MOLECULAR AND CELLULAR BIOLOGY, Oct. 1991, p. 5147–5153
0270-7306/91/105147-07$02.00/0

Vol. 11, No. 10

Two Distinct Alpha-Interferon-Dependent Signal Transduction Pathways May Contribute to Activation of Transcription of the Guanylate-Binding Protein Gene

THOMAS DECKER,† DANIEL J. LEW,‡ AND JAMES E. DARNELL, JR.*
The Rockefeller University, 1230 York Avenue, New York, New York 10021

Received 16 April 1991/Accepted 4 July 1991

The promoter of the gene encoding a cytoplasmic guanylate-binding protein (GBP) contains two overlapping elements: the interferon stimulation response element (ISRE), which mediates alpha interferon (IFN-α)-dependent transcription, and the IFN-γ activation site (GAS), which is required for IFN-γ-mediated stimulation. The ISRE binds a factor called ISGF-3 that is activated by IFN-α but not by IFN-γ. The GAS binds a protein that is activated by IFN-γ, which we have termed GAF (IFN-γ activation factor; T. Decker, D. J. Lew, J. Mirkovitch, and J. E. Darnell, Jr., EMBO J., in press; D. J. Lew, T. Decker, I. Strehlow, and J. E. Darnell, Jr., Mol. Cell. Biol. 11:182–191, 1991). We now find that the GAS is also an IFN-α-responsive element in vivo and that IFN-α (in addition to activating ISGF-3) rapidly activates a GAS-binding factor, the IFN-α activation factor (AAF). The AAF has characteristics very similar to those of the previously described GAF. Through the use of inhibitors of protein synthesis and inhibitors of protein kinases, the activation conditions of AAF, GAF, and ISGF-3 could be distinguished. Therefore, not only do IFN-α and IFN-γ stimulate transcription of GBP through different receptors linked to different signaling molecules, but occupation of the IFN-α receptor apparently leads to the rapid activation of two different DNA-binding proteins through the use of different intracellular pathways.

Treatment with type I (alpha/beta interferon [IFN-α/β]) or type II (IFN-γ) interferons (IFNs) induces multiple changes in the biology of a cell. Although the effects of the two IFN types on cells are not generally identical, there are functional alterations, such as the antiviral state or an inhibition in growth rate, that can be brought about by either cytokine (reviewed in reference 7). Some genes are stimulated transcriptionally by only one type of IFN, and other genes, like the guanylate-binding protein (GBP) gene, respond to IFN-α and IFN-γ (2, 3, 5, 10, 15, 16, 18, 22, 23, 25, 29, 31). The molecular pathways that allow two different ligand-receptor pairs to cause a similar biological response are of major interest in the study of intracellular signaling.

Some of the interactions between IFN-α- and IFN-γ-induced molecular events are known. For example, IFN-γ can enhance the transcription of IFN-α-responsive genes as well as antagonize IFN-α-induced gene repression (5, 22, 23). Also, there are multiple additive or synergistic interactions of the two IFN types combined in the induction of the GBP gene (5, 23). In order to understand these interactions, we initially concentrated on the primary transcriptional effects of IFNs on gene expression, that is, those events that occur within minutes after treatment and do not require new proteins to be made. In both HeLa cells and diploid fibroblasts, at least one of the protein complexes that are involved in immediate transcriptional activation by IFN-α or IFN-γ is known: ISGF-3 is a major IFN-α-activated transcription factor, and GAF (IFN-γ activation factor) is a major IFN-γ-stimulated transcription factor (4, 6, 13, 17, 20, 21, 24). Both factors are composed of proteins that preexist in the cell cytoplasm and become rapidly activated upon treatment with IFN-α or IFN-γ (4, 6, 21). Any initial interactions between the signaling paths induced by the two cytokines might therefore influence the activity of these DNA-binding protein complexes. In fact, while IFN-γ cannot activate ISGF-3 by itself, we did find that in HeLa cells the IFN-α-dependent activation of ISGF-3 is greatly enhanced after treatment with IFN-γ, owing to an increase in the DNA-binding component (ISGF-3γ) of the ISGF-3 complex (22).

To extend our knowledge about interactive effects of IFNs on transcription factor activation, we tested the effect of IFN-α treatment on the GAF. IFN-α rapidly induces an IFN-γ activation site (GAS)-binding factor with DNA-binding characteristics very similar to those of the GAF, which we term IFN-α activation factor (AAF). Through the time course of activation, the requirements for protein synthesis, and the different susceptibility to kinase inhibitors, we could distinguish between the AAF, the GAF, and ISGF-3. Our results suggest that in the response to IFN-α, two distinct signal transduction pathways rapidly activate different transcriptional factors in addition to supporting the previous conclusion (23) that the molecular pathways originating at the liganded IFN-α and IFN-γ receptors are distinguishable.

MATERIALS AND METHODS

Cells and reagents. HeLa S3 cells were obtained from the American Type Culture Collection (Rockville, Md.). Human diploid fibroblasts (FS2) were a gift from E. Knight (Du Pont, Wilmington, Del.). Both cell types were grown to confluency in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Human recombinant IFN-α_2 was a gift from P. Sorter (Hoffmann-La Roche). Human recombinant IFN-γ was kindly provided by D. Vapnek

* Corresponding author.
† Present address: Fraunhofer Institute for Toxicology and Molecular Biology, Nikola-Fuchsstrasse 1, 3000 Hannover 61, Federal Republic of Germany.
‡ Present address: Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037.

