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Subunit of an Alpha-Interferon-Responsive Transcription Factor Is Related to Interferon Regulatory Factor and Myb Families of DNA-Binding Proteins

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Alpha interferon stimulates transcription by converting the positive transcriptional regulator ISGF3 from a latent to an active form. This receptor-mediated event occurs in the cytoplasm, with subsequent translocation of the activated factor to the nucleus. ISGF3 has two components, termed ISGF3α and ISGF3γ. ISGF3γ serves as the DNA recognition subunit, while ISGF3α, which appears to consist of three polypeptides, is a target for alpha interferon signaling and serves as a regulatory component whose activation is required to form ISGF3. ISGF3γ DNA-binding activity was identified as a 48-kDa polypeptide, and partial amino acid sequence has allowed isolation of cDNA clones. ISGF3γ translated in vitro from recombinant clones bound DNA with a specificity indistinguishable from that of ISGF3γ purified from HeLa cells. Sequencing of ISGF3γ cDNA clones revealed significant similarity to the interferon regulatory factor (IRF) family of DNA binding proteins in the amino-terminal 117 residues of ISGF3γ. The other IRF family proteins bind DNA with a specificity related to but distinct from that of ISGF3γ. We note sequence similarities between the related regions of IRF family proteins and the imperfect tryptophan repeats which constitute the DNA-binding domain of the c-*myb* oncoprotein. These sequence similarities suggest that ISGF3γ and IRF proteins and the c-*myb* oncoprotein use a common structural motif for DNA recognition. Recombinant ISGF3γ, like the natural protein, interacted with HeLa cell ISGF3α to form the mature ISGF3 DNA-binding complex. We suggest that other IRF family members may participate in signaling pathways by interacting with as yet unidentified regulatory subunits analogous to ISGF3α.

Interferons (IFNs) constitute a family of polypeptide cytokines secreted by mammalian cells in response to viral infection. Transcriptional induction of the IFN genes is a response activated by double-stranded RNA produced during viral infection and relies on cellular transcription factors. Newly synthesized IFN interacts with neighboring cells through cell surface receptors, resulting in the synthesis of a group of new cellular proteins (for a review, see reference 12). Many of the new proteins produced early in the response to IFN-α are the products of a set of genes whose transcriptional activity is rapidly but transiently increased to very high levels in a response displaying similarities to, but that is distinct from, the transcriptional induction of the IFN genes themselves (19, 21, 45, 55). Transcriptional stimulation in response to IFN treatment, like induction of IFN genes by virus, is mediated by preexisting, latent cellular proteins that become activated in response to the signaling pathway (11, 18, 22, 44, 51, 53). An important regulatory step unique to the response to IFN-α treatment is activation of a transcription factor that recognizes a conserved *cis*-acting DNA element (the IFN-stimulated response element, or ISRE) located within the regulatory sequences of target genes (9, 50, 61, 66). This transcription factor, termed ISGF3, is the positive activator responsible for the initial stimulation of IFN-α-responsive genes (41).

Active ISGF3 was identified as an ISRE-binding factor present in nuclear extracts from IFN-α-treated, but not untreated, HeLa cells and human fibroblasts (50, 62). The kinetics and extent of ISGF3 activation and the resistance of this process to inhibitors of protein synthesis matched the induction profile of IFN-stimulated transcription. Extensive point mutagenesis of the ISRE showed that protein-DNA contacts between ISGF3 and the ISRE were precisely the nucleotides required for transcriptional activity (41). These features contrast with the characteristics of other ISRE-binding proteins. For example, ISGF1, a constitutive nuclear protein, is unaffected by IFN treatment. ISGF2, an IFN-induced protein, requires protein synthesis for its appearance in cells and accumulates with kinetics considerably delayed from those of IFN-stimulated transcription and ISGF3 activation (50). In addition, this protein, which is the human homolog of a mouse factor termed IRF1 (58, 60), recognizes the central ~9-nucleotide (nt) core region of the ISRE that is shared between the ISRE and PRDI of the IFN-β (41, 62). An IFN-stimulated transcriptional response, on the other hand, is mediated by the full 18-nt ISRE recognized by ISGF3.

Regulation of ISGF3 involves conversion from a latent to an active form capable of binding DNA (50). This conversion occurs in the cytoplasm (10, 49), and only the activated factor is transported to the nucleus. In its latent state, ISGF3 probably exists as two independent components, termed ISGF3α and ISGF3γ, that associate only following exposure of cells to IFN-α. In many human cell lines, ISGF3α is present in excess over the level of ISGF3γ (4, 42, 52). In IFN-γ-treated cells, the abundance of ISGF3γ is increased such that subsequent exposure to IFN-α leads to dramati-

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cally higher levels of transcription of target genes because of formation of correspondingly greater levels of ISGF3 (52).

Partial purification and characterization of ISGF3 led to the identification of four polypeptides potentially composing this factor (23, 42). UV cross-linking of protein to the ISRE and glycerol gradient sedimentation studies indicated that the ISGF3γ component is a 48-kDa polypeptide while the ISGF3α component is composed of three polypeptides of 84, 91, and 113 kDa. The stoichiometry of this interaction has not been established; it is possible that differential association of individual ISGF3α polypeptides led to the distinct ISGF3-DNA complexes observed between cytoplasmic and nuclear extracts (42). ISGF3γ is an intrinsic DNA-binding protein and serves as the ISRE recognition component of the transcription factor. One or more of the ISGF3α polypeptides serves as a target for IFN-α signaling. Activated ISGF3α stabilizes ISGF3γ-DNA interactions, resulting in formation of a ternary complex of approximately 25-fold-greater stability than that of ISGF3γ-DNA alone (42).

We have now obtained ISGF3γ in sufficient purity to identify a single species with the ability to bind DNA and interact with ISGF3α. Partial amino acid sequence obtained from ISGF3γ facilitated isolation of cDNA clones encoding this protein. Its sequence revealed a striking similarity with the DNA-binding domains of the three members of the interferon response factor (IRF) family, IRF1, IRF2, and ICSBP. Members of this group of structurally related DNA-binding proteins accumulate in cells in response to a variety of inducers, including IFNs, and recognize PRDI and the core region of the ISRE (15, 26, 29, 36, 56, 60, 79). The product of cloned ISGF3γ, like the natural protein, bound only full-length ISRE. We note sequence similarities between the shared region of ISGF3γ and IRF proteins and the imperfect repeats which constitute the DNA-binding domain of the c-*myb* protein (27, 33, 38, 54), suggesting a shared structural motif involved in binding DNA.

