex is also induced by βIFN. In contrast, ΔSTAT1α cells did not show any significant increase in surface class I expression when exposed to either γ-IFN or βIFN. This observation suggested that induction of class I molecules by γ-IFN is associated, at least in part, with STAT1α.

To test whether the observed differences in the IFN-inducible expression of endogenous class I genes in STAT1α and p48 mutants was correlated with the enhancer activity of B-IRE, reporter gene bioassays were performed. As shown in Fig. 10, both mutants surprisingly exhibited vigorous enhancer activity of B-IRE in response to γ-IFN comparable with that seen with the wild type cells. As seen previously (41), none of the primate A-IRE variants showed any significant reporter gene activity. This enhancer activity of the B-IRE also correlated with the presence of BIGAF as detected in gel shift assays (Fig. 11). Curiously, the relative intensity of the shifted bands from nuclear extracts of the mutants was significantly greater than those seen with the wild type cells.

**DISCUSSION**

Results of experiments described here showed that the γ-IFN-inducible enhancer activity of the B-IRE is associated with the appearance of a novel nuclear factor minutes after exposure of cells to γ-IFN. The appearance of this factor is unaffected by cycloheximide indicating no requirement for protein synthesis. However, its sensitivity to herbimycin A suggests that it may be activated through a protein kinase pathway, perhaps in manner similar to that observed for other γ-IFN-inducible factors such as STAT1α (44, 45). This factor, referred to as BIGAF, may be distinguished from other γ-IFN-inducible factors (e.g. STAT1α and IRF-1) by four criteria: electrophoretic mobility, antigenic distinction, kinetics of activation, and specificity of binding to DNA sequences.
As shown in various gel shift experiments, BIGAF exhibits an intermediate mobility relative to that of STAT1α and IRF-1. STAT1α has a slow mobility, and IRF-1 has the fastest mobility of the three factors. In all these experiments, the lengths of the binding probes are the same. Thus, the observed differences in the relative mobilities among these nuclear factors likely reflects the molecular sizes of the proteins in protein-DNA complexes.

The antigenic distinction of BIGAF from other IFN-inducible factors is verified in supershift assays. Results of experiments reveal the inability of specific antibodies to STAT1α, IRF-1, and p48 to effect any change in the mobility or binding of BIGAF to the B-IRE sequence. BIGAF is also detected in somatic mutants of STAT1α and p48, and the IFN-inducible enhancer activity of B-IRE is maintained in these cells. These observations strongly indicate a function for BIGAF independent to that of STAT1α and p48, the nuclear factors known to mediate transcriptional activation by γ-IFN and αIFN, respectively (44, 56, 59–61).

Perhaps the most curious feature of BIGAF is its fairly transient appearance. Like STAT1α, BIGAF is detected in nuclear extracts of cells within 10 min of exposure to γ-IFN. But unlike STAT1α which can be detected even after 12 h (albeit the levels of detectable activity decreases with time), the level of BIGAF binding activity peaks between 30 and 45 min, after which it is no longer detectable. On the other hand, IRF-1 does not appear until after 1–1.5 h of exposure to γ-IFN. Induction of IRF-1 by γ-IFN has been previously shown to require de novo protein synthesis (53, 63, 64); hence, its late appearance in the nucleus.

BIGAF may also be distinguished from other γ-IFN-inducible factors, like STAT1α (44, 45, 62) and GAF (51–53), by its specificity of binding to DNA. On the one hand, GAF has been previously shown to exclusively bind GAS elements. DNA binding studies have failed to detect any significant binding of GAF to ISRE sequences (49–53), the regulatory motif to which GAS elements ascribe to binding γ-IFN-inducible enhancer activity of the related IRE-like sequences ISG54, C13, 9/27, 6/16, and oligo-adenylate synthetase, indicates that the A-IRE has measurable affinity for BIGAF as well. These observations indicate that the constitutive A-IRE binding factor is different from the γ-IFN-induced B-IRE binding factor (i.e. BIGAF), even though both factors have very similar relative mobilities. Whether these two factors are biochemically related to each other remains to be elucidated, and the possibility remains that both factors mediate regulatory roles by interacting with the class I IRE element.

The notion that the γ-IFN-inducible enhancer activity B-IRE is associated with the BIGAF may become one of significant interest in light of a recent report describing a constitutive A-IRE binding factor (70). In DNA binding assays, this constitutive factor specifically binds A-IRE, and γ-IFN treatment of cells has no significant effect. In the present study, a similar A-IRE binding factor is also detected using nuclear extracts from unstimulated cells but not from γ-IFN-treated cells. In contrast, vigorous B-IRE binding activity is seen only with extracts from γ-IFN-treated cells but not from unstimulated cells. As the data also show, these specific binding activities are not effectively cross-competed. However, the fact that the A-IRE can compete against BIGAF binding activity of the related IRE-like sequences ISG54, C13, 9/27, 6/16, and oligo-adenylate synthetase, indicates that the A-IRE has measurable affinity for BIGAF as well. These observations indicate that the constitutive A-IRE binding factor is different from the γ-IFN-induced B-IRE binding factor (i.e. BIGAF), even though both factors have very similar relative mobilities. Whether these two factors are biochemically related to each other remains to be elucidated, and the possibility remains that both factors mediate regulatory roles by interacting with the class I IRE element.

