Several distinct type I interferon (IFN)-inducible STAT2-containing complexes have been identified. For the IFN-stimulated gene factor 3 (ISGF3), STAT1 and IRF-9 mediate IFN-stimulated response element (ISRE) binding, whereas STAT2 provides a potent transactivation domain. ISGF3-independent STAT2-containing complexes, specifically STAT2:1 and STAT2:3, bind a γ-activated sequence (GAS)-like element, yet the contribution of each STAT to DNA binding is unknown. Moreover, the contribution of these ISGF3-independent STAT2-containing complexes to IFN-inducible responses is not defined. Accordingly, we generated mutant cDNAs, targeting the DNA-binding domain in STAT2. These cDNAs were introduced by transfection into U6A cells lacking STAT2, resulting in a panel of cell lines expressing mutant STAT2 proteins. Studies assessed the sensitivity of U6A cells reconstituted with intact STAT2 (U6A-2) and cells expressing mutant STAT2s (U6A-2E426A,E427A (EE-AA), U6A-2V453I, U6A-2V454I, U6A-2V454A, U6A-2V453I,V454I(VV-II), U6A-2N458A) to IFN-inducible responses. Our data reveal that none of the mutations in the STAT2 DNA-binding domain affected IFN-inducible ISGF3 activation, and only the VV-II mutation restricted antiviral and growth inhibitory responses to IFN. Indeed, U6A-2VV-II cells are refractory to these IFN-inducible biological activities and also exhibit impaired IFN-inducible GAS-driven transcriptional activation and subsequent gene expression. Chromatin immunoprecipitation assays revealed that the VV-II mutation in STAT2 does not abrogate, but reduces the DNA binding activity of STAT2:1 heterodimers. Taken together, these data suggest a role for the conserved DNA-binding domain of STAT2 specific to the activity of ISGF3-independent STAT2-containing complexes.

Type I interferons (IFNs)1 elicit antiviral, antiproliferative, and immunomodulatory effects in target cells by activating specific, cognate cell surface receptors (1–3). This results in the activation of the receptor-associated Janus kinases (Jaks), Tyk2 and Jak1, the phosphorylation of multiple signaling elements, and the engagement of numerous signaling cascades, including signal transducer and activator of transcription (STAT) pathways (4–6). The Jak-STAT pathways are essential for the transcriptional activation of many IFN-stimulated genes. In particular, STAT2 is a critical component of IFN signaling (7, 8). IFN-inducible activation of STAT2 leads to the formation of two distinct types of STAT2-containing DNA binding complexes: IFN-stimulated gene factor 3 (ISGF3) and ISGF3-independent STAT2:1 and STAT2:3 heterodimers (9–11). ISGF3-independent STAT2-containing complexes translocate into the nucleus and bind γ-activated sequence (GAS)-like palindromic IFN-response element (pIRE) (10, 12). Alternately, STAT2:1 heterodimers may associate with the DNA-binding adaptor protein, IFN regulatory factor 9 (IRF-9), to form the ISGF3 complex that binds the IFN-stimulated response element (ISRE) (1, 13). Together, ISGF3 and ISGF3-independent STAT2-containing complexes mediate transcriptional activation of IFN-sensitive genes (ISGs), including double-stranded RNA-activated protein kinase (PKR), 2′,5′-oligoadenylate (OAS), and IRF-1, involved in mediating the biologic effects of IFNs (10, 14–16).

Within the ISGF3 complex, STAT2 contributes its potent transcriptional activation domain, whereas STAT1 and IRF-9 mediate DNA binding (17–19). The carboxyl-terminal transactivation domain of STAT2 also contributes to the transcriptional activation potential of ISGF3-independent complexes (10). However, the role of STAT2 in ISGF3-independent STAT2:1 heterodimers is not fully defined and the contribution of each STAT protein to DNA binding remains unknown.