MATERIALS AND METHODS

Cell extracts and protein purification. HeLa S3 cells were grown in suspension culture in Joklik's modified Eagle's medium or as monolayer cultures in Dulbecco's modified Eagle's medium, as previously described (42). Recombinant human IFN-γ (Amgen) was added to tissue culture media at 1 ng/ml for 18 to 24 h; recombinant human IFN-α_{2a} (Hoffmann-La Roche) treatments were at 500 IU/ml for 45 to 60 min. Cytoplasmic S100 and nuclear extracts were prepared by a modification of the procedure of Dignam et al., as previously described (14, 42). Total cell extracts were prepared by lysis of cells in a solution of 9.5 M urea, 2% Nonidet P-40, and 2% 2-mercaptoethanol. ISGF3γ was fractionated from S100 extracts of HeLa S3 cells grown in suspension and treated with 1 ng of recombinant human IFN-γ (Amgen) per ml for 18 to 24 h by a modification of previously described procedures, and purification was monitored by gel retardation analysis (42). Protein precipitated by 50% saturated ammonium sulfate was chromatographed on heparin-Sepharose, phenyl-Sepharose, and fast protein liquid chromatography (FPLC) Mono Q resins (Pharmacia-LKB) in 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol buffered with either 20 mM Tris-HCl (pH 7.5) or K^+-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.6) by using gradients of KCl, as indicated below. Protein that bound heparin-Sepharose in 50 mM KCl was eluted with a 5-column-volume, linear 50 to 800 mM KCl gradient in HEPES buffer, and ISGF3γ activity eluted between 400 and 600 mM KCl. This eluate was adjusted to 800 mM KCl and loaded on phenyl-Sepharose. This column was washed with 200 mM KCl buffered with Tris, and ISGF3γ was eluted with Tris-buffered 50% ethylene glycol lacking KCl. After dialysis into Tris-buffered 100 mM KCl, protein was loaded on FPLC Mono Q and developed with a 100 to 500 mM KCl gradient over 20 column volumes which resolved ISGF3γ activity from several major contaminants. ISGF3γ activity eluted between 180 and 250 mM KCl, resulting in an approximately 7,000-fold enrichment in specific activity over crude cytoplasm. Cytoplasmic ISGF3α was prepared from S100 extracts of IFN-α-treated HeLa cells and fractionated as previously described (42). To obtain the amino acid sequence, nuclear ISGF3 was purified to near homogeneity; isolation of individual polypeptides and amino acid sequencing following tryptic digestion in situ are described elsewhere (69). In brief, nuclear ISGF3 was purified from 200 liters of HeLa cells treated for 15 h with 1 ng of IFN-γ per ml and for 1 h with 500 U of IFN-α per ml. After final purification by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), 25 to 50 μg of the 48-kDa ISGF3γ polypeptide was recovered for protein sequencing.

Gel retardation analysis. Gel retardation analysis employed an end-labeled synthetic double-stranded ISRE oligonucleotide (1 to 4 ng per reaction) with the sequence GATCCTCGGGAAAGGGAAACCGAAACTGAAGCC (the recognition site is underlined), as previously described (42). Unlabeled heterologous oligonucleotide competitors were as follows: nonspecific (a mutated version of the ISRE), CCCGG GAAAGGGAAACCCACACTGAAG; and core ISRE (−77 to −54 of the IFN-β promoter), GATCGAGAAGTGAAAGT GGGAAATTCCT (28, 59).

Size fractionation and renaturation of ISGF3γ. ISGF3γ enriched from HeLa cell cytoplasmic extracts was electrophoresed in parallel lanes of an 8.5% SDS gel prerun with 0.1 mM sodium thioglycolate. A lane containing ~150 ng of protein was stained with silver (78), and a second lane loaded with 40-fold-more protein was lightly stained with Coomassie R250 in water to avoid fixation and thus facilitate protein recovery (31). Most bands stained with both reagents. Protein was eluted from the Coomassie-stained gel slices and renatured after acetone precipitation to remove the dye, as previously described (7, 42). Recovered material was analyzed by gel retardation assay.

Isolation of ISGF3γ cDNA clones. Amino acid sequences for tryptic peptides from ISGF3γ purified from nuclei of IFN-treated HeLa cells (69) were obtained as described elsewhere (1) and were used to predict fully degenerate sense and antisense complementary oligonucleotides. For example, a mixture of potential sense primers (ATGGAY GTNGCNGARCC) which partially encoded a 7-amino-acid peptide beginning at residue 103 of ISGF3γ was designed. Similarly, mixed antisense primers (GCYTGYTGYTCNG GNGT) were synthesized on the basis of an 11-amino-acid peptide beginning at residue 377. These primer mixtures were used in reverse polymerase chain reactions (PCR) (46, 67) in standard reactions supplemented with 600 μM tetramethylammonium chloride (34), employing a 70°C annealing temperature, to amplify cDNA fragments from oligo(dT)-primed mRNA isolated from IFN-γ-treated HeLa cells. PCR products were cloned into pBluescript KS− (Stratagene) and sequenced.

An 852-nt PCR product found to encode an ISGF3γ peptide at either end was used to screen an oligo(dT)-primed cDNA library prepared by Stratagene in λZap II from

IFN-γ-treated HeLa cell mRNA (60) by standard procedures. Inserts from plaque-purified recombinants were excised into pBluescript SK(−) by using helper phage according to the manufacturer's instructions. The longest cDNA insert was sequenced on both strands with Sequenase from nested 5′ and 3′ deleted subclones generated with exonuclease III and mung bean nuclease (32). The sequence was assembled, conceptually translated, and compared with published sequences by using GCG System Software (13).

Analysis of ISGF3γ RNA. ISGF3γ RNA was synthesized in vitro from 5 μg of linearized plasmids in 50-μl reactions with 50 U of T7 RNA polymerase (57) and quantitated on the basis of incorporation of trace [α-^{32}P]UTP. In vitro translation reactions used 300 ng of RNA, in 3 μl of water, added to 12 μl of rabbit reticulocyte lysate (Promega) and 1 μl (each) of 1 mM amino acids (minus methionine; Promega) and 10 μCi of [^{35}S]methionine (1,300 Ci/mmol, Dupont) per μl. Mock translations received 3 μl of water instead of RNA. Translation reactions were incubated at 30°C for 1 h and stopped by addition of 2 μl of 1.5 mg of RNase A per ml at 30°C for 10 min. Incorporation of ^{35}S was measured by trichloroacetic acid precipitation and typically was 2.5- to 5-fold greater than mock translations; 25% was used for gel retardation binding reactions, and 6% was analyzed by immunoblotting.

