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Combinatorial association and abundance of components of interferon-stimulated gene factor 3 dictate the selectivity of interferon responses

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ABSTRACT **Genes containing the interferon-stimulated response element (ISRE) enhancer have been characterized as transcriptionally responsive primarily to type I interferons (IFNα/β). Induction is due to activation of a multimeric transcription factor, interferon-stimulated gene factor 3 (ISGF3), which is activated by IFNα/β but not by IFNγ. We found that ISRE-containing genes were induced by IFNγ as well as by IFNα in Vero cells. The IFNγ response was dependent on the ISRE and was accentuated by preexposure of cells to IFNα, a treatment that increases the abundance of ISGF3 components. Overexpression of ISGF3 polypeptides showed that the IFNγ response depended on the DNA-binding protein ISGF3γ(p48) as well as on the 91-kDa protein STAT91 (Stat1α). The transcriptional response to IFNα required the 113-kDa protein STAT113 (Stat2) in addition to STAT91 and p48. Mutant fibrosarcoma cells deficient in each component of ISGF3 were used to confirm that IFNγ induction of an ISRE reporter required p48 and STAT91, but not STAT113. A complex containing p48 and phosphorylated STAT91 but lacking STAT113 bound the ISRE *in vitro*. IFNγ-induced activation of this complex, preferentially formed at high concentrations of p48 and STAT91, may explain some of the overlapping responses to IFNα and IFNγ.**

Interferons (IFNs) are cytokines capable of activating an antiviral state in target cells and affecting proliferation, differentiation, and immune responses. IFNs are classified into two groups: type I (IFNα/β) and type II (IFNγ) function through different cell surface receptors to produce distinct, though partially overlapping, effects on cells. Gene activation in response to IFNs depends on tyrosine phosphorylation of a family of cytoplasmic transcription factors (reviewed in ref. 1). In response to IFNα, the 113-kDa protein STAT113 (also known as Stat2), the 91-kDa protein STAT91 (also called Stat1α), and the 84-kDa product of an alternatively spliced mRNA from the STAT91 gene (STAT84, also called Stat1β) become tyrosine-phosphorylated at a single site (2–4). These proteins combine with the 48-kDa DNA-binding protein ISGF3γ (referred to as p48) (5) to form IFN-stimulated gene factor 3 (ISGF3), a multimeric complex that translocates to the nucleus (6) and displays a distinct DNA-binding specificity for the IFN-stimulated response element (ISRE) found in the promoters of IFNα-stimulated genes (7).

In response to IFNγ, of these proteins only STAT91 becomes tyrosine-phosphorylated (8). Phosphorylated STAT91 is capable of forming a homodimer (9) that displays DNA-binding affinity for the IFNγ-responsive enhancer termed the gamma-activated site (GAS). Involvement of STAT91 in responses to both types of IFN (10) explains some of the overlap in these responses, since genes containing GAS sequences can bind STAT91 activated in response to either IFN type (11). We describe a second mechanism modulating overlapping responses to IFNα and IFNγ in which phosphorylated STAT91 is directed to ISRE-containing genes through association with ISGF3γ (p48) in the absence of activated STAT113.

MATERIALS AND METHODS

Cell Culture. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% calf serum and antibiotics. African green monkey kidney Vero cells (CRL 1586) were obtained from the American Type Culture Collection. Transformed human embryonic kidney 293 cells (12) expressing simian virus 40 large tumor antigen (293T cells), a gift from R. Schneider (New York University), were grown in the presence of G418 at 200 μg/ml. Human fibrosarcoma 2fTGH cells and their IFN-unresponsive derivatives U2A, U3A, and U6A have been described (13–16). Cells were treated with recombinant human IFNα-2a (Hoffmann–La Roche) or IFNγ (Boehringer Mannheim) at 500 units/ml. Cycloheximide (Sigma) was added at 50 μg/ml, as indicated in the figures.

RNase Protection Assays. Total RNA from Vero cells lysed in guanidinium thiocyanate solution was isolated by standard methods (17). IFN-stimulated gene (ISG54) RNA was quantified by hybridization to a 250-bp radiolabeled cRNA probe from exon 2 of the human gene that cross-hybridizes with monkey RNA, followed by digestion with RNase T2, as described (18). ISG54 induction was quantified by PhosphorImager (Molecular Dynamics), with normalization to constitutively expressed γ-actin mRNA (19).