(Amgen). IFN-α was used at 500 antiviral U/ml (15 pM), and IFN-γ was used at 100 antiviral U/ml (290 pM). Cycloheximide was from Sigma and used at 50 μg/ml. The kinase inhibitors H7 (Seikagaku Inc.) and 2-aminopurine (Sigma) were used at concentrations of 30 μM and 9 mM, respectively. Exonuclease III was from Boehringer Mannheim.

Plasmids and oligonucleotides. All recombinant plasmids containing the GBP promoter DNA or point and deletion mutations thereof (with the exception of the ΔGAS mutations, see below) have recently been described (6, 24). The same articles also describe the construction of the 4XGAS-HC plasmid, which contained four copies of the GAS oligonucleotide

GATCCGTCAGTTTCATATTACTCTAAATCCA
GCAGTCAAAGTATAATGAGATTTAGGTCTAG

comprising sequences −125 to −101 from the GBP promoter fused to basal elements from the human immunodeficiency virus (HIV) long terminal repeat (LTR). The ΔGAS mutations were constructed by using the mg1 (triple) and mg3 (double) GAS point mutants (24). These mutations create an *Eco*RI and a unique *Xho*I site, respectively, within the GAS. The Δ9 mutant was constructed through ligation of the *Hin*dIII (−216)-*Eco*RI (−115) GBP promoter fragment from mg1 into mg3 after digestion with *Hin*dIII and *Xho*I (−107) and removal of the *Hin*dIII (8 to 216)-*Xho*I (−107) fragment. Following treatment with 1 U of S1 nuclease (Boehringer) for 30 min at 37°C, the plasmid was covalently closed. The resulting constructs were confirmed by DNA sequencing to contain the GBP promoter fused to the chloramphenicol acetyltransferase (CAT) gene but with GAS sequences −115 to −107 deleted and 2 bp mutated, one retained from mg1, the other from mg3 (see Fig. 8). The Δ7, Δ8, and Δ10 mutations were constructed by using the mg3 mutant. The plasmid was digested with *Xho*I and treated with 1 U of S1 nuclease for 30 min at 37°C. The resulting DNA was religated, and the deletions were analyzed by DNA sequencing. Their extents and positions are given in Fig. 8.

Nuclear extracts. Extraction of nuclei was done by a slight modification (20) of the protocol provided by Dignam et al. (8).

Exo III assay. The exonuclease III (exo III) assay was performed as described previously (6).

Gel retardation assay. The gel retardation assay has been described previously (12, 20).

Transfection of DNA and analysis of reporter gene expression. DNA was transfected into HeLa S3 cells by the DEAE-dextran method (references 6 and 24 and references therein). To control for transfection efficiency, one pool of 10^7 cells was transfected with 15 μg of DNA (unless otherwise stated) and then split equally between control cells and cells for further treatment with IFNs. CAT expression in extracts from transfected cells was determined by standard methods (32).

RESULTS

IFN-α rapidly activates a factor that binds to the GAS. The IFN-γ-stimulated binding of the GAF to the GAS in the GBP gene promoter was originally detected and defined by protection of a labeled promoter DNA fragment against exo III digestion (6). The region of protection was from nucleotides −128 to −99 in the GBP gene promoter, numbered relative to the major initiation site of transcription. The time course of GAF appearance after treatment with IFN-γ differed between cell types. In primary fibroblasts (FS2 cells), the DNA-binding activity appeared within 10 min and declined within 3 to 4 h; in HeLa cells, GAF rose slowly to reach peak levels only after about 6 h.

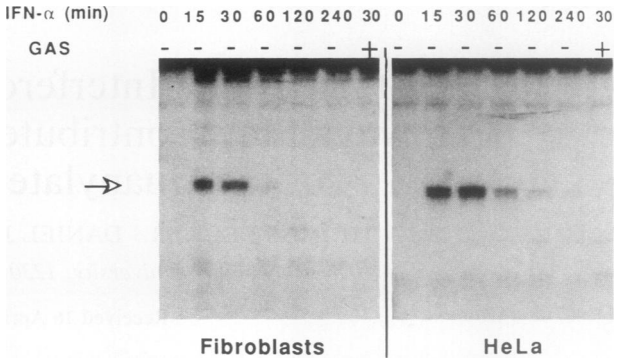


FIG. 1. Time course of AAF induction. Nuclear extracts from cells treated with IFN-α for the indicated periods were incubated with labeled GBP promoter DNA and then subjected to digestion with exo III. The indicated stop corresponds to position −99 in the GBP promoter (6). GAS indicates competition with a 50-fold molar excess of GAS oligonucleotide (see Materials and Methods).