Total cellular mRNA was isolated from HeLa cells disrupted in guanidine isothiocyanate, affinity purified on oligo(dT) cellulose, size fractionated on denaturing agarose gels in the presence of formaldehyde, and blotted to nitrocellulose. RNA blots were hybridized with cDNA clones labeled with [^{32}P]dATP by random primed synthesis.

Immunological methods. The 852-nt cDNA fragment generated by reverse PCR, representing amino acids 103 to 387 of ISGF3γ, was fused in frame with T7 phage gene 10 in the pGEMEX-1 vector (Promega), and the resulting protein was overexpressed in *Escherichia coli* and purified from inclusion bodies (70). The purified fusion protein was used to immunize rabbits by standard procedures (31).

Immunoblots were performed on SDS-PAGE-fractionated protein electroblotted to nitrocellulose (73). Antigen-antibody complexes were detected by using horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin and enhanced chemiluminescence reagents (Amersham). For antibody disruption of gel retardation reactions, binding reactions were incubated at room temperature for 30 min prior to addition of 0.2 volumes of antiserum diluted 1:20 in binding buffer. Incubation was continued for 1 h on ice, and 25% of this material was resolved on polyacrylamide gels. Antibody against human ISGF2 was a kind gift of R. Pine (60).

Nucleotide sequence accession number. The nucleotide sequence for ISGF3γ has been submitted to GenBank under accession number M87503.

RESULTS

Isolation of ISGF3γ cDNA clones. ISGF3γ was identified by fractionation of cytoplasmic extracts of IFN-γ-treated HeLa cells by column chromatography. Fractionation of ISGF3γ employed heparin affinity, hydrophobic interaction, and ion-exchange chromatography and resulted in an approximately 7,000-fold increase in specific activity (on the basis of DNA binding and ISGF3 formation). In previous fractionations, the presence of a 48-kDa protein correlated with ISGF3γ activity (23, 42). To precisely identify the ISGF3γ polypeptide, a protein preparation which could be resolved into individual protein species by SDS-PAGE and silver staining was obtained (Fig. 1A). A parallel gel lane was lightly stained with Coomassie blue in water rather than fixative to facilitate protein recovery for analysis of DNA-binding activity. Protein was eluted from slices corresponding to each band observed by silver staining (most of which were also detected by Coomassie blue) and from regions of the gel between observable bands, covering the size range from approximately 35 to 60 kDa. As shown in Fig. 1B, the majority of protein with ISGF3γ activity detected by gel shift analysis eluted from slice 4, corresponding to a 48-kDa stained protein, with the low amount of activity found in slice 5 probably representing incomplete resolution in the preparative gel of the same protein. ISGF3γ alone, and the ISGF3 complex formed after addition of ISGF3α to the eluted protein, displayed the same DNA-binding specificity as nuclear ISGF3; that is, they bound only to wild-type ISRE and not to mutated or core sequences (not shown).

The ISGF3 complex was purified from nuclear extracts of IFN-γ- and IFN-α-treated HeLa cells by using ion-exchange and affinity column chromatography, as previously described (69). This purification resulted in fractions of ISGF3 of sufficient purity to allow the three previously identified ISGF3α polypeptides (42) and the ISGF3γ polypeptide to be excised as homogeneous proteins from an SDS gel electroblotted to nitrocellulose. These four proteins were individually digested with trypsin in situ, and recovered peptides were subjected to automated Edman degradation. The resulting sequences were used to obtain cDNA clones for ISGF3γ (see below) and for the three ISGF3α polypeptides (69).

Unique amino acid sequences for three ISGF3γ-derived tryptic peptides of 7 to 11 amino acids and mixed sequences for several additional peptides were determined. Fully degenerate sense and antisense primers partially encoding the sequenced ISGF3γ peptides were used in reverse PCR. First-strand cDNA synthesized from oligo(dT)-primed mRNA isolated from IFN-γ-treated HeLa cells served as a template. One combination of primers amplified an 852-nt cDNA fragment that, when cloned and partially sequenced, was found to contain primer sequences at either end, plus additional nucleotides predicted by the known amino acid sequence of one peptide.

This 852-nt cDNA was used to screen an oligo(dT)-primed cDNA library prepared by Stratagene in λZap II from IFN-γ-treated HeLa cell mRNA. Seventy-two of 600,000 clones in a primary screen hybridized the probe; several of these were purified, and their inserts were rescued into plasmid. The longest cDNA insert, designated clone 48, was sequenced after isolation of nested, 5′ and 3′ deleted derivatives by using exonuclease III. This clone contained an open reading frame potentially encoding 377 amino acids and a 371-nt 3′ untranslated region ending with a polyadenylation signal and a 28-nt poly(A) tract. A second cDNA clone, designated clone 38, contained a longer open reading frame that overlapped clone 48 but extended 5′ for an additional 16 amino acids. Another ISGF3γ cDNA clone was isolated from a human diploid fibroblast library (47).

A composite cDNA clone for ISGF3γ was created by fusing portions of clones 38 and 48 to create clone 38/48. This 1,618-nt cDNA contained an open reading frame predicting a 44-kDa protein of 393 amino acids (Fig. 2). All the sequences derived from peptides were included within clone 38/48. Three unique-sequence peptides are boxed in Fig. 2, while three peptides that were derived from partially mixed sequences are underlined. The amino termini of peptides used