In earlier studies we described the preferential binding of STAT2:1 heterodimers to a palindromic GAS-like sequence, suggesting that within this complex, STAT2 contributes to sequence-specific DNA binding activity (12). Indeed, several residues in STAT1s that contribute to DNA binding, namely residues corresponding to arginine 378 (Arg378), valine-threonine (Val426-Thr427), histidine 431 (His431) and asparagine 460 (Asn460) in STAT1, glutamates 434 and 435 (Glu434-Glu435) and valines 461 and 462 (Val461_Val462) in STAT3 and valines 466 and 467 (Val466_Val467) in STAT5, are conserved in STAT2 (20–23). Moreover, the crystal structures of STAT1:1 and STAT3:3 homodimers bound to DNA revealed that each STAT protein interacts with DNA on opposite sides of the double helix (23, 24). Taken together, these data suggest that, in ISGF3-independent STAT2-containing complexes, STAT2 may contribute to DNA binding by virtue of its putative DNA-binding domain.

In the present study we provide evidence that IFN-inducible ISGF3-independent STAT2-containing complexes contribute to IFN-inducible biologic responses in target cells. Specifically, we show that in cells where IFN-inducible ISGF3 activation is...
intact, it is possible to disrupt IFN-inducible transcriptional activation and ISG expression mediated by GAS-like gene elements, effected by STAT2:1-DNA interactions, resulting in blunted antiviral and antiproliferative responses. Using a panel of STAT2 mutants, we provide evidence that specific residues in the putative DNA-binding domain of STAT2 influence STAT2:1-DNA binding activity and transcriptional activation of ISGs.

MATERIALS AND METHODS

Cells and Reagents—Human fibroblasts 2TGH and U6A were obtained from G. Stark (Cleveland Clinic Foundation, Cleveland, OH). Cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen), supplemented with 10% fetal calf serum (HyClone), 100 units/ml penicillin, 100 mg/ml streptomycin (Invitrogen), and 250 μg/ml hypromycin B (Calbiochem), except in the antiviral assays where Dulbecco’s modified Eagle’s medium containing 2% fetal calf serum was used. Human recombinant IFN-alfacon-1 (IFN alfacon-1, specific activity, 3.0 × 10^9 units/ml) was provided by L. Blatt (Intermune, Brisbane, CA). Antibodies against phospho-STAT2 and phospho-STAT1 were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against STAT2 (C-20) and STAT1 (C-136) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against IRF-9 was kindly provided by D. B. Boles (University of Wisconsin, Madison, WI). Primers used: E426A, E427A (5′-CGGTTGATTATTTCCGCCATGAACAGCTCTC and 5′-GAAATAATAATAATAGGGAGGGTGTCCGTTTTC), N458A (5′-GAAATAATAATAATAGGGAGGGTGTCCGTTTTC), V453I, V454I (5′-TGGCGATTATTTCCAACATGAA and 5′-CAG and pIRE, 5′-GGAACAGCGGAGGGTGTCCGTTTTC), V456E, V456I, V456M (5′-TGGCGATTATTTCCAACATGAA and 5′-CAG and pIRE, 5′-GGAACAGCGGAGGGTGTCCGTTTTC), and pIRE (5′-GGAACAGCGGAGGGTGTCCGTTTTC). Genomic DNA harvested from 2TGH cells and water was used as positive and negative PCR controls, respectively. PCR products were resolved in a 1.2% agarose gel and visualized using a Gel Doc apparatus (Bio-Rad).

RNA Preparation, Complementary DNA Synthesis, and Real-time PCR—To harvest RNA, cells were either left untreated or treated with 5 ng/ml IFN alfacon-1 for 6 h at 37 °C. Cells were then lysed and homogenized using Qiazol QIA-shredder columns and RNA isolation was performed using the Qiagen RNeasy mini kit according to the manufacturer’s protocol. The complementary DNA (cDNA) was synthesized using 1 μg of RNA in the presence of random primers and avian myeloblastosis virus reverse transcriptase for 1 h at 42 °C (Promega).

Reaction components for real-time PCR were obtained from the LightCycler® FastStart Plus DNA Master SYBR Green I kit (Roche) and Light Cycler software (Roche) and LightCycler® Quantitation Software were used for all reactions. The PCR was performed in a final volume of 20 μl containing 0.5 μM of each primer and 5 μl of template cDNA (concentration 100 ng/μl). The following primer sets were used: 6–16 (5′-TAAAGAAAGGTTGCCTGGGAGCC-5′ and 5′-GGACGCCGGCATGAAAGGCT-3′), ISG15 (5′-TCTCTGTTGAGAATACACCCAG-3′ and 5′-GTTCTAGCGGCTCGCTAGTCTTGA-3′), IRF-1 (5′-TCTTACCCTCTCCACGCTGGAAGC-3′ and 5′-GTCGACCTTCTCTATCAC-3′), and IRF-3 (5′-GTTGACATCCTTCTGGGAGT-3′ and 5′-ACTGGCATCGTGATGGAC-3′). Standard curves were established for each primer set and both reference (β-actin) and target reactions were performed for each sample.