To test for possible interactions of the two IFN types in the regulation of the GAF, we performed exo III protection assays with a labeled GBP promoter fragment and extracts from both HeLa cells and fibroblasts after treatment with IFN-α for various periods of time. For both cell types, we observed a strong exo III stop on the top strand at position −99 that appeared after treatment with IFN-α (Fig. 1). This exo III stop was coincidental with the downstream stop that had been detected earlier for the GAF after IFN-γ treatment (6). Binding of the IFN-α-induced factor was specific for the GAS because it could be prevented by a 50-fold molar excess of unlabeled GAS oligonucleotide (Fig. 1). However, the pattern of appearance and disappearance was different between the IFN-α-induced exo III stop and GAF. In both HeLa cells and fibroblasts, the IFN-α-induced factor rose within 15 min and declined almost completely within 1 h. This contrasted to the slower appearance and longer persistence of GAF in HeLa cells and also its longer persistence (3 to 4 h) in fibroblasts (6). To account for these differences between the IFN-α- and IFN-γ-induced factors, we call the new IFN-α-induced DNA-binding activity AAF (for IFN-α activation factor) to distinguish it from the GAF.

IFN-α-induced stop does not require protein synthesis in either fibroblasts or HeLa cells. The rapid activation of the AAF suggested that the proteins involved in this process existed in the cell before IFN-α treatment. We tested this assumption by inducing cells with IFN-α in the presence of cycloheximide (CHX). Figure 2 shows that the proteins responsible for the AAF exo III stop were unaffected by the inhibitory action of CHX in either fibroblasts or HeLa cells. In contrast, induction of GAF by IFN-γ in HeLa cells requires ongoing protein synthesis (6). The AAF must thus either be a different protein or consist of the same protein(s) activated through a pathway not requiring the synthesis of new protein.

DNA-binding activities of the AAF and the GAF are indistinguishable. To examine possible differences in physical appearance between AAF and GAF, we compared their DNA-binding properties. We first assayed nuclear extracts from IFN-α- and IFN-γ-treated cells with exo III on both the top and bottom DNA strand and compared the region of the

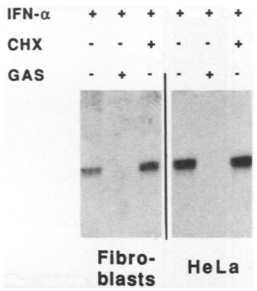


FIG. 2. Effect of CHX on induction of the AAF. Cells were treated for 15 min (FS2 fibroblasts) or 30 min (HeLa) with IFN-α in the presence or absence of CHX. The corresponding nuclear extracts were assayed for AAF activity with exo III in the presence and absence of competing unlabeled GAS oligonucleotides.

GBP gene promoter that was protected from digestion. As indicated above, the exo III stop on the top strand was identical between AAF and GAF at position −99. Figure 3 shows that the specific, GAS-competable exo III stops on the bottom strand were also identical at nucleotide −128 for both AAF and GAF. Thus, within the resolution provided by the exo III assay, the footprint of both factors on the GBP gene promoter is the same. (We noted in our original description of GAF [6] that we were not able to obtain gel mobility shift complexes with this factor and have therefore not done footprint analysis based on the isolation of such complexes. Even with highly purified GAF [34a], we do not obtain retarded gel complexes with labeled GAS DNA).

We next used a 30-fold molar excess of a series of promoter fragments, each with a different point or deletion mutation (6, 24), to compete for AAF or GAF binding to compare their exact sequence requirements for binding. A series of fragments competed virtually identically for the two factors, reproducing the pattern reported previously for the GAF (Fig. 4) (6). Briefly, the 3′ deletions of the GAS site up to nucleotides −109 (which do not compete; Fig. 4, lanes 9) and −102 (which does compete; Fig. 4, lanes 10) define the downstream boundary of the binding site for both factors. Likewise, point mutations starting from nucleotide −121 abolish the ability to compete in the exo III protection assay, thus establishing the 5′ boundary of both the AAF and GAF binding site (Fig. 4, lanes 5). From these results and those in

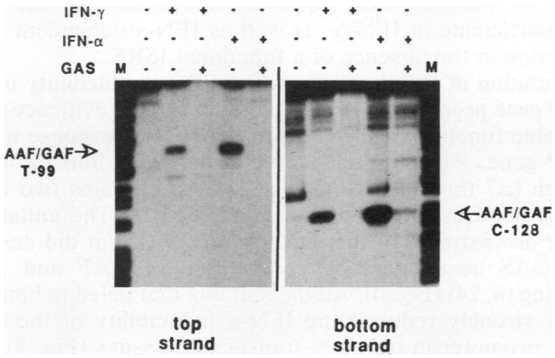


FIG. 3. Exo III protection of the GBP promoter by the AAF and GAF. Nuclear extracts from HeLa cells that were either left untreated or treated with IFN-α for 30 min or with IFN-γ for 4 h were assayed with the top- and bottom-strand probes from the GBP promoter described before (6).