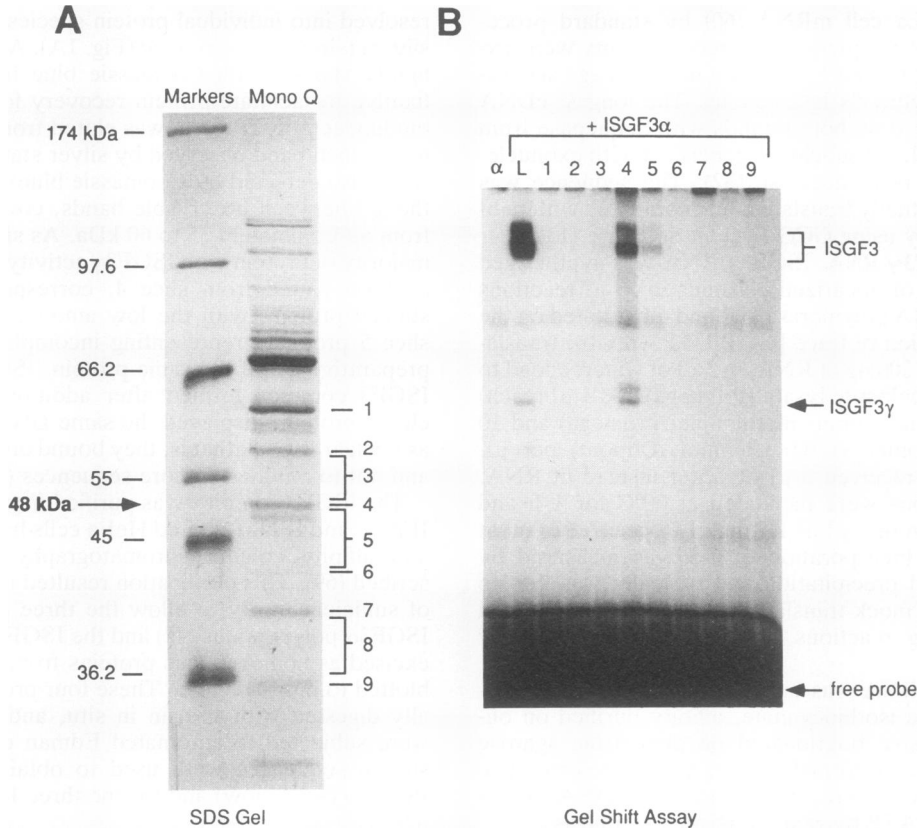

FIG. 1. Identification of the 48-kDa ISGF3γ polypeptide. (A) Cytoplasmic ISGF3γ fractionated from HeLa cells (150 ng) was resolved on an 8.5% acrylamide-SDS gel and stained with silver (lane 2); 6.3 μg of the same material was electrophoresed in a parallel lane and stained without fixation by using Coomassie R250 in water (not shown). Protein was eluted from Coomassie-stained gel slices corresponding to the indicated bands and regions between bands. (B) Partially purified ISGF3α was mixed in DNA-binding reactions with 2.4% of the protein eluted from each gel slice and labeled ISRE, and complexes were resolved on a native polyacrylamide gel. The migrations of ISGF3- and ISGF3γ-DNA complexes and of free probe are indicated. ISGF3γ appears as a doublet, probably because of incomplete renaturation. ISGF3 also appears as a doublet, which is characteristic of cytoplasmic ISGF3α (42) and may be caused by differential utilization of individual ISGF3α polypeptides. ISGF3α alone (α) and 0.03% of the ISGF3γ fraction loaded on the SDS gel mixed with ISGF3α (L) were included for comparison.

to define useful PCR primers were at amino acids 103 and 377.

ISGF3γ synthesized in vitro forms ISGF3. ISGF3γ was initially defined by its ability to associate with ISGF3α to form ISGF3 (49). We have shown that ISGF3γ binds DNA with specificity for the full-length ISRE and with an affinity that increases following association with ISGF3α (42). Its intrinsic specificity for the ISRE led to the conclusion that the ISGF3γ polypeptide is the DNA recognition subunit of ISGF3. Both these ISGF3γ activities, which can be detected by using gel shift analysis, were measured for the protein encoded by clone 38, which contains a full-length open reading frame. RNA was transcribed in vitro from a clone 38 template by using T7 RNA polymerase, and the RNA was translated in rabbit reticulocyte lysates. In vitro-translated ISGF3γ displayed the same sequence specificity and mobility as ISGF3γ present in crude extracts of IFN-γ-treated HeLa cells (not shown) or as ISGF3γ highly enriched by column chromatography (Fig. 3A). Both purified (lane 1) and synthetic (lane 4) ISGF3γ bound an ISRE probe, and this interaction was not significantly subject to competition by excess amounts of a core oligonucleotide representing an IRF1-binding site (lanes 2 and 5) but was substantially blocked by homologous competitor (lanes 3 and 6). Likewise, addition of ISGF3α fractionated from extracts of IFN-α-treated HeLa cells resulted in formation of ISGF3 from both HeLa cell (Fig. 3B, lanes 1 to 3) and synthetic (lanes 4 to 6) ISGF3γ. ISGF3 formed with recombinant protein also displayed ISRE-specific DNA binding (compare lanes 5 and 6). Mock translation reactions lacked detectable ISRE-specific DNA binding, and addition of ISGF3α did not lead to formation of significant ISGF3 (lanes 7 to 9), although a weak, ISGF3-like shifted band could be detected after prolonged autoradiography. This may indicate trace presence of a rabbit homolog of ISGF3γ in the reticulocyte lysates.

Antibodies raised against recombinant ISGF3γ recognize an IFN-γ-induced protein. Antibodies were raised against recombinant ISGF3γ produced in bacteria. Amino acids 103 to 387, encoded by the PCR-generated fragment of ISGF3γ, were fused in frame with the amino-terminal 260 amino acids of the phage T7 gene 10 product and were overexpressed in *E. coli*. Antisera obtained from rabbits immunized against this material were tested for their effects on ISRE-specific protein-DNA complexes in gel shift assays (Fig. 4). Crude extracts from IFN-treated HeLa cells were allowed to bind ISRE probes in the absence of added rabbit serum (Fig. 4, NS) or in the presence of serum obtained prior to (Fig. 4,

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          10         20         30         40         50         60         70         80
  1 MASGRARCTR KLRNWVVEQV ESGQFPGVCW DDTAKTMFRI  PWKHAGKQDF REDQDAAFFK AWAIFKGKYK EGDTGGPAVW 80
 81 KTRLRCALNK SSEFKEVPER GRMDVAEPYK VYQLLPPGIV  SGQPGTQKVP SKRQHSSVSS ERKEEEDAMQ NCTLSPSVLQ 160
161 DSLNNEEEGA SGGAVHSDIG SSSSSSSPEP QEVTDTTEAP  FQGDQRSLEF LLPPEPDYSL LLTFIYNGRV VGEAQVQSLD 240
241 CRLVAEPSGS ESSMEQVLFP KPGPLEPTQR LLSQLERGIL  VASNPRGLFV QRLCPIPISW NAPQAPPGPG PHLLPSNECV 320
321 ELFRTAYFCR DLVRYFQGLG PPPKFQVTLN FWEESHGSSH  TPQNLITVKM EQAFARYLLE QTPEQQAAIL SLV        393
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FIG. 2. Amino acid sequence of ISGF3γ. Amino acid sequence predicted by the open reading frame in clone 38/48 is shown, with sequences of tryptic peptides obtained from purified ISGF3γ indicated. Unique sequence peptides are boxed, and sequences obtained from mixed peptides are underlined. The primers used successfully in PCR were derived from peptides beginning at amino acids 103 and 377.

