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Tyrosine-phosphorylated Stat1 and Stat2 plus a 48-kDa protein all contact DNA in forming interferon-stimulated-gene factor 3

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ABSTRACT **Interferon α induction of transcription operates through interferon-stimulated-gene factor 3 (ISGF), a transcription factor two components of which are members of the newly characterized Stat family of transcription factors. Interferon α induces tyrosine phosphorylation of Stat1 and Stat2 proteins that associate and, together with a 48-kDa protein, form ISGF3. Evidence is presented that a heterodimer of Stat1 and Stat2 is present in ISGF3 and that Stat1 and the 48-kDa protein make precise contact, while Stat2 makes general contact, with the interferon-stimulated response element, the binding site of the ISGF3.**

Cytokine attachment to cell surface receptors triggers gene activation in the cell nucleus (1). Many of these extracellular polypeptides cause their intracellular changes through the Jak–Stat pathway (2). The Jak proteins are protein-tyrosine kinases associated with cell surface receptors that are activated by receptor occupation. The Stat proteins serve the dual function of signal transduction and activation of transcription. The first polypeptide ligand recognized to use this pathway was interferon α (IFN-α), which leads to activation of a nuclear DNA-binding complex called interferon-stimulated-gene factor 3 (ISGF3) (3, 4), which upon purification proved to contain four protein species, 113, 91, 84, and 48 kDa in size (5). The first three of these were the proteins that yielded sequence information establishing the sequence similarity in the Stat family (6, 7); it was also demonstrated that the 113-, 91-, and 84-kDa proteins (renamed Stat2, Stat1α, and Stat1β, respectively) were activated by phosphorylation on a single similarly located tyrosine (8–10). (The 91- and 84-kDa proteins differ only in a 38-aa carboxyl-terminal extension in the 91-kDa protein.) The 48-kDa protein, p48, is not a Stat family member but a member of a separate DNA-binding family (11, 12). By itself p48 protein has a low affinity for the interferon-stimulated response element (ISRE) but together with the other proteins forms a stable protein–DNA complex (11, 12). Early studies using UV crosslinking showed p48 protein to contact DNA, but results on DNA contacts by the other proteins were inconclusive (5, 11, 13).

In addition to the copurification of these proteins as ISGF3, antibodies against Stat2 coprecipitated Stat1α and Stat1β after IFN-α activation but not before, indicating a physical association (8). The conclusions from the biochemical experiments on the composition of ISGF3 were corroborated by somatic cell genetic experiments. The U3A cell line, which lacks Stat1α and -1β, is unresponsive to IFN-α and can be restored to IFN-α responsiveness and ISGF3 formation with an expression construct encoding either Stat1α or Stat1β (14). More recently, Stat2 has also been proved genetically to be required: U6A cells are nonresponsive to IFN-α, lack Stat2, and can be restored to IFN-α responsiveness and ISGF3 formation by a Stat2 expression construct (15).

In this paper we describe further biochemical experimentation on the composition of ISGF3 and the DNA contacts made by each of the proteins in the complex. We conclude that a heterodimer of Stat2 and either Stat1α or Stat1β plus p48, all contacting DNA within a 10-bp region, constitutes ISGF3.

MATERIALS AND METHODS

Cell Lines and Preparation, Fractionation, and Purification of HeLa Cell Extract. HeLa S3 (American Type Culture Collection) and U6A (kindly provided by S. Leung and G. Stark, Cleveland Clinic Foundation, Cleveland) (15) were maintained in Dulbecco's modified Eagle's medium plus 10% calf serum (HyClone). Cytoplasmic and nuclear extracts were prepared as described (4). Cytoplasmic extract of IFN-α-treated HeLa cells was concentrated with Centricon 30 filtration units (Amicon), and 2 ml of the concentrate (equivalent of 2.5×10^8 HeLa cells) was applied to a Pharmacia Sephacryl S300 column (100 cm × 2.5 cm) equilibrated with buffer A [20 mM Hepes, pH 7.9/100 mM KCl/1 mM EDTA/0.5 mM EGTA/0.5 mM phenylmethanesulfonyl fluoride/0.05% Nonidet P-40/10% (vol/vol) glycerol]. Fractionation was performed in the same buffer and after the first 50 ml (void volume, V_o) was discarded, about 100 fractions of 300 μl were collected. Molecular weight standards were run under the same conditions and their elution profiles were determined by protein–dye binding assay (Bio-Rad). The ISGF3 activity from HeLa cell nuclear extract was partially purified as described (5). Broad fractions were pooled, resulting in only about 2000-fold purification of ISGF3 activity. Peak fractions containing ISGF3 activity were used for protein–DNA crosslinking studies described.