Expression Plasmids. Chloramphenicol acetyltransferase (CAT) plasmids containing the hamster ISG54 promoter with wild-type or modified ISRE sequences have been described (20). Full-length cDNA expression constructs for ISGF3γ (p48) (5), STAT113 (3), STAT91 (2), and Tpr-Met (21) (details available on request) were driven by the cytomegalovirus promoter (22). Cells were transfected with calcium phosphate/DNA coprecipitates (23) or, for 2fTGH and U2A, by the DEAE-dextran method (24). For CAT assays, cells were treated with IFN for 24 hr one day after transfection, and cell extracts were assayed for CAT activity by standard methods (25).

Electrophoretic Mobility-Shift Assays. Cytoplasmic and nuclear protein extracts were prepared from transiently transfected 293T cells and assayed for ISRE-binding activity using the high-affinity ISG15 ISRE sequence, as described (26). Cells in a six-well dish were transfected with expression plas-

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Abbreviations: IFN, interferon; ISGF3, IFN-stimulated gene factor 3; ISRE, IFN-stimulated response element; CAT, chloramphenicol acetyltransferase; GAS, gamma-activated site.
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mids directing the production of p48 and STAT91 (2 μg), and ligand-independent activation of STAT91 was achieved by cotransfection with 0.2 μg of an expression plasmid for the Tpr-Met tyrosine kinase. Antibodies against ISGF3 proteins (4, 5) were used at a dilution of 1:200. ISG15 ISRE (27), a high-affinity binding site for ISGF3 complexes *in vitro* (7), PRDI from the IFNβ promoter (28) that fails to bind ISGF3 (6), or a synthetic, high-affinity GAS sequence that binds STAT91 (29) were used as competitors at 20-fold molar excess.

RESULTS

Induction of ISG54 by Both IFNα and IFNγ in Vero Cells. Transcription of the ISG54 gene is induced by IFNα but not by IFNγ in HeLa cells (30, 31). A different pattern was observed in Vero cells, where expression was induced by both types of IFN (Fig. 1). ISG54 induction was rapid and transient in response to IFNα (Fig. 1*A*, lanes 2–6). Maximal expression was observed after 4 hr (Fig. 1*A*, lane 3, 33-fold induction), but little ISG54 mRNA remained in cells exposed to IFNα for 24 hr (Fig. 1*A*, lane 5, 8-fold induction; see also Fig. 1*B*, lane 2). Inhibition of protein synthesis by cycloheximide had no effect on the induction of ISG54 by IFNα (Fig. 1*A*, compare lanes 4 and 6, 22- and 23-fold induction, respectively). IFNγ also induced expression of ISG54 mRNA in these cells (lanes 7–10). IFNγ-induced expression was slower, reaching peak levels only after 8 hr (lane 8, 22-fold induction), and did not decline following prolonged (24 hr) exposure of cells to IFNγ (lane 9, 21-fold induction). Similar results were obtained with ISG15 (data not shown), another gene containing an ISRE (27).

Maximal expression of ISG54 in response to IFNγ was sensitive to cycloheximide (Fig. 1*A*, compare 8, 22-fold induction, with lane 10, 11-fold induction), indicating that newly synthesized proteins contributed to the IFNγ response, in contrast to induction by IFNα. Prolonged treatment of Vero cells with IFNα followed by treatment with IFNγ produced a profound "superinduction" of ISG54 expression (Fig. 1*B*, compare lane 2, 5-fold induction, with lane 4, 40-fold induction). This superinduction demonstrated that at least some of the newly synthesized components required for the induction of ISG54 in response to IFNγ were themselves IFN-inducible. Induction of ISG54 by IFNγ once these proteins had accumulated was no longer sensitive to cycloheximide (Fig. 1*B*, lane 5, 48-fold induction). Vero cells lack genes for the IFNα/β family (32); therefore, secretion of IFNα itself cannot explain the response of ISG54 to IFNγ.