Pre-imm.) or following (Fig. 4, Immune) immunization. Cytoplasmic extracts from IFN-γ-treated HeLa cells (lanes 1 to 6) were used as a source of ISGF3γ, and cytoplasm from cells pretreated with IFNγ and subsequently stimulated with IFN-α (lanes 13 to 18) was used as a source of intact ISGF3. As a control for the specificity of the antiserum, nuclear extracts from IFN-γ-treated HeLa cells (lanes 7 to 12) were analyzed for ISGF2. Preimmune serum had no effect on protein-DNA complex formation, while antisera against ISGF3γ prevented the ISGF3γ-specific (lane 5) and ISGF3-specific (lane 17) complexes from entering the polyacrylamide gel without affecting other protein-DNA complexes. In contrast, antiserum reactive with human ISGF2 (Fig. 4, Anti-ISGF2) severely retarded the normal mobility of the ISGF2-DNA complex (lane 12) but had no effect on ISGF3γ or ISGF3 (lanes 6, 12, and 18).

IFN-γ pretreatment of HeLa cells potentiates the subsequent transcriptional induction of IFN-α-stimulated genes because of increased abundance of ISGF3γ activity (4, 49, 52, 72). This increased sensitivity to IFN-α requires ongoing protein and RNA synthesis during the IFN-γ treatment, suggesting that ISGF3γ is being synthesized during this period. ISGF3γ protein levels were assayed in HeLa cell extracts by immunoblotting (Fig. 5A). ISGF3γ protein levels were increased approximately 10-fold after overnight treatment of cells with IFN-γ (compare lanes 1 and 2).

ISGF3γ cDNA encodes a full-length protein. The nucleotide sequence encoded by clone 38/48 does not contain an in-frame termination codon preceding the first methionine, making it difficult to establish with certainty the translational initiation site used in vivo. However, the similar sizes of the synthetic product and the protein from cell extracts indicated that no more than a few additional amino-terminal residues could be present in ISGF3γ prior to the first methionine present in clone 38 (Fig. 5A). No difference was detected between the migration on SDS-PAGE of HeLa cell ISGF3γ from total lysates of untreated or IFN-γ-treated cells (lanes 1 and 2) or from partially purified fractions (lane 3). Likewise, the major in vitro translation product produced by using RNA transcribed from clone 38 (lane 4) was indistinguishable in length. In contrast, protein expressed from the truncated clone 48 cDNA, which initiates from the second methionine (codon 37), was clearly resolved from full-length ISGF3γ (lane 5). A shorter, approximately 45-kDa in vitro product was also observed by using RNA transcribed from either clone 38 or clone 48. However, this protein failed to bind DNA (not shown) and most likely is derived from translational initiation at an internal AUG codon. No immunoreactive proteins comigrating with full-length ISGF3γ were detected in reticulocyte lysates from mock translation reactions (lane 6). The heterogeneous appearance of synthetic ISGF3γ is not understood, but all forms close in size to the major, 48-kDa translation product were capable of binding the ISRE, while the smaller product of clone 48 was not (not shown).

ISGF3γ cDNA clones hybridized a single mRNA from HeLa cells on RNA blots (Fig. 5B). This RNA was approximately 1.9 kb in length and was induced by treatment of cells with either IFN-γ (lane 2) or IFN-α (not shown) in parallel with increased DNA-binding activity (48). The 1,618-bp length of composite clone 38/48, which contains some 5′ untranslated sequence and the entire 3′ region up to the polyadenylation site, indicates that little poly(A) and 5′ untranslated sequence is missing from this clone.

ISGF3γ is related to the IRF and *myb* families of DNA-binding proteins. The nucleotide and predicted amino acid sequences of ISGF3γ were compared with all sequences in

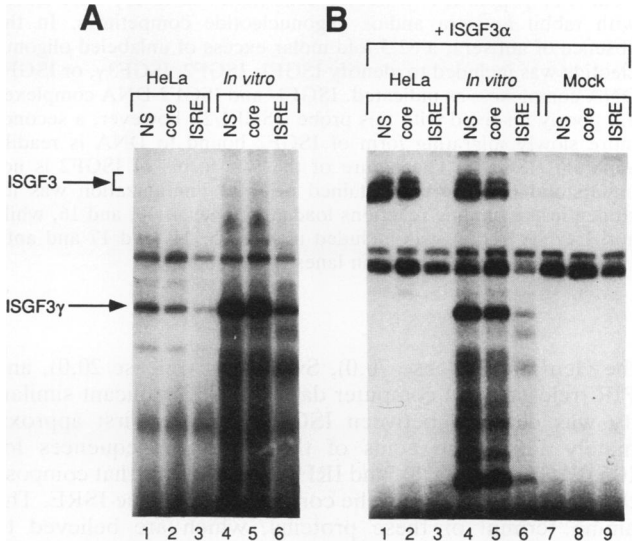

FIG. 3. In vitro-translated ISGF3γ binds DNA and forms ISGF3. (A) Enriched ISGF3γ from cytoplasmic HeLa cell extracts (lanes 1 to 3) or in vitro-translated ISGF3γ from clone 38 (lanes 4 to 6) was bound to an end-labeled ISRE oligonucleotide and resolved by gel retardation analysis. DNA binding by both HeLa ISGF3γ and synthetic ISGF3γ was subject to competition by full-length ISRE (lanes 3 and 6) but not by mutated (NS; lanes 1 and 4) or core (lanes 2 and 5) oligonucleotides. (B) ISGF3 was formed by mixing partially purified cytoplasmic ISGF3α with cytoplasmic ISGF3γ (lanes 1 to 3) or in vitro-translated, recombinant ISGF3γ (lanes 4 to 6). ISGF3 formed by association with either HeLa or recombinant ISGF3γ had specificity similar to that of ISGF3γ binding to DNA alone; full-length ISRE (lanes 3, 6, and 9) competed with binding but mutated (lanes 1, 4, and 7) or core (lanes 2, 5, and 8) oligonucleotides did not. Mock translation reactions were loaded in lanes 7 to 9. The protein-DNA complexes with in vitro-translated protein migrating more quickly than ISGF3γ probably represent incomplete translation products.