RESULTS

Mutational Analysis of Conserved DNA-binding Domain of STAT2—The DNA-binding activity of STAT1 has been characterized and mutagenesis studies have identified residues in STAT1 that are critical for DNA binding activity (22, 23, 34). We undertook a comprehensive analysis of the crystal structure of STAT1 bound to DNA, using molecular modeling and visualization software (SYBYL, version 6.9, Tripos Inc.). Using SYBYL, we imported the STAT1 RCSB Protein Data Bank coordinates (Protein Data Bank code 1BF5) and rendered the protein to highlight the DNA-binding domain and examine key residues involved in mediating DNA binding. This approach allowed us to identify several residues that appear crucial for mediating DNA binding and for maintaining the structure of the DNA-binding domain (Fig. 1). Specifically, Glu258 lies in the long loop between β8 and β9, that interacts with the minor groove and makes phosphate contacts with the major groove of the DNA molecule. This residue forms a hydrogen bond with His311, an interaction that contributes to the stability of this region. Similarly, Glu258 stabilizes this area by forming a hydrogen bond with the DNA base. Another key segment is the region wherein Asn460 is located. Similarly, Glu428 stabilizes this area by forming a hydrogen bond with another DNA base. This residue forms a hydrogen bond with the DNA molecule. This residue forms a hydrogen bond with another DNA base. This residue forms a hydrogen bond with the DNA molecule. This residue forms a hydrogen bond with another DNA base. This residue forms a hydrogen bond with the DNA molecule. This residue forms a hydrogen bond with another DNA base. This residue forms a hydrogen bond with the DNA molecule.

Each of these residues is conserved in STAT2 and occupies positions Glu246, Glu247, Val453, and Asn428. Based on crystal structure analysis, we infer that substitution of Glu246, Glu247 to alanines (AA) may disrupt DNA binding by reducing the rigidity and support of DNA-contacting loops. This mutation may abolish hydrogen bonds stabilizing the loop between β8 and β9. Additionally, mutation of one or both Val453 and isoelucines (II) may alter the structure of β-sheet 11, destabilizing the loop between β-sheet 11 and α-helix 6 and perhaps preventing Asn428 from making direct contact with the DNA DNA.
molecule. A single Val\textsuperscript{454} to Ala mutation targets the second, more critical residue of this VV motif. Also, mutation of Asn\textsuperscript{458} to Ala may have a detrimental effect on DNA binding by impairing specific STAT-DNA interactions.

Therefore, to assess the function of the conserved putative DNA-binding domain of STAT2, the following mutations were introduced into the STAT2 protein: E426A, E427A (EE-AA), V453I, V454I, V454A, V453I, V454I (VV-II), and N458A (Table I). cDNAs encoding the STAT2 mutants were introduced by stable transfection into U6A cells lacking STAT2 to generate a panel of cell lines expressing these mutant STAT2 proteins. A cDNA construct encoding intact STAT2 was also stably introduced into U6A cells, generating the U6A-2 cell line.

IFN Alfacon-1 Treatment of U6A-2 Cells Induces GAS-driven Luciferase Activity—In earlier studies, we reported that treatment of cells with 5 ng/ml IFN alfacon-1 resulted in ISGF-3-independent STAT2-containing complexes (9, 12). To establish a dosage appropriate for further studies, we treated U6A cells reconstituted with intact STAT2 (U6A-2) with varying doses of IFN alfacon-1 (data not shown). The data show that 0.5 ng/ml is sufficient for maximal levels of IFN-inducible STAT2 tyrosine phosphorylation. Dose-response studies for IFN-inducible GAS-mediated transcriptional activation were performed and the results indicate a dose-dependent increase in luciferase activity. Indeed, a dose of 5 ng/ml IFN alfacon-1 is required to achieve optimal GAS-driven transcriptional activity in U6A-2 cells (data not shown). Accordingly, to assess the relevance of IFN-inducible, GAS-binding ISGF3-independent STAT2-containing complexes, a dose of 5 ng/ml IFN was routinely used in all subsequent experiments.