ISG54 Expression Requires ISRE Sequences and ISGF3 Components for Responses to Both IFNs. The monkey ISG54 promoter is highly homologous to its human, mouse, and hamster counterparts, including the characteristic duplicated ISRE sequence that responds to IFNα (20, 33). To study the role of the ISRE sequence in responses to IFNγ, Vero cells were transfected with a series of CAT reporter genes driven by the hamster ISG54 promoter (Fig. 2). The wild-type promoter (nt −429 to +31, pH1) responded strongly to both IFNα and IFNγ, showing that response to both IFNs was a transcriptional effect dependent on the promoter. Clustered mutations introduced into this promoter outside the ISRE (pH2 and pH8) had no effect on induction in response to either IFN, whereas mutations introduced into ISRE I (pH5, pH6) greatly reduced the response of the ISG54 promoter to both IFNα and IFNγ. Mutations introduced into the less active ISRE II (20) had modest effects on expression (pH3 and pH4), whereas a double mutation introduced into both ISRE sequences (pH7) completely inactivated the promoter. Promoters with two ISRE II sequences (II/II) showed a diminished response while promoters bearing two ISRE I sequences (I/I) showed an enhanced response to both IFNs. Importantly, no mutation was found that differentially affected the response to only one type of IFN. This demonstrated an essential role for ISRE sequences in responses to both IFNα and IFNγ and indicated that the same or very similar transcription factors were responsible for ISRE-dependent transcription in response to both IFNs.

All the components of the IFNα-responsive transcription factor ISGF3 have been identified. The role of these proteins in the response of ISG54 to IFNγ was studied by using a cotransfection assay. Vero cells were transfected with the wild-type ISG54-CAT construct (pH1) in combination with vectors driving constitutive expression of p48, STAT91, and STAT113 (Fig. 3). Introduction of p48 or STAT91 accentuated the response of ISG54-CAT to both IFNα and IFNγ. Introduction of STAT113, in contrast, affected only the response to IFNα. Combined expression of p48 with STAT91 led to greatly enhanced responses to both IFNα and IFNγ, whereas combined expression of p48 and STAT113 enhanced only the response to IFNα. The response to IFNγ in cells cotransfected with p48 and STAT113 expression constructs was no different from that of cells expressing p48 alone. Likewise, cotransfection of cells with STAT91 and STAT113 constructs led to an exaggerated response to IFNα, but the response to IFNγ was no different from that of cells transfected with the STAT91 construct alone. Combined expression of all three ISGF3 components (p48, STAT91, and STAT113) led to maximal IFNα responsiveness, while the response to IFNγ was similar to the response of cells transfected with p48 plus STAT91. Thus, the response to IFNγ utilized only p48 and STAT91 proteins of the ISGF3 complex.

Response of ISG54 to IFNγ in Human Fibrosarcoma Cells Requires ISGF3 Components. Expression of endogenous ISG54 in human fibroblasts and fibrosarcoma cells is highly induced in response to IFNα but is only marginally affected by IFNγ (14, 30). However, with the highly sensitive ISG54-CAT reporter assay, a modest response to IFNγ was detected in the human fibrosarcoma cell line 2fTGH (Fig. 4). IFN-unresponsive derivatives of the 2fTGH cell line (13–16) specifically deficient in p48 (U2A), STAT91/84 (U3A), or STAT113 (U6A) were tested for their ability to induce ISG54-CAT in response to IFNs. The U2A cell line, expressing only truncated forms of p48 (ref. 14 and unpublished data), failed to induce ISG54-CAT in response to either IFNα or IFNγ. However, cotransfection of ISG54-CAT with the p48 expression vector restored full response to both IFNα and IFNγ. Likewise, the STAT91-defective cell line U3A was unable to induce ISG54-CAT in response to either IFN. Similar to results for GAS-containing genes (10), the defect in IFN responsiveness for ISG54-CAT could be overcome by complementation with the STAT91 expression plasmid. These results are consistent with the results obtained in Vero cells that IFNγ