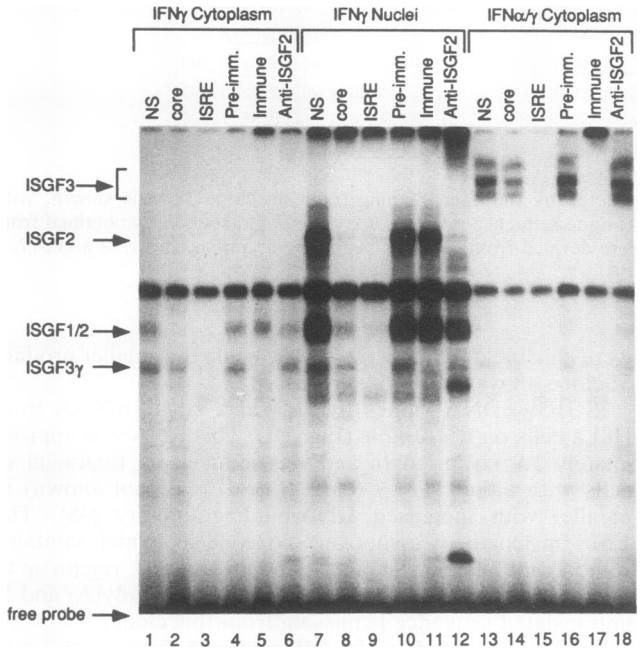

FIG. 4. Antibodies raised against recombinant protein recognize HeLa ISGF3γ. DNA-binding reactions with 50 μg of HeLa cytoplasmic extract (lanes 1 to 6), 25 μg of nuclear extract from IFN-γ-treated cells (lanes 7 to 12), or 36 μg of cytoplasmic extract from IFN-γ- plus IFN-α-treated cells (lanes 13 to 18) were mixed with rabbit antisera and/or oligonucleotide competitors. In the absence of antisera, a 62.5-fold molar excess of unlabeled oligonucleotide was included to identify ISGF1, ISGF2, ISGF3γ, or ISGF3 DNA complexes, as indicated. ISGF1- and ISGF2-DNA complexes are poorly resolved with this probe (ISGF1/2); however, a second, more slowly migrating form of ISGF2 bound to DNA is readily apparent (ISGF2). The nature of the two forms of ISGF2 is not understood (60). Serum obtained prior to immunization was included in the binding reactions loaded in lanes 4, 10, and 16, while anti-ISGF3γ serum was included in lanes 5, 11, and 17 and anti-ISGF2 serum was included in lanes 6, 12, and 18.

the GenBank (release 70.0), Swiss-Prot (release 20.0), and PIR (release 30.0) computer data banks. Significant similarity was detected between ISGF3γ and the first approximately 120 amino acids of the predicted sequences for ICSBP (15), IRF1 (58), and IRF2 (29), proteins that compose the IRF family and bind the core sequence of the ISRE. The amino termini of these proteins, which are believed to encode DNA-binding domains, are compared in Fig. 6. Amino acid identity in this region was closest between ISGF3γ and ICSBP, showing 59% identity with no gaps and functionally related amino acids at 40% of the remaining positions. By comparison, identity between ISGF3γ and mouse IRF1 or mouse IRF2 was approximately 38% in this region, allowing for a one-amino-acid gap, while ICSBP/IRF1 identity was 46% and IRF1/IRF2 identity was 76%. Human and rat IRF1 sequences, which display ~97% cross-species identity (56, 79), were also approximately 37% identical to ISGF3γ. Binding of ISGF3γ (49) and ISGF2 (not shown) to DNA is sensitive to protein alkylation by *N*-ethylmaleimide; the presence of a cysteine at residue 86 within a block of conserved amino acids may indicate that this region is in contact with DNA.

ISGF3γ and ICSBP were also related over three short, interspersed stretches in sequences located carboxyl terminal to the region compared in Fig. 6, revealing approximately 25% identity in this region. This is similar in extent to the relationship between IRF1 and IRF2, which have carboxyl-terminal sequences unrelated to ICSBP. Despite the extent of similarity, human ISGF3γ and mouse ICSBP are unlikely to be homologs of one another. ISGF3γ and a cDNA probe for mouse ICSBP (kindly provided by K. Ozato) hybridized distinct restriction fragments in mouse genomic DNA (not shown). In addition, a mouse ISGF3γ cDNA clone which is distinct from ICSBP has been isolated (16).

Each of the IRF family members has five conserved tryptophan residues in the amino-terminal region. This pattern of tryptophan spacing is reminiscent of the tryptophan repeats clustered in the DNA-binding regions of the c-*myb* and c-*ets* oncoproteins (2, 27, 33, 39, 54). As shown in Fig. 6, the three tryptophans of each of the 51- to 52-amino-acid c-*myb* repeats (R1, R2, and R3) could be aligned with the third through fifth tryptophans of the IRF family amino termini. In addition, this alignment brought into register conserved sites of hydrophobic amino acids at positions 37,