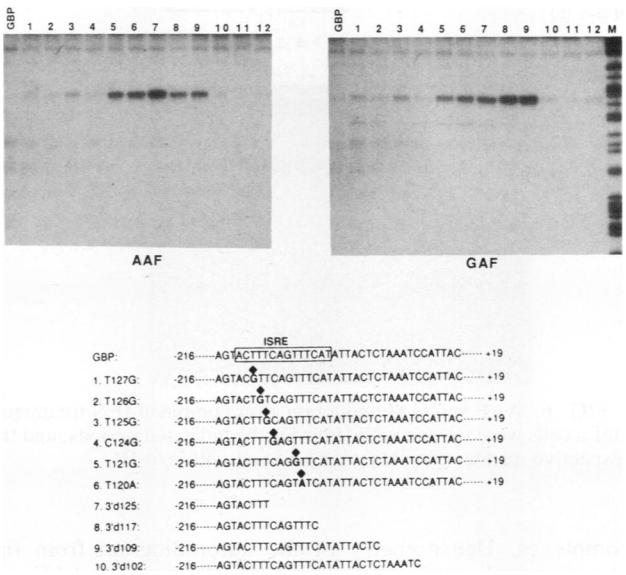


FIG. 4. Competition with AAF and GAF by mutated GBP promoter DNA. Nuclear extracts from HeLa cells treated for 30 min with IFN-α (left) or for 4 h with IFN-γ (right) were assayed with exo III in the presence of a 30-fold molar excess of the indicated DNA. Mutants in lanes 5 to 9 failed to compete and define the AAF and GAF binding sites.

Fig. 3, the sequence requirements for AAF and GAF are identical or at least very similar.

We then tried to distinguish AAF and GAF on the basis of their relative binding affinity to the GAS. Nuclear extracts from IFN-α- or IFN-γ-treated HeLa cells were incubated with labeled GBP gene promoter DNA and, after a binding period of 30 min, were challenged with a 100-fold molar excess of unlabeled GAS oligonucleotide. At the indicated times (Fig. 5), aliquots were taken from the reaction mix and subjected to digestion with exo III. This experiment explored the stability of preformed (off-times) protein-DNA

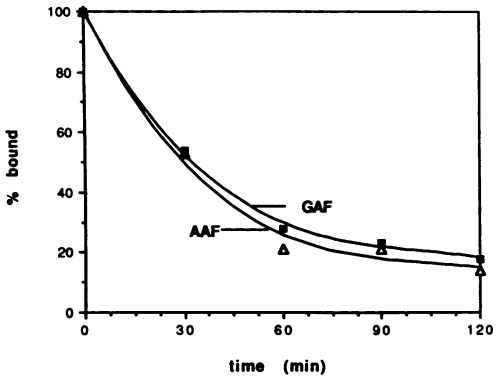


FIG. 5. Relative dissociation rates of AAF and GAF. Labeled GBP promoter DNA was incubated with HeLa nuclear extracts from cells treated with either IFN-α (AAF) for 30 min or IFN-γ (GAF) for 4 h. After 30 min at room temperature to allow AAF and GAF binding to the template, the protein-DNA complexes were challenged with a 100-fold molar excess of GAS oligonucleotide. Aliquots taken at the indicated times were digested with exo III. The AAF (△) and GAF (■) bands on the resulting autoradiogram were evaluated by densitometry and normalized to the signal obtained from a reaction with a noncompetitive oligonucleotide.

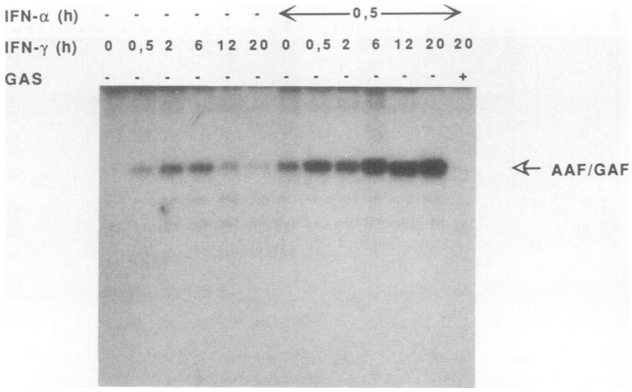


FIG. 6. AAF and GAF induction after combined IFN treatment. HeLa cells were treated with IFNs for the indicated periods, and the respective nuclear extracts were assayed with exo III.

complexes. Densitometry of the autoradiogram from the experiment showed indistinguishable off-times for AAF and GAF. Taken together, the DNA-binding data (Fig. 3 through 5) suggested a close physical relationship between the AAF and the GAF, presumably at least in their DNA-binding component.

IFN-α and IFN-γ synergize in AAF and GAF induction. Since both IFN-α and IFN-γ induced similar GAS-binding factors, we determined the effect of combined IFN treatment on factor production. HeLa cells were either treated for different amounts of time with IFN-γ or received IFN-α during the last 30 min of IFN-γ treatment. Cell extracts at every time point showed that the short IFN-α treatment produced an increase in exo III protection (Fig. 6). In cells treated with IFN-γ exclusively, in which GAF had begun to disappear (12 and 24 h), a 30-min IFN-α exposure caused the reappearance of a maximal amount of exo III protection activity.