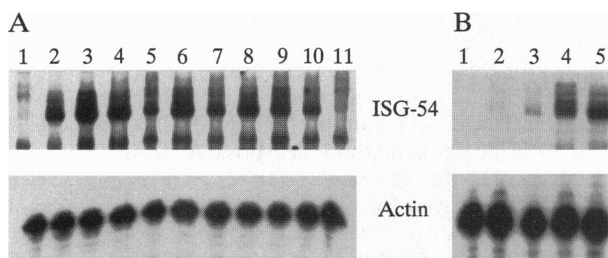

FIG. 1. Induction of ISG54 in response to both IFNα and IFNγ. RNA from Vero cells was analyzed for ISG54 (*Upper*) and γ-actin (*Lower*) expression by RNase protection after various treatments. (*A*) Lane 1, untreated; lanes 2–5, IFNα for 2, 4, 8, or 24 hr, respectively; lane 6, IFNα and cycloheximide for 8 hr; lanes 7–9, IFNγ for 4, 8, or 24 hr, respectively; lane 10, IFNγ and cycloheximide for 8 hr; lane 11, cycloheximide for 8 hr. (*B*) Lane 1, untreated; lane 2, IFNγ for 1 hr; lane 3, IFNα for 24 hr; lane 4, IFNα for 24 hr and IFNγ for 1 hr; lane 5, IFNα for 24 hr, IFNγ for 1 hr, and cycloheximide for 1 hr.

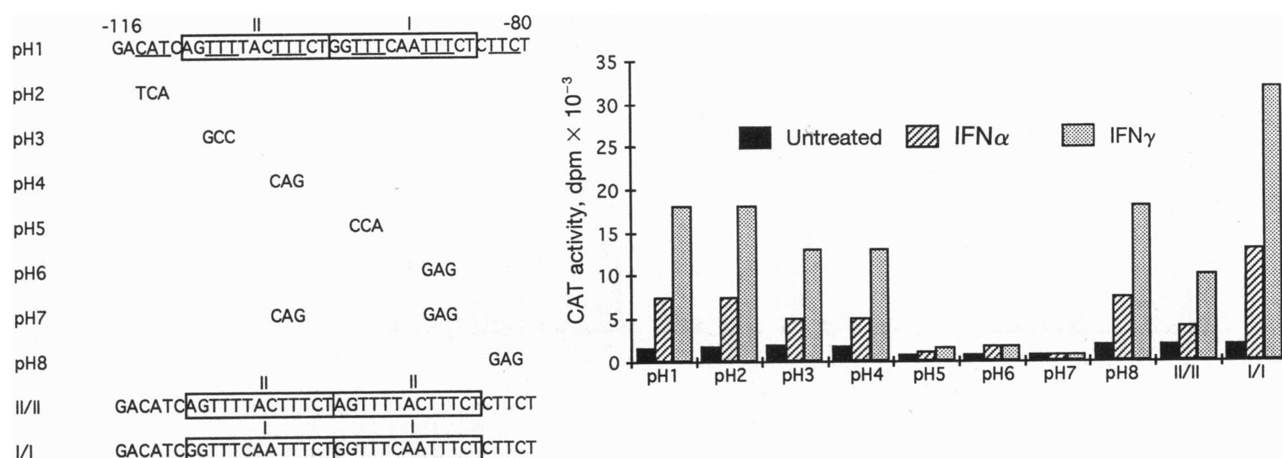

FIG. 2. Induction of ISG54 by both IFNα and IFNγ requires an intact ISRE. Vero cells were transfected with ISG54-CAT constructs containing the wild-type hamster ISG54 ISRE (pH1) or the indicated mutations within or adjacent to the ISRE sequence. Cells were treated with IFN for 24 hr as indicated and extracts were prepared and analyzed as described (20). CAT assays were quantified by liquid scintillation counting, and the results presented represent the averages of three or four experiments.

responsiveness of the ISG54 promoter requires functional p48 and STAT91 proteins.

U6A cells lacking STAT113 (16) also failed to induce ISG54-CAT in response to IFNα and were only marginally responsive to IFNγ (Fig. 4). Complementation of these cells with a STAT113 expression plasmid restored the response to IFNα but had no effect on the response to IFNγ. However, cotransfection of U6A cells with p48 or STAT91 expression plasmid enhanced the response of ISG54-CAT to IFNγ without affecting its lack of response to IFNα. Combined expression of p48 and STAT91 enhanced the IFNγ response but was unable to restore a response to IFNα in the absence of STAT113. As was true for Vero cells, these results demonstrated that STAT113 is involved only in responses to IFNα. The diminished response of ISG54-CAT to IFNγ observed in U6A cells, which could not be complemented by introduction of intact STAT113, has not been further characterized. Response of endogenous 9-27 and major histocompatibility complex class II genes to IFNγ is also diminished in U6A cells but is not related to the absence of STAT113 (16).