Antibodies, Immunoprecipitation, and Western Blotting. Stat1n, -m, and -c as well as Stat2n and -m antibodies have been described (6, 7). Stat2c antibody was raised in rabbits immunized with a glutathione *S*-transferase–Stat2-(750–850) fusion protein. Immunoprecipitation and Western blotting of Stat proteins have been described (8). When required, the Western filters were stripped of previous antibody by incubation in 62.5 mM Tris Cl, pH 6.7/2% SDS/100 mM 2-mercaptoethanol at 50°C for 30 min.

UV Crosslinking of Proteins to DNA. Oligodeoxynucleotides corresponding to an ISRE from the ISG15 gene (16) were synthesized by modified phosphoramidite chemistry (Glen Research, Sterling, VA) on a DNA synthesizer (Applied Biosystems). In substituted oligodeoxynucleotides prepared for crosslinking studies we used 5-bromo-2′-deoxyuridylate for thymidylate, 5-bromo-2′-deoxycytidylate for 2′-deoxycytidylate, and 8-bromo-2′-deoxyguanylate for 2′-deoxyguanylate. Double-stranded oligonucleotides were produced by annealing a nonsubstituted and a substituted oligonucleotide. Oligonucleotides were designed such that only the substituted strand would be labeled when filled in with ^{32}P-labeled nucleotides.

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Abbreviations: IFN, interferon; ISGF3, IFN-stimulated-gene factor 3; ISRE, IFN-stimulated response element.

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These labeled double-stranded oligonucleotides were used in an electrophoretic mobility-shift assay (EMSA) with a partially purified fraction of HeLa nuclear extract (see above). A scale-up EMSA reaction was fractionated by electrophoresis in a 4.5% polyacrylamide gel. After electrophoresis, wet gels were wrapped in SaranWrap; to crosslink labeled oligonucleotide to protein the gels were exposed to long-wave UV light for 60 min and then exposed to x-ray film to locate the position of ISGF3. The ISGF3 bands were cut out, equilibrated in 0.4 M Tris Cl, pH 6.6/2% SDS/100 mM 2-merceptoethenol, and loaded onto an SDS/polyacrylamide gel (13-cm 4.5% resolving gel and 4-cm 4% stacking gel cast between 20 cm × 20 cm plates). Wells were flushed with loading buffer and electrophoresis was performed at 50 V for 20 hr, after which the gels were fixed in 20% methanol/7% acetic acid for 30 min at room temperature, dried, and exposed to x-ray film at −80°C with an intensifying screen.

RESULTS

IFN-α Induces Tyrosine Phosphorylation and Association of Stat1 and Stat2 Proteins. Treatment of IFN-α-responsive cells leads to tyrosine phosphorylation of Stat1 and Stat2 (8). An antibody against Stat2 coprecipitated Stat1, but only from cells that had been treated with IFN-α (8, 17). Previous attempts with available antisera against Stat1 did not coprecipitate the putative Stat1/Stat2 complex. We have now extended this early experiment with samples of a number of different antibodies raised against three different regions of each Stat protein (Fig. 1*A*). These were used to precipitate Stat proteins from HeLa cell lysates prepared from untreated cells or from cells treated with IFN-α (Fig. 1*B*). Each of these antibodies precipitated its respective Stat protein from extracts of untreated cells, as expected (Fig. 1, c lanes). In addition, although there were distinct quantitative differences, each of the antibodies also precipitated the other Stat protein from extracts of IFN-α-treated cells but not untreated cells (Fig. 1, α lanes). To rule out the possibility that Stat2 antibodies crossreacted with Stat1 protein after but not before it was phosphorylated on tyrosine, we immunoprecipitated Stat1 and Stat2 proteins from IFN-γ treated cell extracts, a situation where Stat1 but not Stat2 is phosphorylated. Even though IFN-γ induces tyrosine phosphorylation of Stat1 at the same site as IFN-α (18), Stat2 antibodies failed to precipitate Stat1 from extracts of IFN-γ-treated cells, and Stat1 antibodies precipitated only Stat1 from these extracts (data not shown). These results established the usefulness of immunoprecipitation assay for the study of protein interaction during ISGF3 formation and demonstrated that Stat1 and Stat2 form a complex after IFN-α treatment.