GAS as an IFN-α-responsive element in transient transfection. The identification of DNA-binding activity does not prove that such factors have any necessary function in gene activation. To test for a possible role of AAF in IFN-α-dependent transcription of transfected DNA templates, various recombinant constructs were transfected in HeLa cells, and expression of the test gene (CAT) was assayed. The data for those experiments are shown in Fig. 7 and 8. In the first experiment, we wished to establish whether the GAS, in the absence of a functional ISRE, could drive transcription in response to IFN-α. The recombinant construct (4XGAS-HC; see Materials and Methods) contained four copies of the GAS oligonucleotide fused upstream to a basal promoter (the HIV LTR), followed by a sequence encoding the bacterial CAT gene. The sequence contained on this promoter begins at −125 and extends to −101. This sequence lacks the critical first four bases of the ISRE (20, 24) and has no ISGF-3 binding activity (22a). Different amounts of plasmid were transfected into HeLa cells, and the transfected cultures were split into equal portions and either left untreated or treated with IFN-α or IFN-γ. The transcription of the CAT reporter gene was stimulated by both cytokines (Fig. 7). This response suggested the possibility that AAF can participate in IFN-α- as well as IFN-γ-dependent transcription in the absence of a functional ISRE.

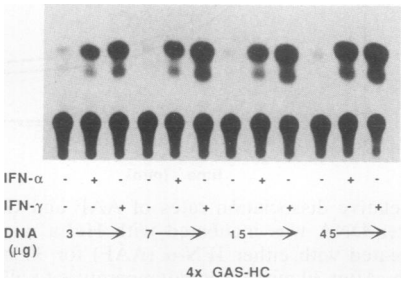
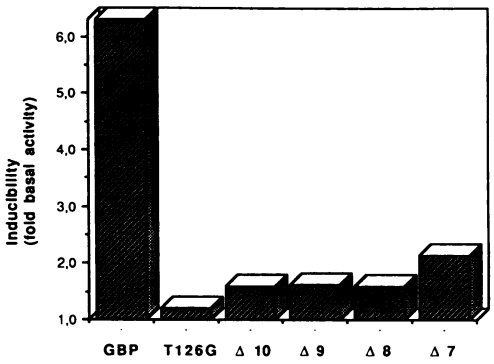


FIG. 7. IFN responsiveness of the GAS in transient transfection. Indicated amounts of the 4XGAS-HC DNA construct (see text) were transfected into HeLa cells. After a 24-h period, the transfected cells were treated with IFN-α or IFN-γ for 15 h. CAT activity in the resulting extracts indicates the GAS-mediated IFN responsiveness of basal promoter elements from the HIV LTR (6, 24).



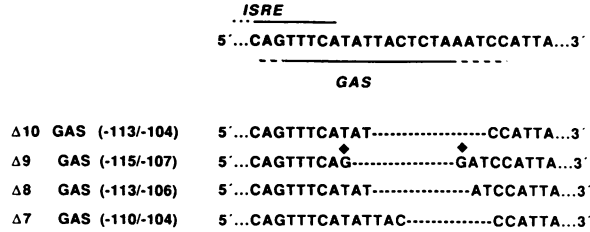


FIG. 8. Effect of GAS deletion on the IFN-α inducibility of the GBP gene promoter. HeLa cells were transfected with 15 μg of the indicated plasmids. CAT activity was determined after treating the transfected cells with IFN-α for 15 h. Quantitation of CAT activity was achieved by cutting ^{14}C-labeled, acetylated chloramphenicol from the thin-layer chromatogram and measuring its radioactivity by scintillation counting.

Mutation of the GAS affects the IFN-α inducibility of the GBP gene promoter in vivo. To obtain further evidence for a possible function of the GAS in the IFN-α response of the GBP gene, we introduced several deletions into the GAS region (Δ7 through Δ10 GAS; Δ9 GAS contains two additional point mutations as indicated; Fig. 8). The mutations were downstream of the intact 14-bp ISRE but did destroy the GAS as assayed by competition of GAF and AAF binding (6, 24) (Fig. 4). All the mutants that failed to bind the AAF strongly reduced the IFN-α inducibility of the GBP gene promoter in transient-transfection assays (Fig. 8). Selective mutation of the ISRE also abolished the response to IFN-α, as demonstrated with the T126G mutant (Fig. 3 and 8). Thus, the action of ISGF-3, at least on this construct, during a transfection has a greater effect than does AAF. Nevertheless, these data, together with those in Fig. 7,

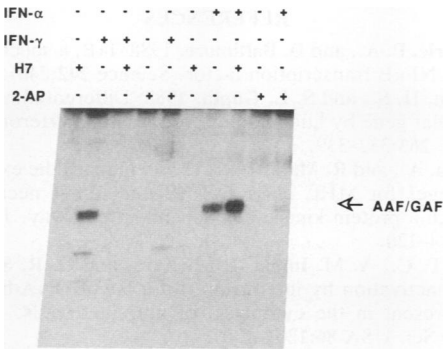


FIG. 9. Effects of kinase inhibitors on the induction of AAF and GAF. HeLa cells were treated with IFN-α for 30 min or IFN-γ for 4 h in the presence of H7 or 2-aminopurine (2-AP). AAF and GAF activity in nuclear extracts was determined in the exo III assay.