A p48/STAT91 Protein Complex Binds the ISRE. ISGF3 proteins function through direct interaction with DNA. The STAT91/STAT113 complex formed in response to IFNα displays no affinity for the ISRE (6); however, in combination with p48, a sequence-specific DNA-binding protein (5), the multimeric ISGF3 complex is directed to the ISRE (26). We examined the ability of STAT91 to bind ISRE sequences in the absence of STAT113. The ISRE sequence from the ISG15 gene was used because of its higher affinity for p48 *in vitro* and because similar results to those shown in Fig. 3 were obtained by using a reporter construct containing this ISRE (data not shown). As a source of ISGF3 proteins, 293T cells were transfected with expression plasmids for STAT91 or p48, and cell extracts were assayed for ISRE binding by electrophoretic mobility shift using an ISRE probe (Fig. 5). The STAT91 plasmid was cotransfected with an expression construct for the constitutively active Tpr-Met tyrosine kinase to provide efficient, ligand-independent activation of this protein through phosphorylation of the same tyrosine residue modified in response to IFN (data not shown). Cell extracts containing activated STAT91 displayed no ISRE-binding activity (lane 1), whereas extracts from p48-transfected cells displayed a rapidly migrating complex characteristic of ISGF3γ (lane 2). Mixing of extracts containing STAT91 with extracts containing p48 (lane 3) produced a novel, slowly migrating complex reminiscent of ISGF3 (6, 26). The composition of this complex was identified by use of specific antibodies: the slow-mobility complex, as well as the p48 complex, was supershifted into a very slowly migrating complex with antibodies specific for p48 (lane 4). Likewise, the slowly migrating complex, but not the p48 complex, was disrupted by antibodies against STAT91 (lane 5). In contrast, antibodies against STAT113 (lane 6) had no effect.

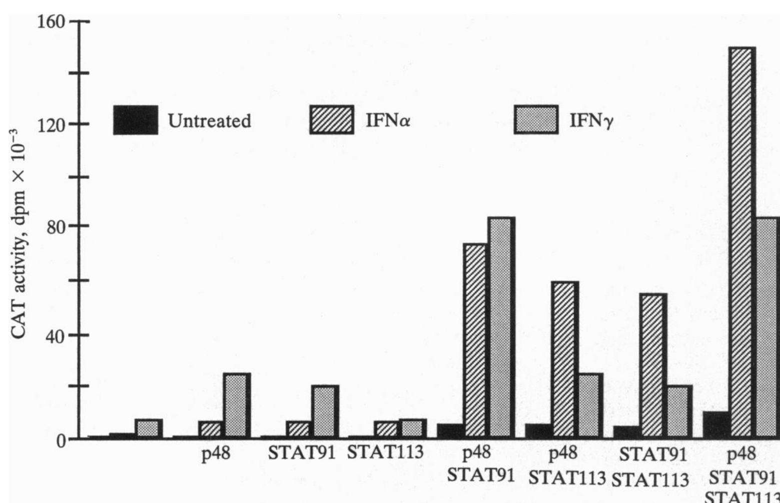

FIG. 3. IFNγ-induced expression through the ISRE is accentuated by p48 and STAT91, but not by STAT113. Vero cells were transfected with the wild-type ISG54-CAT construct (pH1) alone or together with expression plasmids directing the synthesis of ISGF3 proteins, as indicated. Cells in 60-mm dishes were transfected with 2.5 μg of ISG54-CAT reporter along with 2.5 μg of each expression plasmid; the total amount of DNA was kept constant by substituting empty vector DNA in transfections not requiring expression constructs. Cells were treated with IFNα or IFNγ as described in *Materials and Methods.*