Phosphorylated Stat1/Stat2 Complex That Can Participate in ISGF3 Formation. To examine the stability and properties of the Stat1/Stat2 complex formed after IFN-α treatment, we subjected extracts of IFN-α-treated cells to gel filtration on a Sephacryl S300 column. As tested by immunoblots of total protein in the column fractions (Fig. 2*A*), the majority of Stat1 (91-kDa protein) was eluted from the gel filtration column later than Stat2 (113-kD protein) (fractions 70–85 compared with fractions 66–75), consistent with its smaller mass (738 aa for Stat1α compared with 851 aa for Stat2). The distribution of tyrosine-phosphorylated Stat1 and Stat2 was then assayed by immunoprecipitation with a mixture of Stat1c and Stat2m antibodies, followed by gel electrophoresis and phosphotyrosine antibody exposure (Fig. 2*B*). Almost all of the Stat1 and Stat2 phosphoproteins emerged from the gel filtration column considerably ahead of (i.e., as if they were larger) the unphosphorylated form of the proteins. Thus, all phospho-Stat1 and -Stat2 were in apparent complexes. While there was phospho-Stat1 together with phospho-Stat2 in fractions 50–69, there was more phospho-Stat1 in fractions 62–71, which probably represented the Stat1/Stat1 complex (as will be seen from Fig. 3). (Comparisons of relative intensity of Stat1 and Stat2 should not be taken as quantitative, because the phosphotyrosine antibody used in this experiment, PY20 (Upstate Biotechnology, Lake Placid, NY), reacts less well with phospho-Stat2 than with phospho-Stat1.)

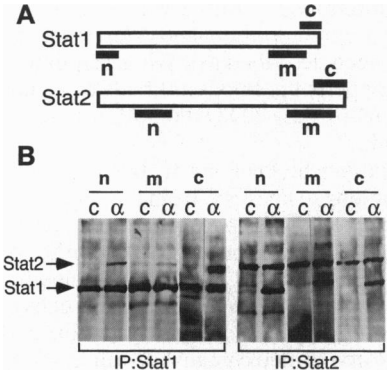

FIG. 1. Immunoprecipitation of Stat1 and Stat2 from HeLa cells. (*A*) Regions of Stat proteins used to raise antibodies. Precise boundaries have been described (6, 7). (*B*) Western blot analysis of Stat1 and Stat2 in immunoprecipitates of untreated (lanes c) or IFN-α-treated (lanes α) HeLa cell lysates. Lysates were immunoprecipitated (IP) with antibodies (**n**, **m**, **c**) to Stat1 (*Left*) or Stat2 (*Right*) fractionated by SDS/7.5% PAGE and Western blotted with Stat1c and Stat2m antibodies to detect Stat1 and Stat2 proteins.