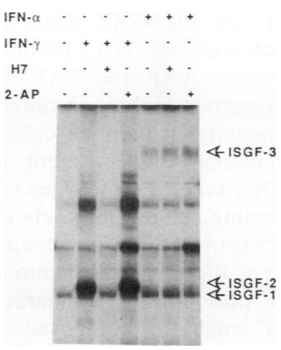


FIG. 10. Effect of kinase inhibitors on ISGF-3 induction. The same extracts used in the experiment shown in Fig. 9 were subjected to gel retardation assay analysis with an ISRE-containing oligonucleotide corresponding to the sequence found in the promoter of the ISG 15 (20, 29).

provide evidence for the GAS as a second IFN-α-responsive element in the GBP gene promoter. Since, in this promoter, GAS lies next to the ISRE, the AAF may act together with ISGF-3 in IFN-α-induced transcription. We should be careful to note that the experiments with transfected constructs do not establish which sites or factors are most important in the endogenous gene. Furthermore, it is reasonable that only the genes with both a GAS and an ISRE could possibly respond to AAF. Finally, examination of additional genes with GAS sequences to determine whether GAS is always adjacent to an ISRE would help settle whether the two sites can act in independent fashion.

Kinase inhibitors distinguish the activation of AAF, GAF, and ISGF-3. In a previous study, we suggested that the signals from the IFN-α and IFN-γ receptors employed different intracellular proteins in signal transduction, based on differential effects of kinase inhibitors (23). H7, an in vitro inhibitor of several kinases, nearly abolished the IFN-γ-stimulated transcriptional induction of the GBP gene, leaving the response to IFN-α largely intact. 2-Aminopurine, which inhibits the double-stranded RNA-dependent kinase in vitro (11), also abolishes the GBP gene's transcriptional response to IFN-γ and reduces that to IFN-α by about twofold (23). We now tested the effects of these kinase inhibitors on the proteins that are involved in the transcriptional regulation of the GBP gene.

HeLa cells were treated with the kinase inhibitors for 10 min and then treated with either IFN-α for 30 min or IFN-γ for 4 h in the continued presence of the kinase inhibitors. More than 90% of GAF activity was blocked by H7, and virtually no GAF appeared in cells when 2-aminopurine was present during IFN-γ induction (Fig. 9). These data correlate well with the earlier transcriptional study showing that these two drugs block induction of the GBP gene by IFN-γ (23), and they provide physiological evidence for the importance of the GAF in the process of transcriptional activation in response to IFN-γ. In contrast to the GAF, the appearance of the AAF in response to IFN-α was totally unaffected by the presence of H7. However, 2-aminopurine blocked AAF activation by at least 90%. GAF and AAF are thus activated through different receptor-ligand interactions that act through intracellular proteins with different inhibitor sensitivities. These results also suggest that the approximately 50% inhibition of IFN-α-induced GBP transcription that we had reported earlier (23) might be due to the inhibition of AAF, assigning a possible role to this factor in the transcriptional induction of the endogenous GBP gene.

Since IFN-α also activates ISGF-3, the same extracts that were used to assay AAF and GAF in the exo III assay were now used to perform a mobility shift assay with a labeled ISRE oligonucleotide (20). Figure 10 shows that neither H7 nor 2-aminopurine caused significant changes in ISGF-3 activity in extracts from IFN-α-induced cells. Both inhibitors therefore apparently distinguish between intracellular signaling pathways that are rapidly activated in response to IFN-α treatment and independently cause the very similar kinetics of appearance of the two DNA-binding factors AAF and ISGF-3. Moreover, the lack of effect on ISGF-3 and the decrease in AAF brought about by 2-aminopurine again strengthens the assumption that inhibition of AAF might be responsible for the 50% drop in GBP gene transcription in response to the combination of IFN-α and 2-aminopurine.

DISCUSSION

Our initial studies on IFN-α-stimulated transcription were done with genes that are not responsive to IFN-γ (18, 20, 29). That work led to the definition of the ISRE as an IFN-α-responsive DNA element and of ISGF-3 as a major transcription factor in the IFN-α-induced stimulation of genes. Because of its rapid and protein synthesis-independent transcriptional response to IFN-γ, we used the GBP gene to study the immediate response to this cytokine (in spite of the fact that the GBP gene is also stimulated by IFN-α [5]). The GAF was detected as binding to the GBP gene promoter and, as noted earlier, rises rapidly in parallel with IFN-α-induced GBP transcription in fibroblasts and more slowly in parallel with the transcriptional response in HeLa cells (6). We concluded that ISGF-3 and GAF might be the two major factors governing the transcriptional response to IFNs. Therefore, any interactions known to occur between the two IFN types might depend on interactions between the intracellular events leading to the activation of ISGF-3 and GAF. This possibility was earlier found to be correct for ISGF-3; while this factor is activated only after IFN-α treatment, the levels of latent factor can be increased in HeLa cells through induction of its DNA-binding subunit by IFN-γ (22). With these results in mind, we tested for any effect of IFN-α on factors that bind to the GAS and detected AAF, an IFN-α-dependent factor that binds to the GAS and not the ISRE.