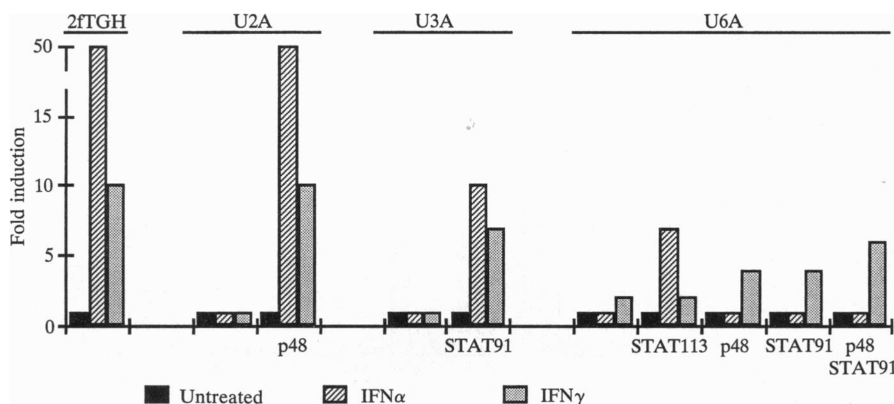

FIG. 4. IFNγ induction of ISG54 in human fibroblasts requires p48 and STAT91, but not STAT113. ISG54-CAT expression was measured in wild-type 2fTGH cells and in the IFN-unresponsive derivatives U2A, U3A, and U6A alone or together with expression plasmids directing synthesis of ISGF3 proteins, as indicated. Fold induction in response to IFNα and IFNγ within each cell line was compared with CAT activity in untreated cells.

The sequence recognition specificity of this novel complex was examined by competition assay. A 20-fold molar excess of unlabeled ISRE abrogated all binding to the labeled probe (Fig. 5, lane 7). However, a related-sequence oligonucleotide containing only the central 9 nt of the ISRE (PRDI from the IFNβ promoter) failed to compete for binding of either complex (lane 8). Interestingly, a GAS site with a high affinity for STAT91 homodimers was capable of disrupting the slowly migrating complex without affecting the ability of p48 to recognize the ISRE (lane 9). Taken together, these results indicate that the slowly migrating complex was composed of STAT91 and p48 bound to the ISRE oligonucleotide.

DISCUSSION

ISG54, which is inducible by IFNα in many cell types, was found also to be inducible by IFNγ in Vero cells. Induction by both IFNs required an intact ISRE and utilized ISGF3 proteins. However, induction in response to IFNγ was dependent on protein synthesis and was delayed in comparison to the IFNα response. Cotransfection experiments in Vero cells as well as in mutant human fibroblasts lacking specific ISGF3 proteins demonstrated that the IFNγ response required STAT91 and p48, but not STAT113. It is likely that the protein-synthesis dependence of this response, as well as the superinduction following IFNα pretreatment, reflects a requirement for synthesis of abundant p48 for a maximal response (30). The p48 protein accumulates to high levels in Vero cells in response to IFNα (unpublished data).

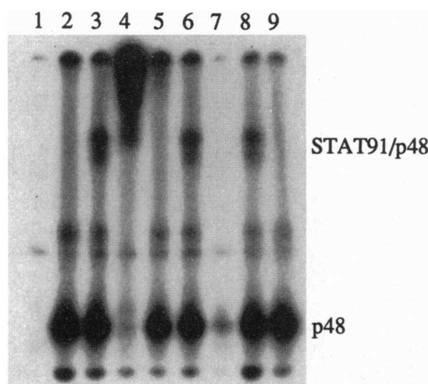

FIG. 5. p48 facilitates STAT91 binding to the ISRE. Cytoplasmic extracts from 293T cells overexpressing p48 or activated STAT91 phosphorylated by Tpr-Met were analyzed by mobility shift assay using a labeled ISG15 ISRE probe. Antibodies specific for p48, STAT91, or STAT113 were added at a final dilution of 1:200. Extracts were derived from cells expressing STAT91 (lane 1) or p48 (lane 2), or these extracts were mixed *in vitro* (lanes 3–9). The STAT91/p48 complex was challenged with antibodies against p48 (lane 4), STAT91 (lane 5), or STAT113 (lane 6) or with a 20-fold excess of unlabeled ISRE (lane 7), PRDI (lane 8), or GAS (lane 9) oligonucleotides.

Another potential factor regulating transcription in response to both IFNs would be the ISRE-binding protein IRF1. This protein, induced to high levels in response to IFNα and IFNγ, has been ascribed a maintenance role in prolonging transcription initiated in response to ISGF3 activation (14, 34). However, it is unlikely that IRF1 expression is the protein synthesis-dependent step responsible for the induction of ISG54 in Vero cells. Unlike results observed with ISGF3 proteins, cotransfection of IRF1 expression plasmids with ISG54-CAT led to increased basal expression but had no effect on inducibility in response to either IFNα or IFNγ (data not shown).