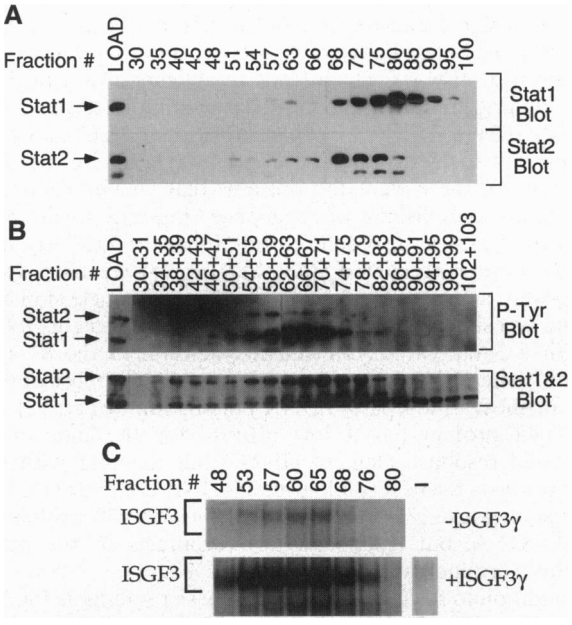

FIG. 2. Fractionation of Stat1 and Stat2 proteins by gel filtration chromatography. Cytoplasmic extract from HeLa cells was fractionated on a Sephacryl S300 column. (*A*) Western blot analysis of column fractions. Six microliters of each column fraction was Western blotted and probed first with Stat1c (Stat1 blot) antibody and then with Stat2m antibody (Stat2 blot). LOAD, a portion of the sample before loading on the gel filtration column. Positions of Stat1 and Stat2 are shown. (*B*) Immunoprecipitation and detection of tyrosine-phosphorylated proteins in column fractions. Two of the indicated fractions were pooled and immunoprecipitated with a mixture of Stat1c and Stat2m antibodies. After blotting, the filters were first probed with anti-phosphotyrosine antibody PY20 (P-Tyr blot) and then probed with a mixture of Stat1c and Stat2m antibodies (Stat1&2 blot). (*C*) ISGF3 activity in column fractions. Six microliters of each fraction was subjected to EMSA with a ^{32}P-labeled ISRE as probe with (+ISGF3γ) or without (−ISGF3γ) the addition of HeLa cytoplasmic extract as a source of p48 (4). Lane (−), reaction with ISGF3γ extract only.

To test the functionality in DNA binding of the associated Stat1/Stat2 proteins, samples of column fractions 48–80 were exposed to a labeled DNA probe containing a high-affinity ISRE binding site from the ISG15 gene (19). The binding reaction mixtures were either supplemented or not with a protein fraction containing p48 (labeled ISGF3γ in Fig. 2*C*), a necessary component of ISGF3 (5, 12). Electrophoresis of the protein–DNA complexes then identified fractions 48–68 as the principal fractions capable of forming ISGF3, but only when these fractions were supplemented with a source of p48 (Fig. 2*C*). Thus the phosphorylated Stat1 and Stat2 proteins capable of forming ISGF3 clearly separated from the mass of the nonphosphorylated Stat protein.

Heterodimer of Stat1 and Stat2 in Association with p48 Assembles on DNA to Form ISGF3. Earlier work identified by sedimentation analysis a Stat1/Stat1 homodimer in cells treated with IFN-γ (18), and we wished to test for a Stat1/Stat2 heterodimer in IFN-α-treated cells. However, we failed to recover Stat1/Stat2 protein that was still associated (C. M. Horvath and J.E.D., unpublished observations). This failure accords with early experiments in which, in the absence of DNA, p48 and the larger proteins that participated in ISGF3 were found in two distinct peaks of ≈50 and ≈100 kDa (11, 13). We then used gel filtration chromatography to test the approximate size of the IFN-α-induced Stat1/Stat2 complexes. A partially purified nuclear protein preparation from IFN-α-treated cells (as described in *Materials and Methods*) was passed over a Sephacryl S300 column and tested as in Fig. 2 (by adding a source of p48) for ISGF3 binding activity. In addition, a second DNA binding site, the GAS oligonucleotide, which binds Stat1/Stat1 homodimers, was also used to assay DNA-binding activity. The fractions competent to produce ISGF3 were eluted slightly earlier than the fractions competent to bind the GAS element (Fig. 3*A*). The IFN-α-induced factor that binds the GAS element was previously called AAF (20) and is indistinguishable in behavior from the IFN-α-induced factor GAF, which is a homodimer of Stat1 (18). Thus the Stat1/Stat2 complex that can participate in forming ISGF3 is only slightly larger by exclusion chromatography than the 180-kDa homodimer of Stat1. To rule out the possibility that fractions containing the ISGF3 activity contained homodimers of Stat1 and homodimers of Stat2, the proteins from fractions 12–36 of Fig. 3*A* were bound to and eluted from a phosphocellulose (Whatman P11) column. This resulted in separation of GAF and ISGF3 activities (Fig. 3*B*). We therefore conclude that the ISGF3 precursor in fractions 12–28 in Fig. 3*A* is a Stat1/Stat2 heterodimer. [We used conventional molecular size markers to attempt a size estimation on these column fractions but obtained an unreasonable result: the GAF DNA-binding activity, known to be a Stat1 homodimer (180 kDa), peaked in fraction 24–28, the same fractions as ferritin, which is 440 kDa. Earlier sedimentation analysis with the entire ISGF3 complex, including DNA, indicated a size for ISGF3 of about 250 kDa, which is smaller than ferritin (11). Thus we believe the standard molecular size markers to be inaccurate in gel filtration analysis of the Stat protein complexes. In other recent experiments (U. Vinkemeier and J.E.D., unpublished observations), highly purified Stat1α monomers have also been found not to be eluted at ≈100 kDa in gel filtration as are the globular protein markers.]