This result was somewhat surprising because ISGF-3 acting through the ISRE seemed sufficient to explain the

IFN-α inducibility of the GBP gene. All present data, including functional analyses, DNA binding, and affinity, fail to distinguish between AAF and GAF. However, the two factors are clearly activated through different receptors and have different longevity in induced cells, different protein synthesis requirements, and different inhibitor profiles. Their identical DNA-binding properties could result from a common DNA-binding subunit that acts in association with different accessory proteins. Such a speculation is especially tempting because of the multimeric composition of ISGF-3 (13, 17) and our preliminary biochemical results, which suggest that GAF might be composed of more than one protein (34a). A heterooligomeric structure which combines DNA binding with regulatory subunits is a property of several transcriptional activators (overview in reference 33). Examples include the HAP2/3/4 complex in yeast cells (26), the Fos/Jun heterodimer of AP-1 (27, 28), and, perhaps, the multiprotein complex mediating the serum response of the *fos* gene (34) and NFκB (1). Like AAF, GAF, and ISGF-3, all of these transcriptional activators mediate responses to external stimuli.

Inhibitors of protein kinases that act both in cell extracts and with purified enzymes affect the activation of AAF, GAF, and ISGF-3 differently. ISGF-3 appearance in response to IFN-α is not influenced by either H7 or 2-aminopurine; GAF activation is inhibited by both inhibitors, and AAF activation is blocked by 2-aminopurine but unaffected by H7. From all these results, it is clear that IFN-α can activate DNA-binding factors through at least two distinguishable pathways, one leading to AAF and the other to ISGF-3. These signaling paths would appear to employ different kinases or different kinase-substrate interactions. Their nature is at present unclear because the components that constitute the entire IFN-responsive signaling paths leading to transcriptional activation are still poorly characterized. Because of inhibition with staurosporine, which inhibits protein kinase C (PKC), among other kinases, it was suggested that PKC acts during IFN-α signaling (9, 30). However, more recently, cells in which PKC α, β, and γ are removed have been shown to respond normally to IFN-α (16a). Inhibitors of arachidonic acid metabolism have been shown to block IFN-α activation (14), but agonists of this purported second-messenger pathway are not capable of activating ISG transcription. Thus, while it seems likely that some kinase action will follow IFN attachment to the cell surface, we still suggest (see reference 19) that any participating kinase will be associated directly with a receptor recognition complex that involves the waiting cytoplasmic components of transcription factors. If this formulation is correct, the IFN-α receptor must be capable of recognizing two (at least) different sets of waiting proteins, one leading to ISGF-3 and the other to AAF.

Finally, we should note that the GBP gene is equipped with a formidable array of signal-responsive elements, all within a 30-bp region between −130 and −100 within its promoter. The two overlapping DNA elements ISRE and GAS can bind at least three different factors, AAF, GAF, and ISGF-3. The presence of two separate IFN-α-responsive activation mechanisms could mean that careful regulation of the concentration of this protein of unknown function may be important. We are therefore constructing cells in which the GBP gene is mutated to test its possible role in the IFN response.

REFERENCES

1. **Baeuerle, P. A., and D. Baltimore.** 1988. IκB, a specific inhibitor of the NFκB transcription factor. Science **242**:540–546.
2. **Caplen, H. S., and S. L. Gupta.** 1988. Differential regulation of a cellular gene by human interferon-α and interferon-γ. J. Biol. Chem. **263**:332–339.
3. **Celada, A., and R. Maki.** 1991. IFN-γ induces the expression of the genes for MHC class II I-Aβ and tumor necrosis factor through a protein kinase C-independent pathway. J. Immunol. **146**:114–120.
4. **Dale, T. C., A. M. Imam, I. M. Kerr, and G. R. Stark.** 1989. Rapid activation by interferon-α of a latent DNA-binding protein present in the cytoplasm of untreated cells. Proc. Natl. Acad. Sci. USA **86**:1203–1207.
5. **Decker, T., D. J. Lew, Y.-S. E. Cheng, D. Levy, and J. E. Darnell, Jr.** 1989. Interactions of α- and γ-interferon in the transcriptional regulation of the gene encoding a guanylate-binding protein. EMBO J. **8**:2009–2014.
6. **Decker, T., D. J. Lew, J. Mirkovitch, and J. E. Darnell, Jr.** Cytoplasmic activation of GAF, an IFN-γ-regulated DNA-binding factor. EMBO J., in press.
7. **De Maeyer, E., and J. De Maeyer-Guignard.** 1988. Interferons and other regulatory cytokines. John Wiley & Sons, Inc., New York.
8. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
9. **Fan, X.-D., M. Goldberg, and B. R. Bloom.** 1988. Interferon-γ-induced transcriptional activation is mediated by protein kinase C. Proc. Natl. Acad. Sci. USA **85**:5122–5125.
10. **Fan, X.-D., G. R. Stark, and B. R. Bloom.** 1989. Molecular cloning of a gene induced selectively by gamma interferon from human macrophage cell line U937. Mol. Cell. Biol. **9**:1922–1928.
11. **Farrell, P. J., K. Balkow, T. Hunt, and R. J. Jackson.** 1977. Phosphorylation of initiation factor eIF-2 and the control of reticulocyte synthesis. Cell **11**:187–200.
12. **Fried, A., and D. M. Crothers.** 1981. Equilibria and kinetics of lac repressor-operator interaction by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9**:6505–6525.
13. **Fu, X.-Y., D. S. Kessler, S. A. Veals, D. E. Levy, and J. E. Darnell, Jr.** 1990. ISGF-3, the transcriptional activator induced by IFN-α, consists of multiple interacting polypeptide chains. Proc. Natl. Acad. Sci. USA **87**:8555–8559.
14. **Hannigan, G. E., and B. R. G. Williams.** 1991. Signal transduction by IFN-α through archidonic acid metabolism. Science **251**:204–207.
15. **Hug, H. M., M. Costas, P. Staeheli, M. Aebi, and C. Weissmann.** 1988. Organization of the murine *Mx* gene and characterization of its interferon- and virus-inducible promoter. Mol. Cell. Biol. **8**:3065–3079.
16. **Kelly, J. M., C. S. Gilbert, G. R. Stark, and I. Kerr.** 1985. Differential regulation of interferon-induced mRNAs and c-myc by α- and γ-interferons. Eur. J. Biochem. **153**:367–371.