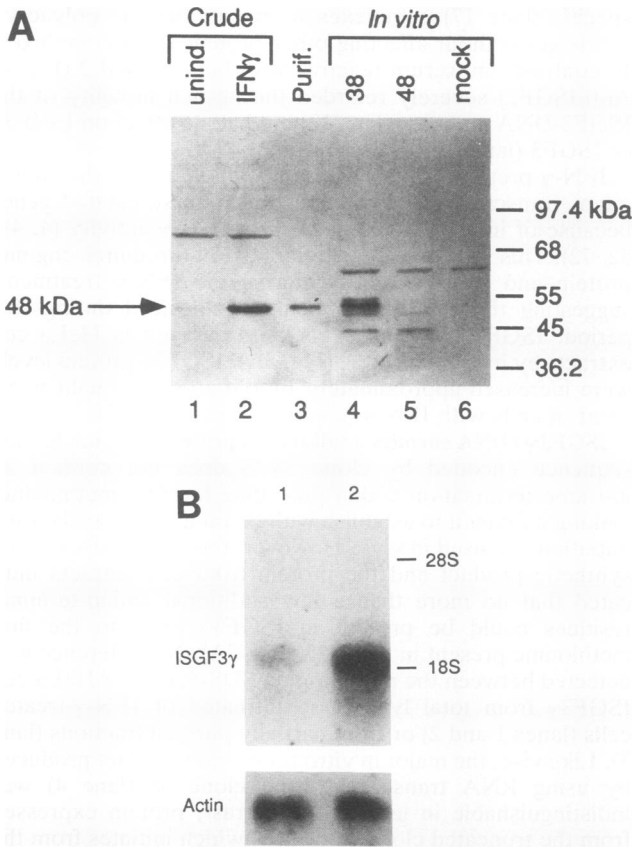

FIG. 5. ISGF3γ cDNA clone 38 encodes a full-length, IFN-γ-induced protein. (A) Total cell lysates (10 μg) from uninduced and IFN-γ-treated HeLa cells (lanes 1 and 2), 4 ng of an enriched fraction of cytoplasmic ISGF3γ (lane 3), in vitro translation reactions programmed with RNA transcribed from clone 38 (lane 4) or clone 48 (lane 5), and mock translations (lane 6) were electrophoresed in an 8.5% acrylamide–SDS gel, electroblotted to nitrocellulose, and developed with antiserum from rabbits immunized with recombinant ISGF3γ. (B) Formaldehyde-agarose gels with 2 μg of poly(A)-selected RNA from untreated (lane 1) or IFN-γ-treated HeLa cells (lane 2) were transferred to nitrocellulose and hybridized with probes for ISGF3γ (top) or human γ-actin (bottom).

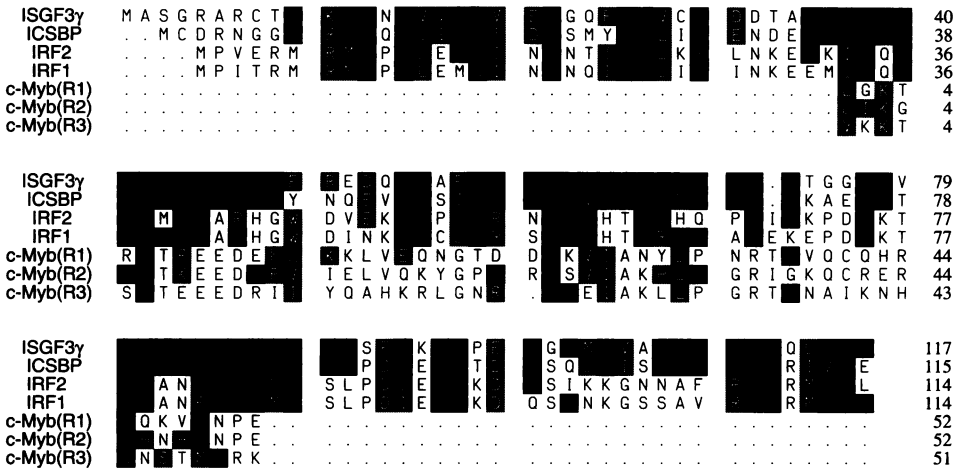

FIG. 6. IRF family members are related to c-*myb*. The conserved amino-terminal sequences of ISGF3γ, mouse ICSBP (15), mouse IRF2 (29), and mouse IRF1 (25) were aligned with the three imperfect repeats (R1, amino acids 38 to 89; R2, amino acids 90 to 141; and R3, amino acids 142 to 192) of mouse c-*myb* (5). Black shading indicates amino acid residues identical to the ISGF3γ sequence, and gray shading indicates amino acids with similar hydrophobicity or charge.

50, 64, 69, and 84 of ISGF3γ and of charged amino acids at positions 44, 47, 55, 60, and 73. A comparison with the tryptophan repeats found in c-*ets*-1 and related proteins did not show significant conservation of spacing with the IRF family proteins.

DISCUSSION

We have cloned, sequenced, and expressed a cDNA encoding human ISGF3γ, the DNA recognition subunit of the IFN-α-stimulated transcriptional activator ISGF3. In gel shift assays, the protein expressed from the cDNA by in vitro transcription and translation exhibited mobility and DNA sequence specificity identical to those of HeLa cell-derived ISGF3γ. The synthetic protein associated with ISGF3α to form the intact ISGF3 complex. Additionally, antiserum raised against recombinant protein recognized ISGF3γ in HeLa cell extracts. The cDNA clone, although lacking a portion of the 5′ untranslated sequence of the mRNA, probably encodes a complete protein because the recombinant and HeLa cell proteins comigrated on SDS-PAGE and displayed similar DNA- and protein-interaction characteristics.

The first 117 amino acids of the predicted sequence of ISGF3γ displayed striking similarity to the N termini of the DNA-binding proteins collectively known as the IRF family (Fig. 6). The IRF proteins (ICSBP, ISGF2/IRF1, and IRF2) all share a DNA-binding specificity related to but distinct from that of ISGF3γ. In spite of this functional similarity, ICSBP is more closely related to ISGF3γ than it is to IRF1 and IRF2.

ISGF3γ serves as the DNA recognition subunit of ISGF3, and as such is required for transactivation of IFN-α-stimulated promoters. The functions of previously cloned IRF proteins, on the other hand, are not well understood, but ISGF2/IRF1 and IRF2 have been implicated as positive and negative regulators, respectively, of the IFN-β gene (24–26, 29, 58, 74). ISGF2 was first identified as an IFN-induced nuclear protein capable of binding the ISRE (50), but its role in regulation of IFN-responsive genes remains obscure. Its induction profile in response to IFN does not correspond with activation of IFN-responsive genes. Its accumulation late during the transient cycle of IFN-α-stimulated transcription has been correlated with both prolongation and suppression of active transcription (35, 50). Protein sequence from purified ISGF2 identified it as the human homolog of IRF1 (60), a cDNA clone of which had been isolated on the basis of the ability of recombinant IRF1 protein to recognize the PRDI site within the IFN-β promoter (58). Overexpression of IRF1 in vivo leads to inefficient activation of endogenous IFN-α and IFN-β genes, although artificial constructs containing multiple IFN-β promoter elements are efficiently *trans*-activated (24, 30). The role of IRF1 in regulation, particularly with respect to IFN-α genes, remains controversial (3, 65, 68). IRF2, identified by sequence homology to IRF1, appears to act as a silencer of unactivated IFN-β genes, since it will effectively antagonize transactivation of reporter constructs by IRF1 (29). ICSBP, which is IFN-γ inducible in lymphoid tissues, also binds PRDI and the core sequence of the ISRE, but its role in transcriptional regulation has not been established (15).