To demonstrate an association between the phosphorylated Stat1 and Stat2 proteins that survived gel filtration, samples from fraction 63 of the Sephacryl S300 column (Fig. 3*A*) and from fraction 31 of the P11 column (Fig. 3*B*) were subjected to precipitation with Stat1c or Stat2m antibodies, followed by immunoblotting using anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology). Fraction 63, which gave no ISGF3 or GAF activity, contained no phosphoprotein in the precipitates (Fig. 3*C Left*, lanes 1 and 2) whereas fraction 31, which gave ISGF3 activity, did (Fig. 3*C Left*, lanes 3 and 4). With the anti-phosphotyrosine antibody 4G10, approximately equally strong Stat1 and Stat2 signals were observed [the stronger Stat1 signal in lane 4, precipitated with Stat1c antiserum, was presumably due to Stat1 homodimers, as indicated by GAF activity present in fraction 31 (Fig. 3*B*), which would not be precipitated by Stat2m antiserum]. Stat2m antiserum precip-

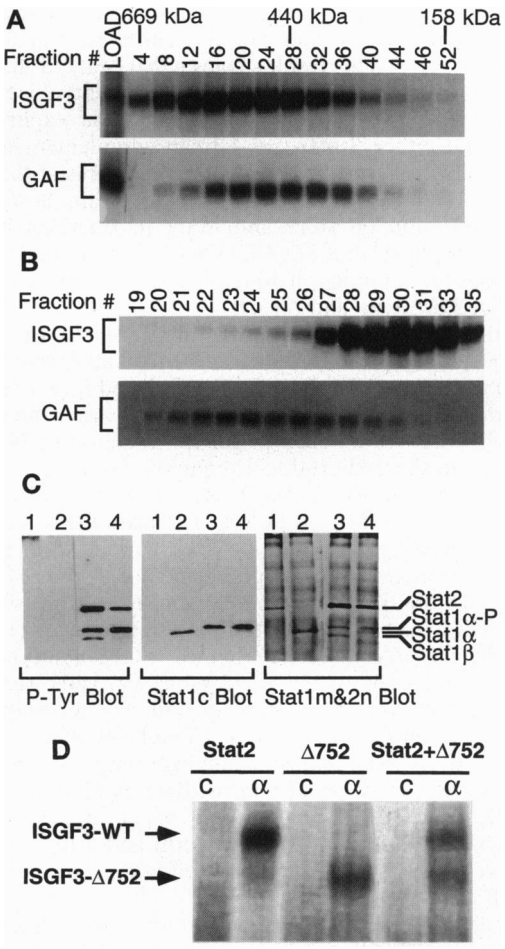

FIG. 3. Molecular size of the ISGF3 complex. Nuclear extract from IFN-α-treated HeLa cells was partially purified as described in *Materials and Methods* before fractionation on a Sephacryl S300 gel filtration column. The same column was used to determine the elution profile of molecular size standards. (*A*) Elution profile of the ISGF3 and GAF (AAF) activity in column fractions. The ISGF3 and GAF activity in the column fractions was examined by EMSA using ^{32}P-labeled ISRE and GAS oligonucleotides as probes, respectively. LOAD, EMSA with a portion of the sample before loading onto the Sephacryl column. Positions of size markers are indicated at the top: thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa); position of bovine serum albumin (65 kDa) is not shown here. (*B*) Separation of the ISGF3 and GAF activities. Fractions 8–40 of *A* were pooled and bound to a phosphocellulose (Whatman P11) column. After removal of the unbound proteins, the bound proteins were eluted from the column with a linear gradient of 0.15–1.0 M KCl and analyzed by EMSA for ISGF3 and GAF activities. (*C*) Coimmunoprecipitation of tyrosine-phosphorylated Stat proteins from column fractions. Stat1 and Stat2 were immunoprecipitated from fraction 63 of the Sephacryl S300 column (lanes 1 and 2) or fraction 31 of the P11 column (lanes 3 and 4) with Stat1c antibody (lanes 2 and 4) or Stat2m antibody (lanes 1 and 3) and, after Western blotting, probed first with anti-phosphotyrosine antibody 4G10 (P-Tyr blot), then with Stat1c antibody (Stat1c blot), and finally with a mixture of Stat1m and Stat2n antibodies (Stat1m & 2n blot). Stat1α-P, phosphorylated Stat1α. (*D*) The ISGF3 activity in cell lines expressing Stat2 (Stat2), a truncated Stat2 (Δ752), or both (Stat2+Δ752). Whole cell extracts (17) from cells left untreated (lanes c) or treated with IFN-α (lanes α) for 45 min were subjected to EMSA with the ISRE as probe.

