Distinct Transcriptional Activation Functions of STAT1α and STAT1β on DNA and Chromatin Templates*

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Interferon-induced transcription depends upon tyrosine phosphorylation, subsequent dimerization, and binding to DNA of STAT1. Other factors, including but not necessarily limited to CBP/p300, then bind within the C-terminal 38 amino acid transactivation domain (TAD) to activate transcription. We show that both tyrosine-phosphorylated STAT1α (full-length wild-type protein) and STAT1β (lacking the TAD) stimulate in vitro transcription on a naked DNA template. Furthermore, in a system with purified proteins and naked DNA, STAT1α- and STAT1β-dependent transcription is stimulated by the TRAP/Mediator co-activator complex. Thus STAT1, through some site other than the C-terminal TAD, can interact with TRAP/Mediator or some intermediate protein. Although both STAT1α and STAT1β bind to known STAT sites within in vitro assembled chromatin templates, only STAT1α, and not STAT1β, in cooperation with p300 and acetyl-CoA, stimulated in vitro transcription from chromatin. After interferon-γ treatment, cells recruit STAT1α or -β to the chromosomal interferon-1 gene, but only STAT1α-containing cells recruit p300 and stimulate transcription. We conclude that chromatin remodeling by p300 in vivo makes TRAP/Mediator effective in stimulating transcription.

The signal transducers and activators of transcription (STAT) proteins were discovered as interferon (IFN)-induced DNA-binding proteins that bound to sequences upstream of IFN-induced genes (1–3). These binding sites are capable of directing IFN-induced transcription of synthetic reporter genes in transfection experiments. Extensive studies of this family of transcription factors by mutagenesis and by reintroduction of IFN-induced proteins were modified by phosphorylation on Tyr-701 by the T4 endonuclease V-protease to remove His6 tags. All STAT proteins were expressed in Escherichia coli and purified by Ni²⁺-agarose batch binding and elution followed by MonoQ column chromatography (18).

FLAG-tagged ISWI and ACF1 were co-expressed via baculovirus vectors in Sf9 cells and co-purified as a complex (ACF) by anti-FLAG affinity batch binding and elution as described previously (19). His6-tagged p300 and CBP were expressed and purified from baculovirus-infected Sf9 cells.

DNA Templates—Transcription reactions with STATs were carried out with the test plasmid pLy6E/G-less, which contained three STAT-binding sites from the mouse Ly6E gene (20). These three IFN-γ-activated sites were placed upstream of the adenovirus major late core promoter (21) followed by a G-less cassette; the construct is referred to as pLy6EML53G or pLy6EB. The control plasmid, pML53G, lacked activator binding sites and contained the adenovirus major late promoter followed by a G-less cassette shorter than that in the Ly6E plasmid.

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¶§ The abbreviations used are: STAT, signal transducers and activators of transcription; IFN, interferon; CBP, cAMP-response element-binding protein; HNF, hepatocyte nuclear factor; TF, transcription factor; USA, upstream activator; TRAP, thyroid hormone receptor-associated protein; ChIP, chromatin immunoprecipitation; IRF-1, IFN regulatory factor-1.

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The plasmid pA4xMLΔS3 (22) contains four cognate sites for HNF-4 upstream of the same core promoter.

Chromatin Assembly—The assembly of chromatin with purified recombinant ACF, purified recombinant DNAP1, purified core histones, and TFIIA, TFIIIB, and Pol II is described (26, 27). The assembly of chromatin with the Drosophila S190 extract was carried out as described (23). The resulting chromatin samples were subjected to micrococcal nuclease digestion analysis to confirm the appropriate distribution of periodic arrays.

In Vitro Transcription—In vitro transcription reactions other than those shown in Fig. 3 were performed with HeLa nuclear extract (24). For reactions with naked DNA, STAT1 proteins were incubated with pLy6E/G-less plasmid DNA, and ATP was performed as described (19). The assembly of chromatin with the Drosophila S190 extract was carried out as described (23). The resulting chromatin samples were subjected to micrococcal nuclease digestion analysis to confirm the appropriate distribution of periodic arrays.

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the eluates at 65 °C for 4 h. 10 µl of 0.5 M EDTA, 20 µl of 1 M Tris-HCl, pH 6.8, and 2 µl of 10 mg/ml proteinase K were added to the eluates, and samples were incubated for 1 h at 45 °C. DNA was recovered by phenol/chloroform extraction and ethanol precipitation using 20 µg of glycerol as a carrier. DNA samples were then analyzed with 28 cycles of PCR to amplify IRF-1 promoter sequences. The nucleotide sequences of the primers used for the PCR reaction were 5'-CTTCCGGCTCTAC-3' and 5'-GCTCGGGTGCGCCCTC-3'.