The IRF DNA-binding domain may form a tryptophan-cluster helix-turn-helix. The sequence similarity of IRF family members is limited to the amino-terminal 120 amino acids, suggesting that this region is responsible for their ability to specifically bind DNA. Indeed, the first 168 amino acids of IRF1 and 160 amino acids of IRF2, when swapped with the carboxyl terminus of the other protein, retain the ability to bind DNA, as do the first 188 amino acids of IRF1 when expressed alone (24, 29). The truncated ISGF3γ protein encoded by clone 48, which contains amino acids 37 to 393, fails to bind DNA (data not shown), indicating that the extreme amino terminus is required. A similar observation has been reported for ICSBP (75).

The striking conservation of five tryptophan residues among IRF proteins prompted us to examine their relationship to the c-*myb* and c-*ets* oncoproteins, two families of DNA-binding proteins that contain conserved tryptophan residues in their DNA-binding domains. Although the c-*ets* product has been reported to bind to the ISRE from the oligoadenylate synthetase promoter (77), alignment of IRF family and *ets*-related protein sequences required introduction of several substantial gaps. The c-*myb* protein, on the other hand, showed significant similarity (Fig. 6). c-*myb*,

which acts as a transcriptional regulator during hematopoietic cell differentiation (54), has a DNA-binding domain conserved among *myb*-related proteins and which consists of related stretches of 51 to 52 amino acids repeated three times (20, 27, 33, 38). Each repeat contains a triplet of conserved tryptophan residues spaced at intervals of 18 to 19 amino acids, with 13 amino acids between the tryptophans from adjacent repeats. This triplet of tryptophans aligns with tryptophan residues in the IRF proteins. Alignment of the *myb* and IRF tryptophan residues revealed several other positions with conserved hydrophobicity or charge. In addition, the IRF proteins have conserved tryptophans located 12 residues amino terminal to the triplet (residue 30 of ISGF3γ), reminiscent of the 13-amino-acid spacing between tryptophans in adjacent *myb* repeats.

A tri-α-helical structure with the second and third helices displaying similarity to helix-turn-helix motifs has been proposed for each imperfect repeat of the *myb* DNA-binding domain. Repeats 2 and 3 appear sufficient for sequence-specific DNA binding (20, 27, 33, 38). Amino acid sequence of the similar region from the IRF proteins is permissive for formation of three α-helices (8, 64), predicting that a helix-turn-helix structure may be central to the IRF DNA-binding domain. The 6-bp sequence YAACKG recognized by c-*myb* (6) bears no obvious resemblance to the longer ISRE or core sequences. The additional, highly conserved regions outside the region of *myb* similarity presumably also play a role in DNA binding, contributing to the larger recognition site. Possibly, the two conserved amino-terminal tryptophans upstream of the *myb* homology represent remnants of another *myb*-like repeat.

Despite the striking similarity of DNA-binding regions of the IRF proteins, ISGF3γ displays a distinct DNA-binding specificity. This protein, either alone or in combination with ISGF3α, interacts with the entire ISRE sequence but fails to bind PRDI. ICSBP, IRF1, and IRF2, on the other hand, bind a shorter target, PRDI and the core sequence of the ISRE. There are relatively few regions where ISGF3γ differs from a consensus shared by the other three proteins (e.g., positions 32, 34, 76, 79, 92, 101, and 113). There are additional positions where all four proteins differ, as well as positions where both ISGF3γ and ICSBP differ from an IRF1 and IRF2 consensus. This may reflect a more recent divergence of IRF1 and IRF2 from a common ancestral gene or, perhaps, that ICSBP has a specificity intermediate between that of ISGF3γ and IRF1 and IRF2. The most divergent regions are found at the extreme amino terminus, amino acids 32 to 36, and amino acids 74 to 79; perhaps one or more of these regions are determinants of specificity. Interestingly, none of these regions fall within predicted *myb*-like helices. Therefore, if IRF proteins indeed form a helix-turn-helix structure, residues outside this structure may also contact DNA.

Regulatory protein-protein interactions involving IRF family members. ISGF3γ must associate with ISGF3α to form the transcriptionally active ISGF3 complex. Thus, in addition to a DNA-binding domain, ISGF3γ is expected to have a domain which allows for specific protein-protein interactions. This protein interaction domain could be intimately associated with the DNA-binding domain, as in other common motifs, such as basic region/helix-loop-helix and basic domain/leucine zipper proteins (37). Alternatively, the protein interactions may be governed by a separate domain located distal to the DNA-binding domain. For example, both the positive and negative regulatory domains of c-*myb* are separated from the protein's amino-terminal DNA-binding domain and may act through association with specific proteins (76). The increase in affinity that results from ISGF3α-ISGF3γ interaction could also indicate that a new or modified DNA-binding domain is formed through protein-protein interaction, as has been suggested for the *Notch*- and *ets*-related GA-binding proteins (43, 71).

The sequence similarities between ISGF3γ and the other IRF family members lead us to speculate that the complete function(s) of ICSBP, ISGF2/IRF1, and/or IRF2 could depend on their ability to form complexes with yet unidentified oligomerization partners analogous to the regulatory ISGF3α subunit. Absence of such an activated regulator may explain the limited activity observed following overexpression of transfected IRF1. Several observations point to a potential, virally activated protein component required for efficient transcriptional activation of the IFN-β gene. In most cell lines, a virus-dependent, posttranslational event resistant to the action of cycloheximide has been demonstrated to be a critical step (17, 28, 80). Recently, such a virus-induced regulatory step has been shown to be required even in cells overexpressing IRF1. Although this step could be inhibited by the serine/threonine kinase inhibitor staurosporine (74), the phosphorylation state of ISGF2/IRF1 itself does not appear to change during infection (60), suggesting that this protein is not the direct target for virus-induced phosphorylation. Rather, we suggest that an additional preexisting protein component becomes activated and associates with ISGF2/IRF1 on DNA in virus-treated cells. By analogy with ISGF3, the ternary complex thus formed would stabilize ISGF2/IRF1 binding, even at low-affinity sites, and would provide additional *trans*-activating functions. It is worth noting that in addition to its effect on virus induction of IFN-β transcription, staurosporine inhibits IFN-α-induced cytoplasmic activation of ISGF3α and subsequent ISG transcriptional induction (40, 63). A family of regulatory proteins, sensitive to viral, IFN-α, or perhaps IFN-γ activation, may exist in the cytoplasm, and combinatorial interaction of these regulatory subunits with distinct DNA-binding proteins of the IRF family could provide a regulatory network mediating antiviral responses.

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