itated all three proteins, Stat2, Stat1α, and Stat1β, while the Stat1c antisera (which does not recognize Stat1β) did not precipitate Stat1β. This result suggests that Stat1/Stat2 complexes, presumably dimeric, can contain only Stat1α or Stat1β, which accords with the somatic cell genetic experiments showing that either Stat1α or -1β can complement mutant U3A cells in IFN-α responses (14). Western blots of the same filters followed the experiments of Fig. 1 and again showed lack of coprecipitation of Stat1 and Stat2 in fractions lacking tyrosine-phosphorylated Stat1 and Stat2 and coprecipitation in fractions containing tyrosine-phosphorylated Stat1 and Stat2 (Fig. 3*C Center* and *Right*).

A further test of the composition of the Stat1/Stat2 interacting species that participates in ISGF3 formation was carried out with a truncated Stat2 protein introduced into a cell line (U6) that otherwise lacks Stat2 (15). We had earlier shown that intermediates could be seen in gel shifts migrating between long/long and short/short dimers composed of long and short versions of activated Stat1 (18). This shorter version (Δ752) of Stat2 is 752 aa instead of 851 aa long. Extracts of IFN-α treated U6 cells expressing the wild-type Stat2 produced an IFN-α-dependent protein–DNA complex identical to the ISGF3 in normal cells (ISGF3-WT), whereas the cells expressing the truncated Stat2 produced a faster migrating complex (ISGF3-Δ752) (Fig. 3*D*). As a test of whether ISGF3 could contain more than one copy of the Stat2 molecule, cells were also transfected with vectors expressing both the wild-type and truncated proteins (Stat2+Δ752). Complexes that migrated like either the slower or faster moving complexes were observed, but no complexes of intermediate mobility were seen (Fig. 3*D*, Stat2+Δ752 lanes). This result argues that no complexes containing both a short form and a long form of Stat2 were formed, again consistent with one copy of Stat2 in the ISGF3 complex.

From all of these studies we consider it most likely that ISGF3 consists of one molecule of phosphorylated Stat1, one of phosphorylated Stat2, and one of p48. This molecular size of about 230 kDa accords well with the original sedimentation size of the ISGF3 protein–DNA complex (DNA of 20 kDa), which was stable to sedimentation and gave an estimated size of 250–400 kDa.

Stat1/Stat2 Heterodimer and ISGF3γ (p48) Bind to Distinct Sites on ISRE. Knowing that three proteins are part of ISGF3, we next turned to a reexamination of contact points on the DNA, using a much more extensive series of crosslinking experiments than had been carried out earlier. We synthesized a series of modified oligodeoxynucleotides corresponding to an ISRE from the ISG15 gene (Fig. 4*A* and ref. 19). Substitution of bases that promote UV crosslinking to bound protein was carried out for each of 14 different sites (11 on one strand, 3 on the other) which have earlier been examined by mutagenesis for participation in ISRE function (21). We replaced thymidine with bromodeoxyuridine, guanosine with bromodeoxyguanosine, and cytidine with bromodeoxycitidine. Exposure of these different oligonucleotides to partially purified ISGF3 preparations from IFN-α-activated cells was followed by preparative gel-shift separation, UV crosslinking, and identification of the now crosslinked protein–DNA complexes by an ISGF3 marker, followed by SDS/PAGE analysis of crosslinked products. Sizing of crosslinked products was carried out in the presence of the attached oligonucleotides; as expected, the 48-kDa, 91- and 84-kDa, and 113-kDa proteins migrated somewhat more slowly, with nominal molecular masses of about 60 kDa, 120 kDa, and 130 kDa, respectively.