**RT-PCR**—Total RNA was isolated from U3a cells using TRIzol reagent (Invitrogen). The PCR conditions to detect IRF-1 mRNA were as follows: 94 °C, 30 s; 50 °C, 1 min; 72 °C, 30 s; and repeat 22 cycles. Primers used were as described (30).

**DNase I Primer Extension Footprinting on Chromatin**—600 ng of an ACF-assembled chromatin template was incubated with or without 150 ng of phospho-STAT1 followed by DNase I digestion and primer extension reaction as described previously (31). The 5'-end of the primer used for the extension reaction corresponds to the −210 position of the template. The three STAT binding sites are located at the −110, −130, and −155 positions.

**RESULTS**

**In Vitro Function of STATs on DNA versus Chromatin Templates**—STATs must be phosphorylated on tyrosine (residue 701 in STAT1) to dimerize and bind DNA (21, 32). In addition, maximal in vivo transcriptional activity requires phosphorylation of a C-terminal serine residue (Ser-727 in STAT1) (33). Purified, tyrosine-phosphorylated full-length STAT1 can be most efficiently prepared in large quantities by production in baculovirus-infected insect cells and subsequent tyrosine phosphorylation by activated epidermal growth factor receptor (6). STAT1 prepared in this fashion is completely and exclusively phosphorylated on Tyr-701, with no evidence of Ser-727 phosphorylation (estimated limit of detection is ~5%) from mass spectrometric analysis (data not shown). Because transcription is only reduced and not ablated in cells when Ser-727 is changed to alanine (33), we performed in vitro transcriptional experiments with the purified, tyrosine phosphorylated STAT1 without attempting to phosphorylate Ser-727.

The test DNA template contained three STAT1 binding sites upstream of the adenovirus major late core promoter (20, 21) followed by a G-less cassette, whereas a control template contained only the adenovirus core promoter (~55 to +10) and a shorter G-less cassette. The STAT1 binding sites on the plasmid (pLy6) were from the IFN-γ-induced murine Ly6E gene and were shown previously to function on model templates in vivo (21).

Two transcription systems were employed to analyze the coactivator-dependence of the transcriptional activity of STAT proteins. One system (26) consisted of the following: highly purified RNA polymerase II from HeLa cells; affinity purified preparations of recombinant general transcription factors TFIIA, TFIIIB, TFIIIE, and TFIIF; an enriched chromatographic fraction from HeLa cell nuclear extract containing TFIIH; and purified RNA polymerase II from HeLa cells; affinity purified proteins. One system (26) consisted of the following: highly purified RNA polymerase II from HeLa cells; affinity purified proteins. One system (26) consisted of the following: highly purified RNA polymerase II from HeLa cells; affinity purified proteins. The test DNA template contained three STAT1 binding sites downstream of the CMV promoter (−55 to +10) and a shorter G-less cassette. The STAT1 binding sites on the plasmid (pLy6) were from the IFN-γ-induced murine Ly6E gene and were shown previously to function on model templates in vivo (21).

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A second transcription system used to compare STAT and coactivator-dependent transcription from DNA and chromatin templates employed HeLa cell nuclear extract as a source of all necessary components of the transcriptional machinery. The protocols and the order of addition of reagents are shown in Fig. 1A (DNA) and Fig. 1B (Chromatin). Chromatin templates for all experiments except those seen in Fig. 5 were prepared according to Ito et al. (18), and samples of chromatin preparations digested with micrococcal nuclease are shown in Fig. 1, C and D.

STAT1α is the wild-type full-length transcriptional activator, and STAT1β is a naturally occurring splice variant (40) that lacks the C-terminal transcriptional activation domain which can bind the histone acetyltransferase p300/CBP (14, 15). Both STAT1α and STAT1β bind DNA, with STAT1α showing a stronger tendency to form dimer-dimer complexes (Ref. 6 and Fig. 2A). In crude nuclear extracts containing the general transcription machinery and various cofactors, tyrosine-phosphorylated STAT1α and STAT1β both stimulated transcription (above the basal level) from the test DNA template (pLy6) but not from the control (pMLΔ53) template (Fig. 2B).