**Mutations in the STAT2 DNA-binding Domain Do Not Disrupt IFN-inducible STAT Tyrosine Phosphorylation**—Following introduction of the STAT2 cDNA constructs into U6A cells, Western blot analysis was performed to determine cellular levels of STAT proteins. All transfectants express comparable levels of both STAT1 and STAT2, with the exception of cells expressing the N458A form of STAT2, in which STAT2 levels are lower (Fig. 2). To confirm that the mutations introduced into STAT2 have not affected the ability of STAT2 to be inducibly phosphorylated upon IFN stimulation, lysates of either untreated or IFN-treated transfectants were prepared. Lysates from IFN-treated cells were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted with an antibody against the phosphorylated/activated form of STAT2. The data reveal that mutations in the putative DNA-binding domain of STAT2 do not interfere with IFN-inducible tyrosine phosphorylation of STAT2 (Fig. 2A).

The extent of IFN-inducible STAT1 phosphorylation was also examined in the different transfectants. Studies have demonstrated that STAT2 is required for IFN-induced STAT1 tyrosine phosphorylation, suggesting there is a sequential activation of STAT proteins at the IFN receptor (7, 17). However, STAT1 activation by IFN has been detected in STAT2-deficient cells (35). Therefore, Western immunoblot analysis was performed to determine whether the mutations in the DNA-binding domain of STAT2 affected the ability of STAT1 to be tyrosine phosphorylated upon IFN stimulation (Fig. 2B). IFN-induced STAT1 tyrosine phosphorylation was detected in all transfectants, albeit more weakly in cells expressing the N458A STAT2 mutant. Interestingly, in U6A cells lacking STAT2, the extent of STAT1 tyrosine phosphorylation was similar to that observed in other cell types.

**Cells Expressing the VV-II STAT2 Mutant Exhibit Diminished IFN-inducible Responses**—To determine whether mutations in the putative DNA-binding domain of STAT2 have affected the biological outcome of IFN-inducible signaling, we...
examined IFN-inducible antiviral activity against EMCV in the different transfectants. U6A cells lacking STAT2 are completely unresponsive to the antiviral effects of IFN (Fig. 3A). By contrast, cells expressing intact STAT2 respond to IFN with the appropriate antiviral response. U6A-2EE-AA, U6A-2V453I, U6A-2V454I, U6A-2V454A, and U6A-2N458A cells are also able to mount a full antiviral response upon IFN stimulation (Fig. 3A). However, cells expressing the VV-II STAT2 mutant fail to mount a robust IFN-inducible antiviral response (Fig. 3A).

Transfectants were also examined for IFN-inducible growth inhibitory responses using the cell proliferation assay. STAT2-deficient U6A cells do not exhibit a growth inhibitory response to IFN (Fig. 3B). In contrast, IFN treatment of U6A-2 cells expressing intact STAT2 results in growth inhibition (Fig. 3B). Cells expressing the EE-AA, V453I, V454I, V454A, and N458A mutant forms of STAT2, likewise exhibit normal levels of IFN-inducible growth inhibition (Fig. 3B). Similar to our results in the antiviral assays, cells expressing the VV-II STAT2 mutant are impaired in their ability to respond to the growth inhibitory effects of IFN (Fig. 3B). Together, these data suggest that the VV-II mutation disrupts STAT2-mediated signal transduction such that IFN-inducible antiviral and growth inhibitory responses are diminished.

Cells Expressing the VV-II Mutant Form of STAT2 Exhibit Diminished IFN-inducible GAS-dependent Luciferase Activity—
To examine IFN-inducible transcriptional activation in cells expressing the mutant STAT2 proteins, ISRE- and GAS-luciferase gene reporter assays were conducted. ISRE-luciferase or GAS-luciferase constructs were introduced into the different cell types by transfection. Following a 6-h 5 ng/ml IFN alfa-con-1 treatment, luciferase activity was measured. In U6A cells, IFN-stimulated STAT2-dependent luciferase activity mediated by ISRE or GAS elements was absent (Figs. 3, C and D). In cells expressing either the intact or any of the mutant forms of STAT2, except U6A-2VV-II, IFN-inducible GAS-driven luciferase activity was detected (Fig. 3D). However, IFN-stimulated luciferase activity mediated by the 8\times\text{GAS} element was significantly reduced in cells expressing the VV-II mutant form of STAT2.