16a. **Kessler, D., and D. E. Levy.** Unpublished data.

17. **Kessler, D. S., S. A. Veals, X.-Y. Fu, and D. E. Levy.** 1990. Interferon-α regulates nuclear translocation and DNA binding affinity of ISGF-3, a multimeric transcriptional activator. Genes Dev. **4**:1753–1765.
18. **Larner, A. C., A. Chaudhuri, and J. E. Darnell.** 1986. Transcriptional induction by interferon. J. Biol. Chem. **261**:453–459.
19. **Levy, D. E., and J. E. Darnell.** 1990. Interferon-dependent transcriptional activation: signal transduction without second messenger involvement? New Biol. **2**:923–928.
20. **Levy, D. E., D. S. Kessler, R. Pine, N. Reich, and J. E. Darnell.** 1988. Interferon-induced nuclear factors that bind a shared promoter element correlate with positive and negative control. Genes Dev. **2**:383–393.
21. **Levy, D. E., D. S. Kessler, R. Pine, and J. E. Darnell.** 1989. Cytoplasmic activation of ISGF-3, the positive regulator of interferon-α-stimulated transcription, reconstituted *in vitro*. Genes Dev. **3**:1362–1371.
22. **Levy, D. E., D. J. Lew, T. Decker, D. S. Kessler, and J. E.**

Darnell, Jr. 1990. Synergistic interaction between interferon-α and interferon-γ through induced synthesis of one subunit of the transcription factor ISGF3. EMBO J. **9**:1105–1129.

22a. **Lew, D. J., and J. E. Darnell, Jr.** Unpublished data.

23. **Lew, D. J., T. Decker, and J. E. Darnell, Jr.** 1989. Alpha interferon and gamma interferon stimulate transcription of a single gene through different signal transduction pathways. Mol. Cell. Biol. **9**:5404–5411.

24. **Lew, D. J., T. Decker, I. Strehlow, and J. E. Darnell, Jr.** 1991. Overlapping elements mediate induction of the guanylate-binding protein gene promoter by alpha and gamma interferon. Mol. Cell. Biol. **11**:182–191.

25. **Luster, A. D., J. C. Unkeless, and J. V. Ravetch.** 1985. γ-Interferon transcriptionally regulates an early response gene containing homology to platelet proteins. Nature (London) **315**:672–676.

26. **Oleson, J. T., and L. Guarente.** 1990. The HAP-2 subunit of yeast CCAAT transcriptional activator contains adjacent domains for subunit association and DNA recognition: model for the HAP2/3/4 complex. Genes Dev. **4**:1714–1729.

27. **Rauscher, F. J., III, D. R. Cohen, T. Curran, T. J. Bos, P. J. Vogt, D. Bohman, R. Tjian, and B. R. Franza.** 1988. Fos-associated protein p39 is the product of the jun oncoprotein. Science **240**:1010–1016.

28. **Rauscher, F. J., III, P. J. Voulalas, B. R. Franza, and T. Curran.** 1988. Fos and Jun bind cooperatively to the AP-1 site: reconstitution *in vitro*. Genes Dev. **2**:1687–1699.

29. **Reich, N. C., B. Evans, D. E. Levy, D. E. Fahey, E. Knight, and J. E. Darnell.** 1987. Interferon-induced transcription of a gene encoding a 15KDa protein depends on an upstream enhancer element. Proc. Natl. Acad. Sci. USA **84**:6394–6398.

30. **Reich, N. C., and L. M. Pfeffer.** 1990. Evidence for involvement of protein kinase C in the cellular response to interferon-α. Proc. Natl. Acad. Sci. USA **87**:8761–8765.

31. **Reid, L. E., A. H. Brasnett, C. S. Gilbert, A. C. G. Porter, D. R. Gewert, G. R. Stark, and I. Kerr.** 1989. A single DNA response element can confer inducibility by both α- and γ-interferons. Proc. Natl. Acad. Sci. USA **86**:840–844.

32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

33. **Shaw, P. E.** 1990. Multicomponent transcription factor complexes: the exception or the rule? New Biol. **2**:111–118.

34. **Shaw, P. E., H. Schröter, and A. Nordheim.** 1989. The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human c-fos promoter. Cell **56**:563–572.

34a. **Shuai, A., T. Decker, and J. E. Darnell, Jr.** Unpublished data.

35. **Yan, C., P. B. Seghal, and I. Tamm.** 1989. Signal transduction pathways in the induction of the 2′-5′-oligoadenylate synthetase gene expression by interferon α/β. Proc. Natl. Acad. Sci. USA **86**:2243–2247.