These results provide an additional twist to the original model of IFNα and IFNγ responsiveness (Fig. 6). IFNα stimulates the tyrosine phosphorylation of both STAT91 and STAT113 (35–38). STAT91/STAT113 heterodimers, in combination with p48, interact with ISRE sequences to activate gene expression (6, 7), while STAT91 homodimers interact with GAS sequences (9, 11, 39). IFNγ, which activates STAT91 but not STAT113 phosphorylation (8, 40), has been thought to exclusively target genes containing GAS sequences that bind STAT91 homodimers. However, as we show here, STAT91 in the presence of p48 also binds the ISRE, allowing ISRE-containing genes to respond to IFNγ in the absence of phosphorylated STAT113.

Given the overlapping use of signaling pathways and transcription factors by the two types of IFN, one might expect all ISRE-containing and GAS-containing genes to respond identically to both IFNα and IFNγ. However, this is not the case. In addition, different cell types display markedly different responses. For example, ISG54 is induced strongly by both IFNα and IFNγ in Vero cells, whereas it is only marginally induced by IFNγ in diploid human fibroblasts. These differences can be ascribed to three factors. First, the affinity of different transcription factor complexes toward ISRE sequences varies. The STAT91/p48 complex displays a lower affinity for the ISG15 ISRE than does classical ISGF3. For instance, the STAT91/p48 complex was efficiently detected *in*

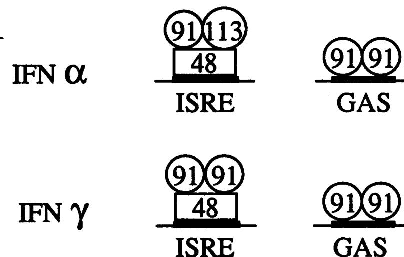

FIG. 6. STAT transcription factor complexes formed in response to IFNα and IFNγ.

vitro as a DNA-binding complex only under conditions of overexpression of the two polypeptides (Fig. 5). Indeed, a high-affinity STAT91 binding site effectively competed for formation of the STAT91/p48 complex on the ISRE (Fig. 5), but had no effect on an ISGF3 complex stabilized by STAT113 (data not shown). This higher affinity of classical ISGF3 allows ISRE-containing genes to respond to IFNα even at low abundances of the ISGF3 proteins. A maximal IFNγ response requires higher protein levels, particularly of p48, due to the low affinity of the STAT91/p48 complex. Therefore, many ISRE-containing genes may respond to IFNγ only following accumulation of high levels of p48 after prolonged exposure to IFNγ.

A second aspect determining differential responses could involve relative affinities of distinct ISRE sequences for ISGF3 complexes. For example, the 9-27 gene responds to both IFNα and IFNγ, whereas the 6-16 gene is largely restricted to IFNα responsiveness in its endogenous context (41). The differential responses of these genes depend on sequence variation at the ISRE (41, 42), correlating with differential binding affinities for ISGF3 components. The 9-27 ISRE displays a heightened affinity for p48 relative to the 6-16 ISRE (34). The data presented here suggest that this differential affinity would allow a productive transcription complex of p48 and STAT91 to form on the 9-27 ISRE in response to IFNγ, whereas the 6-16 ISRE would require phosphorylated STAT113 to form a functional complex, thus restricting its expression to IFNα responses.

A third factor affecting differential responses to IFNα and IFNγ involves differences in the kinetics of STAT activation between cell lines. For the above scenario of IFNγ induction of ISG54 to occur, sufficient activated STAT91 must remain following prolonged exposure to IFNγ to interact with the p48 that accumulated over time. In many cell types, including human fibroblasts, STAT91 phosphorylation in response to IFNγ is transient, with little active protein remaining after 8 hr of incubation with IFNγ (29). In contrast, Vero cells accumulate high levels of activated STAT91 that are retained for >18 hr (data not shown). Therefore, an overnight incubation of Vero cells with IFNγ results in high levels of both activated STAT91 and p48, allowing formation of this low-affinity complex on DNA. Overall levels of gene expression in response to IFNs thus depend on the duration of tyrosine-phosphorylated STAT proteins as well as on the accumulation of DNA-binding adaptor proteins.

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