Mutations in the Putative DNA-binding Domain of STAT2 Do Not Affect IFN-inducible STAT Complex Formation or DNA Binding in Vitro—To examine whether the STAT2 mutations interfered with STAT complex formation and DNA binding, a series of EMSAs were performed. Data obtained from antiviral, growth inhibitory and luciferase gene reporter assays suggested only the VV-II mutation in STAT2 disrupted IFN signaling. Therefore, we analyzed IFN-induced STAT complex formation and DNA binding in U6A-2VV-II cells and in two transfectants that respond normally to IFN, namely U6A-2EE-AA and U6A-2V454A cells. At the outset, we conducted EMSAs using an ISRE probe. Within the ISGF3 complex, STAT2 associates with STAT1 via its phosphotyrosine-Src homology 2 domain and with IRF-9 by means of its coiled-coil domain, residues 138–230 (36). Although we showed that our mutations to STAT2 did not affect IFN-inducible tyrosine phosphorylation of STAT1 or STAT2 (Fig. 2), we undertook experiments to examine the effects of these residue changes in STAT2 on the formation of ISGF3 complexes and their ability to bind ISRE. Cells were left untreated or treated with IFN alfacon-1 for 15 min. Nuclear extracts were prepared, incubated with an ISRE probe and resolved by native gel electrophoresis. The precipitated chromatin was analyzed using primers specific for a 6–16 ISRE and an IRF-1 GAS. Primers for \(\beta\)-actin were used to confirm the immunoprecipitation is specific for STAT2. D, the signal intensity of each band was determined. Histograms representing signal intensity ratios of each ChIP sample band to its corresponding input band for the 6–16 and IRF-1 primer sets are provided. Data are representative of three independent experiments.
ent transfectants. IFN treatment of cells expressing the EE-AA and V454A mutant forms of STAT2 results in levels of ISGF3-IRF-1 gene expression, equivalent to a 1.3-fold induction compared with U6A-2 cells. In U6A cells we also observed a 4-fold induction of IRF-1 gene expression following treatment with IFN, as a series of quantitative real-time PCR was performed. Following a 6-h 5 ng/ml IFN alfacon-1 treatment, ISRE-mediated gene expression was assessed using primers specific for the 6–16 and ISG15 genes and GAS-driven gene expression was examined using primers designed for the IRF-1 gene. The data show that, upon IFN treatment, cells expressing the VV-II mutant form of STAT2 induce comparable levels of 6–16 and ISG15 gene expression compared with IFN-treated U6A-2 cells (Fig. 5). In U6A cells, as expected, IFN-induced ISRE-mediated gene expression is abrogated (Fig. 5). IFN treatment of U6A cells induced low levels of IRF-1 gene expression, equivalent to a 1.3-fold induction. In U6A-2 cells a 4-fold induction of IRF-1 was observed following IFN treatment. We infer that this IRF-1 gene expression is regulated by STAT1 and STAT3 homo- and heterodimers, and by STAT2:1. Notably, only a 2.1-fold induction was reported in U6A-2VII-II cells, likely because of the absence of appropriate STAT2:STAT1 DNA interactions. Thus, in addition to reducing the DNA binding activity of STAT2-containing heterodimers to GAS elements (Fig. 4D), the VV-II mutation in STAT2 also has an effect on IFN-inducible GAS-driven gene expression.

**DISCUSSION**

IFN-α activation of STAT2 is pivotal for an IFN response in target cells. Considerable attention has focused on STAT2 in the context of ISGF3 complexes (7, 8, 17), yet little is understood about the role of STAT2 in ISGF3-independent complexes. Although STAT2 contains a conserved 170-residue putative DNA-binding domain, the functional activity of this domain has not been examined until now. In this report we provide evidence for IFN-activated STAT2 contributing to IFN-inducible biological responses that are independent of ISGF3 activity. Specifically, our mutagenesis studies suggest that STAT2 forms a heterodimer with STAT1 that will bind to chromatin and determine transcriptional activation that contributes to IFN-inducible biological responses.