Very clear results were obtained with substitutions of the thymidines that occur in two triplets, $T^{90}T^{91}T^{92}$ and $T^{96}T^{97}T^{98}$ [Fig. 4*B*; the numbers correspond to positions upstream from the RNA start site in the ISG15 gene (19)]. All of the thymidine residues have been found to be required for DNA binding and transactivation in earlier work (21). The two Stat1 proteins, α and β (based on the approximate size of 118 or 122 kDa), crosslinked strongly to T^{91}, very weakly to T^{90} and T^{92}, and essentially not at all to the T^{96}, T^{97}, and T^{98}. In addition, Stat1α and -1β also were not crosslinked to any of the C- or G-substituted oligonucleotides. p48 interacted strongly with the substitutions at T^{97} and T^{98} and to a lesser extent with virtually every substituted base (Fig. 4 *B* and *C*). Because of the strength of the signal, we interpret the contacts of p48 at T^{97} and T^{98} as specific and conclude that perhaps this protein has a general DNA-binding ability as well as a specific one.

While the thymidine substitutions did not give any indication of Stat2 binding, we did observe weaker crosslinking signals of Stat2 with G- and C-substituted oligonucleotides (Fig. 4*C*). (The exposure time for Fig. 4*C* was three times that of Fig. 4*B*). The strongest Stat2 crosslinking occurred to G^{95}, with some possible specific interaction with G^{99}. Both these sites are also among those shown by mutagenesis to be required for ISGF3 binding and for function in transfections (21). While the results were clear in identifying specific base contacts for Stat1

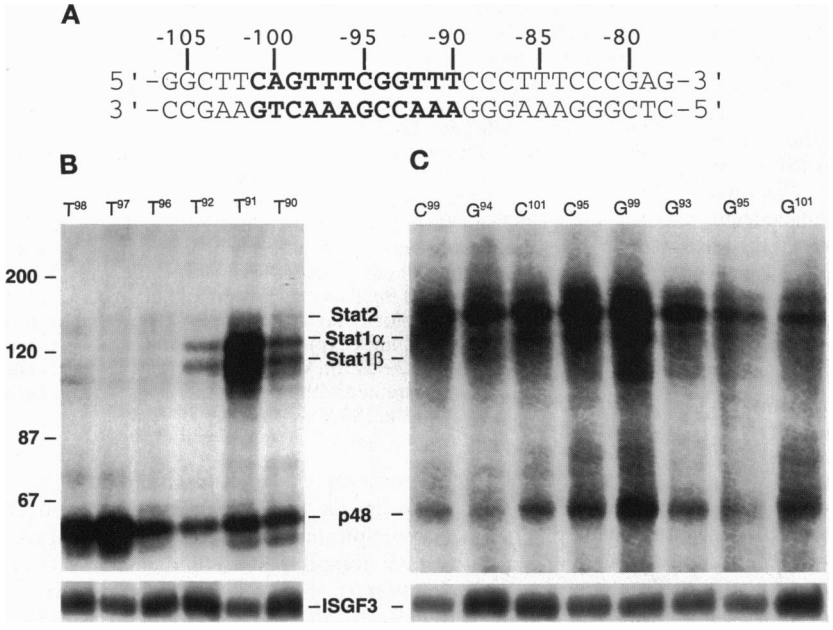

FIG. 4. UV crosslinking of the ISGF3 proteins to the DNA. ISRE-containing oligonucleotides with substitution at only one position were ^{32}P-labeled and used to perform EMSA, gels were exposed to UV crosslinking, and the ISGF3 bands were identified and then resolved by SDS/PAGE. Positions of the appropriately sized proteins were identified by autoradiography. (*A*) ISRE sequence of the ISG15 gene used in this assay; numbering is as described (21). ISRE is shown in bold. (*B*) Crosslinking of bromodeoxyuridine-substituted ISREs to ISGF3 proteins. (*C*) Crosslinking of bromodeoxyguanosine- or bromodeoxycytidine-substituted ISREs to ISGF3 proteins. Superscript numbers indicate the position of the substitution in the ISRE. *B* and *C Lower* show the ISGF3 bands for each of the lanes above that were cut out after UV crosslinking of ISGF3 proteins to the ISREs for fractionation by SDS/PAGE.

and p48 presumably indicating a strong interaction, the Stat2 interactions were definitely less distinct.

DISCUSSION

This work is concerned with a more precise definition of the composition of the multiprotein DNA-binding factor ISGF3—known to contain Stat1 (α or β), Stat2, and p48—and the contacts made by these proteins with their cognate DNA sequence element, the ISRE (5, 6).