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FIG. 10. STAT1α and p48 somatic mutants maintained B-IRE enhancer activity. Luciferase reporter constructs containing various IRE sequences were used in transient transfection assays using the indicated cell lines. Sequences of the respective IREs, procedure for transfection, measurement, and normalization of luciferase activity were as described previously (41). Data shown are duplicate transfections in each experiment. B-locus, B-IRE; HuCh-A, human/chimpanzee A-IRE; Gogo-A, gorilla A-IRE; Popy-A, gibbon/orangutan A-IRE; None, luciferase vector; SIE, γ-IFN- and c- sis-inducible element in the c-fos promoter (45, 62).

be determined.

Results presented also show that the constitutive A-IRE binding factor appears to be dependent on the A-specific nucleotides in the middle of the core IRE sequence as well as specific flanking residues. The relative intensity of the binding signal is higher with longer 28-mer A-IRE probes that contain the core A-IRE sequence flanked by additional A-specific nucleotides. This binding activity is decreased when such flanking residues are deleted. The A-IRE probes show only marginal binding activity with nuclear extracts from γ-IFN-treated cells. In contrast, the B-IRE probes show low constitutive factor binding regardless of the presence of flanking B-specific residues. Both long (28-mer) and short (17-mer) versions of B-IRE probes show equivalent binding activity for BIGAF as shown by the cross-competition between these probes. Taken together, these observations suggest that BIGAF binding to B-IRE is dependent on the B-specific nucleotides in the middle of the core IRE sequence, whereas constitutive factor binding to A-IRE requires A-specific residues in the middle of the core IRE and the immediate flanking A-specific residues.

While data presented here show that the γ-IFN-inducible enhancer activity of the MHC B-IRE is associated with the appearance of the novel factor, BIGAF, the role of this factor in the transcriptional activation of class I genes in the context of the whole promoter remains to be examined. In addition, the roles of two other IFN-responsive factors, namely STAT1α and p48, in class I gene transcription are implicated in this study, suggesting the interplay of various factors in the induction of class I genes by IFN. Inasmuch as protein expression is a late event, STAT1α and p48 may be acting downstream from BIGAF in the cascade of events leading to class I expression on the cell surface. Consistent with this view is the reported requirement of STAT1α in the γ-IFN-induced de novo synthesis of IRF-1 through the binding of STAT1α to the GAS element in the IRF-1 promoter (71, 72). This is confirmed in the targeted deletion of STAT1 in mice in that there is a total loss of γ-IFN induction of the murine IRF-1 homologue in STAT1-deficient animals (73). Thus, the suggested role of IRF-1 in the induction class I genes by γ-IFN (35, 67) may be related to STAT1α-mediated IRF-1 up-regulation. This may explain the observation that γ-IFN failed to elicit additional increases in the levels of expression of surface class I HLA proteins in somatic mutants of STAT1α. Whether IRF-1 is necessary for class I up-regulation by γ-IFN in 2FGTH cells (the parental cell line of the somatic mutants of STAT1α) is not known. It must be noted, however, that IRF-1 may not always be required for the inducible expression of class I genes. In mice, targeted deletion of IRF-1 does not affect the responsiveness of class I genes to γ-IFN (74). Thus, the specific factor(s) that governs the γ-IFN-inducible expression of class I genes may have yet to be determined. Further studies examining the significance of the early and transient activation of BIGAF upon exposure of cells to γ-IFN may provide insight on the initial events that eventually lead to class I gene transcription.

Finally, the fact that BIGAF has demonstrable binding activity for related elements of the IRE family opens the possibility that this factor also contributes to the regulation of genes outside the class I MHC multigene family. This would not be surprising since there are many examples of DNA binding proteins that interact with regulatory element families that are dispersed in the genome.

Acknowledgments—We thank Dr. Robert Abraham and Gregg Brunn (Mayo Clinic) for technical advice and for providing various reagents; Dr. George Stark (Cleveland Clinic Research Institute) for providing the cell lines 2FGTH, U2A, and U3A; Drs. David Levy (NYU Medical Center) and Richard Pine (Public Health Research Institute, NY) for the antisera to p48 and IRF-1, respectively; and Kathleen Allen for technical assistance.