Our findings with the U6A cells that lack STAT2 indicate that STAT2 expression is a critical determinant for IFN responsiveness in target cells. In the absence of STAT2, cells are unresponsive to the antiviral and growth inhibitory effects of IFN (Fig. 3, A and B). IFN-inducible ISGF3 formation, DNA binding, and transcriptional activation were unaffected in cells expressing any of the STAT2 proteins carrying mutations in their DNA-binding domain. Moreover, these STAT2 mutations did not interfere with the formation of IFN-inducible ISGF3-independent STAT2:1 heterodimers. Notably, the mutations that we introduced into STAT2 did not fully abrogate STAT2:1-DNA interactions as gauged by EMSA or ChIP assay. A single mutant STAT2 carrying the VV-II mutation affected a decrease in IFN-inducible STAT2-GAS binding on chromatin, resulting in diminished GAS-driven transcriptional activation that was reflected in blunted antiviral and growth inhibitory responses to IFN. This STAT2 mutation reduced GAS-mediated gene expression of the IRF-1 gene. Notably, U6A-2VII-II cells exhibit diminished IFN-stimulated IRF-1 gene expression compared with U6A-2 cells. In U6A cells we also observed minimal IFN-induced IRF-1 expression. Studies have determined that, in addition to STAT2:1 heterodimers, both
STAT1:1 and STAT3:3 complexes can bind the GAS-like element found within the promoter of the IRF-1 gene (9, 10). Our finding therefore suggests that although STAT1 and STAT3 homodimers may contribute to its transcriptional activation, STAT2 is required for full IRF-1 expression. Analysis of the crystal structure of STAT1 bound to DNA identified valines 453 and 454 in STAT2 as important for mediating DNA binding (23, 24). These VV residues do not mediate direct DNA contact, but provide structural support for the DNA-binding loop carrying Asn458. The Val to Ile mutations were designed to be structurally disruptive by virtue of the introduction of a larger aliphatic side chain. As observed, single mutations, V453I or V454I, were not sufficient to disturb the function of this region of the STAT2 protein in the context of DNA binding or transcriptional activation. The double VV-II mutation significantly impaired the ability of STAT2, within ISGF3-independent STAT2-containing complexes, to mediate IFN-inducible responses. Although the EMSA results do not suggest a difference in the STAT2:1-GAS DNA binding for the VV-II mutant compared with intact STAT2 (Fig. 4B), the ChIP data identify that binding of IFN-inducible STAT2-containing complexes that contain the VV-II mutant STAT2 to GAS elements on chromatin, is reduced compared with complexes containing intact STAT2 (Fig. 4D). Moreover, the VV-II mutation in STAT2 affected IFN-inducible GAS-driven transcriptional activation, in further support that this region of STAT2 is important for DNA interactions. The implications are that STAT2:1 complexes interact with chromatin DNA in a manner distinct from their interaction with the minimal pIRE in an EMSA, and that there is a region in STAT2, encompassing residues in the DNA-binding loop associated with Asn458, that is critical for these STAT-DNA interactions.

Studies examining the DNA binding activity of STATs have demonstrated that particular residues in the DNA-binding domain of different STAT proteins may have distinct roles. For instance, residues Glu434 and Glu435 are critical for STAT3-DNA binding but are non-essential for the DNA binding activity of STAT5b (20, 37). Mutation of Val461-Val462-Val463 to AAA in STAT3 disrupts DNA binding but does not have an effect on nuclear localization (20). However, alanine substitutions of the corresponding residues in STAT5b (V466I,V467I,V468I,V469I) prevent DNA binding and also abrogate growth hormone-induced nuclear import of the STAT5b complexes (21). A recent study suggested that although STAT-DNA binding activity regulates nuclear accumulation, it is not an absolute requirement for entry into the nucleus (34). Indeed, mutations in STAT1 (E428A, E429A, and N460A) abolish DNA binding but do not affect STAT1 nuclear translocation (38, 39). Our data...
suggest that the VV-II mutation in STAT2 did not affect STAT2
cytoplasmic transport. In the ISRE and pIRE EMSA
series, nuclear extracts were used, indicating that entry of
STAT2-containing complexes into the nucleus was intact in all
cell types, including cells expressing the VV-II STAT2 mutant.
ChIP data confirmed that IFN-inducible STAT2-containing
complexes carrying the VV-II mutation enter the nucleus and
bind chromatin. Moreover, we have evidence that nuclear import
is unaffected by the VV-II mutation.2