Several results indicate that after IFN-α treatment, phospho-Stat1 and -2 form a heterodimer unassociated with p48 until DNA binding. Further, all three proteins make DNA contact within a 9-bp region, TTTCGGTTT, that differs from the palindromic GAS site in which Stat1 homodimers bind (TTNCNNNAA). (*i*) Antisera to each of three portions of each Stat protein (1 and 2) coprecipitated both Stat1 and Stat2 from cell extracts of IFN-α-treated but not untreated cells (Fig. 1). (*ii*) Gel filtration chromatography of partially purified ISGF3 proteins showed that the phosphorylated Stat proteins separated from the nonphosphorylated proteins as if they were of larger mass. In the fractions with phosphorylated protein, but not in the fractions with nonphosphorylated protein, single antibody (Stat1 or -2) precipitation resulted in coprecipitation of Stat1 and Stat2 (Figs. 2 and 3). (*iii*) The Stat1/Stat2 complex separated from the majority of p48, so that to reconstitute ISGF3, the Stat1/Stat2 complex required p48 addition (Fig. 2).

The size of the Stat1/Stat2 complex has not been directly determined because of the limited amount of highly purified active protein available from treated cells with which to perform protein crosslinking experiments and the relative instability of the presumed dimeric complex during zonal sedimentation. However, the elution profile of the Stat1/Stat2 complex in gel filtration suggests that it is only slightly larger than the Stat1/Stat1 homodimer (Fig. 3). Another type of experiment also showed that ISGF3 bound to DNA very probably contained one Stat2 molecule. In ISGF3, an 851-aa Stat2 can be replaced by a 752-aa Stat2 with the result that a single faster migrating ISGF3 complex is formed. No intermediate-sized ISGF3 was formed, indicating that Stat2 is not present in more than one copy in ISGF3 bound to DNA. Stat1c antibody, which recognizes only Stat1α, did not precipitate Stat1β, eliminating the possibility of a Stat1α and -β dimer in the ISGF3. Together, the evidence suggests that the Stat1/Stat2 complex that forms part of ISGF3 is a dimer. No stable contact with p48 occurs off the DNA, and we have no definitive evidence of the stoichiometry of p48 in the DNA complex. It could be a single molecule, in conformity with the approximate equimolarity in the originally purified preparations, but it could also possibly be a dimer.

The original crosslinking studies between ISGF3 and the ISRE showed definite evidence of p48 contact with DNA but were inconclusive about the possibility of contact by the larger proteins in the complex (11, 13). In the early experiments only the T^{98} residue was substituted (13). In the present experiments definite evidence of specific contact is now provided for p48 on at least two residues, T^{98} and T^{97}, and for Stat1α and -1β chiefly at residue T^{91}. Thus, as judged by crosslinking, the directly repeated two thymidine triplets of the ISRE, separated by three residues, contain the major contacts for p48 and Stat1 (α or β equally). These directly repeated three thymidine residues are the most characteristic feature of ISREs (2) and are required for protein binding and transactivation (21). Contact between the thymidines on deoxycytidine residue C^{95} by Stat2 was observed, though with lower crosslinking efficiency. The weak contacts with C^{95} are in accord with methylation interference experiments showing that methylation at this site blocked DNA binding by ISGF3 (16). Since the bromo derivatives used in these reactions crosslink in the major groove of DNA, we infer that the proteins make contacts in the major groove.

At present the ISGF3 complex is the only identified Stat protein complex involving a second class of proteins, and the conserved ISRE sequence departs from the palindromic sequence, TTNCNNNAA, that has been found as the core sequence of many natural binding sites for Stat1, -3, -4, -5, and -6, all of which can bind apparently as dimers (18, 22, 23). It is possible that the TTT sequence contacted by Stat1 in the ISRE conforms to half-site binding by this protein, which could be stabilized by either Stat2 or p48. The Stat2 that is part of the proposed dimer may be incapable of binding specifically to DNA but could be stabilized by the neighboring p48 binding to the left half of the ISRE sequence. This would predict a contact between Stat2 and p48. Protein crosslinking studies with highly purified, phosphorylated protein should be possible now with proteins obtained from baculovirus cultures expressing both Jak and Stat proteins and may shed light on these possibilities if ISGF3 can be fully reconstituted from such sources.

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