In the ISGF3 complex, STAT2 contributes its potent trans-
activation domain and thus has an important role in mediating
transcription (17, 18). The carboxyl-terminial STAT2 transac-
tivation domain interacts with a number of different co-factors and
mediators, including CBP/p300 and GCN5, which are re-
quired for ISRE-mediated transcriptional activation (19, 40).
Based on the structure of STAT proteins, it is unlikely that a
disruption in the DNA-binding domain would prevent the
STAT protein from interacting with co-activators. Indeed, we
observed that in cells expressing the VV-II mutant form of
STAT2, IFN-inducible ISRE-driven transcription and gene ex-
pression were intact, confirming this mutation did not affect
transcriptional activation by restricting interactions with co-
factors. We infer that it is unlikely that the VV-II mutation in
STAT2 affected the transactivation domain and attribute the
reduced GAS-mediated transcriptional activation and gene ex-
pression to the decreased binding we observed to GAS elements
on chromatin.

These data establish that the putative DNA-binding domain
of STAT2 has functional relevance and its role is specific to the
activity of ISGF3-independent STAT2-containing complexes.
Analysis of the crystal structure of STAT1 homodimer bound to
DNA revealed that both STAT molecules contact DNA via their
DNA-binding domains (20, 21). In agreement with these data,
our findings suggest that in STAT2:1 heterodimers, STAT2 will
bind DNA. Indeed, the data suggest that, within ISGF3-inde-
pendent STAT2-containing complexes, the function of this pu-
tative DNA-binding domain of STAT2 is to mediate interactions
with GAS-like elements, driving the transcriptional
activation of a subset of ISGs. The challenge of our ongoing
experiments is to distinguish the contribution of this subset of
ISGs to IFN-inducible responses.

REFERENCES
1. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., and Schreiber, R. D.
(1998) Annu. Rev. Biochem. 67, 227–264
2. Brierley, M. M., and Fish, E. N. (2002) J. Interferon Cytokine Res. 22, 835–845
3. Platanias, L. C., and Fish, E. N. (1999) Exp. Hematol. 27, 1583–1592
4. Schindler, C., Shuai, K., Prezioso, V. R., and Darnell, J. E., Jr. (1992) Science
257, 809–813
5. Muller, U., Steinhoff, U., Reis, L. F., Hemmi, S., Pavlovic, J., Zinkernagel,
R. M., and Aguet, M. (1994) Science 264, 1918–1921
6. Colamorici, O., Yan, H., Domanski, P., Handa, R., Smalley, D., Mullersman,
J., Witte, M., Krishnan, K., and Krulowski, J. (1994) Mol. Cell. Biol. 14,
8133–8142
7. Leung, S., Qureshi, S. A., Kerr, I. M., Darnell, J. E., Jr., and Stark, G. R. (1995)
Mol. Cell. Biol. 15, 1312–1317
8. Park, C., Li, S., Cha, E., and Schindler, C. (2000) Immunity 13, 795–804
9. Ghislain, J. J., and Fish, E. N. (1996) J. Biol. Chem. 271, 12408–12413
10. Li, X., Leung, S., Qureshi, S., Darnell, J. E., Jr., and Stark, G. R. (1996) J. Biol.
Chem. 271, 5790–5794
11. Fu, X. Y., Kessler, D. S., Veals, S. A., Levy, D. E., and Darnell, J. E., Jr. (1990)
Proc. Natl. Acad. Sci. U. S. A. 87, 8555–8559
12. Ghishain, J. J., Wong, T., Nguyen, M., and Fish, E. N. (2001) J. Interferon
Cytokine Res. 21, 379–388
13. Darnell, J. E., Jr. (1997) Science 277, 1630–1635
14. Goodbourn, S., Didcock, L., and Randall, R. E. (2000) J. Gen. Virol. 81,
2341–2364
15. Clemens, M. J., and Elia, A. (1997) J. Interferon Cytokine Res. 17, 503–524
16. Reboullat, D., and Hovanessian, A. G. (1999) J. Interferon Cytokine Res. 19,
295–308
17. Qureshi, S. A., Leung, S., Kerr, I. M., Stark, G. R., and Darnell, J. E., Jr. (1996)
Mol. Cell. Biol. 16, 288–293
18. Bluyssen, H. A., and Levy, D. E. (1997) J. Biol. Chem. 272, 4600–4605
19. Paulson, M., Fish, E. N., and Levy, D. E. (1996) J. Biol. Chem. 271, 25343–25349
20. Horvath, C. M., Wen, Z., and Darnell, J. E., Jr. (1995) Genes Dev. 9, 984–994
21. Harrington, J., Rui, L., Luo, G., Yu-Lee, L. Y., and Carter-Su, C. (1999) J. Biol.
Chem. 274, 5138–5145
22. Yang, E., Henrikson, M. A., Schaefer, O., Zakharova, N., and Darnell, J. E., Jr.
(2002) J. Biol. Chem. 277, 13455–13462
23. Chen, X., Vinkemeier, U., Zhao, Y., Jeruzalmi, D., Darnell, J. E., Jr., and
Kuriyan, J. (1998) Cell 93, 827–839
24. Becker, S., Groner, B., and Muller, C. W. (1998) Nature 394, 145–151
25. Masters, J., Hinek, A. A., Uddin, S., Platanias, L. C., Zeng, W., McFadden, G.,
and Fish, E. N. (2001) J. Biol. Chem. 276, 48371–48375
26. Mayer, I. A., Verma, A., Grumbach, I. M., Uddin, S., Lekmine, F., Ravandi, F.,
Majchrzak, B., Fujita, S., Fish, E. N., and Platanias, L. C. (2001) J. Biol.
Chem. 276, 28570–28577
27. Uddin, S., Fish, E. N., Sher, D., Gardziola, C., Colamorici, O. R., Kellum, M.,
Pitha, P. M., White, M. F., and Platanias, L. C. (1997) Blood 90, 2574–2582
28. Uddin, S., Majchrzak, B., Woodsen, J., Arunkumar, P., Alsayed, Y., Pine, R.,
Young, P. R., Fish, E. N., and Platanias, L. C. (1999) J. Biol. Chem. 274,
30127–30131
29. Horvai, A. E., Xu, L., Korzus, E., Brard, G., Kalafus, D., Mullen, T. M., Rose,
D. W., Rosenfeld, M. G., and Glass, C. K. (1997) Proc. Natl. Acad. Sci.
U. S. A. 94, 1074–1079
30. Li, Y., Sassano, A., Majchrzak, B., Deb, D. K., Levy, D. E., Gaestel, M.,
Nebreda, A. R., Fish, E. N., and Platanias, L. C. (2004) J. Biol.
Chem. 279, 970–979
31. Wong, M., and Fish, E. N. (1998) J. Biol. Chem. 273, 309–314
32. Cohen, B., Peretz, D., Vaiman, D., Chebath, J., Witte, M., Krishnan, K., and Krolewski, J. (1994) EMBO J. 13, 1411–1419
33. Huang, M., Qian, F., Hu, Y., Ang, C., Li, Z., and Wen, Z. (2002) Nat. Cell Biol.
4, 774–781
34. Meyer, T., Marg, A., Lemke, P., Wiesner, B., and Vinkemeier, U. (2003) Genes Dev.
17, 1992–2005
35. Mowen, K., and David, M. (1998) J. Biol. Chem. 273, 30073–30076
36. Martinez-Mozgawa, M., Gutch, M. J., French, D. L., and Reich, N. C. (1997)
J. Biol. Chem. 272, 20070–20076
37. Luo, G., and Yu-Lee, L. (1997) J. Biol. Chem. 272, 26841–26849
38. McBride, K. M., McDonald, C., and Reich, N. C. (2000) EMBO J. 19,
6196–6206
39. Lillemeier, B. F., Koster, M., and Kerr, I. M. (2001) EMBO J. 20, 2508–2517
40. Paulson, M., Press, C., Smith, E., Tanes, N., and Levy, D. E. (2002) Nat. Cell
Biol. 4, 140–147

N. Reich, unpublished data.