Using the partially purified transcription system that depends upon the USA coactivator fraction, we tested the ability of STAT1α and STAT1β to activate naked DNA templates (Fig. 3A). Under the conditions of the assay, basal level transcription on naked DNA was seen both from the test (pLy6) plasmid containing STAT binding sites and the reference template lacking these sites (pA4xMLΔ53) (Fig. 3A, lane 1). Consistent with
the necessity of tyrosine phosphorylation of STAT1 for dimerization and DNA binding, neither unphosphorylated STAT1/H9251 (Fig. 3A, lane 2) nor unphosphorylated STAT1/H9252 (Fig. 3A, lane 3) displayed any activity. By contrast, both phosphorylated forms of STAT1 significantly stimulated transcription from the test template (Fig. 3A; compare lanes 4 and 5 with lanes 2 and 3, respectively) in the presence of added USA. As a positive control, the transcriptional activator HNF-4 increased transcription from the reference template, which contained HNF-4 binding sites (Fig. 3A, lane 6) (22).

In a further analysis, the reconstituted transcription system containing pure preparations of all required factors (26) was used to test directly the TRAP/Mediator-dependence of STAT1 function (Fig. 3A, B and C). In the absence of added TRAP/Mediator there was no transcriptional activation either by phosphorylated STAT1a (Fig. 3A, lanes 3 and 4) or by HNF-4 (Fig. 3A, lane 2; used as a positive control). However, when this reconstituted system was supplemented with affinity-purified TRAP/Mediator, activation by both STAT1a (Fig. 3A, lanes 7 and 8 versus lane 5) and HNF-4 (Fig. 3A, lane 6 versus lane 5) was evident. Separately, we further found that phosphorylated STAT1β was also able to activate transcription of the naked DNA template with the aid of the TRAP/Mediator complex (Fig. 3C). Overall, these results strongly implicate TRAP/Mediator in the function of STAT1 on naked DNA templates in vitro, and this stimulation can occur (perhaps less well; Fig. 3A, lanes 4 and 5) without the c-terminal STAT1 transactivation domain.

We next examined the ability of STAT1 to activate transcription on chromatin templates (pLy6 and pΔ53ML). The low level
basal transcription observed with the naked DNA templates was completely repressed on chromatin templates (Fig. 2C, lanes 1 and 2, and Fig. 4, lane 1). Significantly, transcriptional activation was observed with STAT1α but not with STAT1β (Fig. 2C, lanes 3 and 5 versus lane 1). Furthermore, preincubation with p300 (Fig. 2C, lanes 3–6) or CBP (data not shown) prior to adding the transcribing extract stimulated chromatin transcription by STAT1α but not STAT1β. In addition to the stimulation by added p300 (a histone acetyltransferase), further stimulation was achieved upon the addition of acetyl-CoA as an acetyl donor (Fig. 4, lane versus lane 2). These results are in accord with the conclusion of in vivo experiments that at least one function of the carboxyl-terminal STAT1 domain is binding CBP/p300 (11). By analogy with the function of this coactivator in mediating other transcriptional events (Ref. 41 and references therein), these results suggest that transcriptional activation by STAT1 involves p300-mediated acetylation of histones to promote access of the transcriptional machinery.

To compare possible in vitro contributions of the STAT1α C terminus with known in vivo functions, transcriptional stimulation by the wild-type protein was compared with stimulation by two mutant proteins in vitro. First, Ser-727, the known phosphorylation site required for full IFN-γ-induced transcriptional activity of STAT1α, was changed to alanine (S727A). A second mutant (L724A) contained a leucine to alanine change at residue 724. Both of these mutations reduce IFN-γ-induced transcriptional activity ~3-fold when expressed in cells lacking STAT1 (16). The L724A and S727A proteins bound DNA similarly to wild-type STAT1 (Fig. 5A) and, when assayed on a naked DNA template in a nuclear extract, both the L724A and the S727A proteins increased transcription to approximately the same extent as wild-type protein (Fig. 5B).

On similarly assayed chromatin templates, the two STAT1α mutants showed no transcriptional activity (Fig. 5C). However, addition of ectopic CBP to the nuclear extract resulted in significant stimulation of transcription by the mutant proteins, as well as stimulation by wild-type protein. These results agree with the known role of the C terminus in binding CBP/p300 and suggest a decreased affinity of CBP/p300 for the L724A and S727A mutant proteins, as was suggested but not demonstrated earlier (16). The relative activities of the mutant and wild-type proteins in vitro approximate the levels observed in transfection assays in vivo (16).

**Binding of STATs to Specific Sites within Chromatin**—Because STAT1β did not stimulate transcription on a chromatin template, we employed DNasel footprint analysis to determine whether it could bind to its cognate sites in chromatin. After incubation with STAT1α or STAT1β, chromatin templates were treated with DNasel, and the resulting DNA fragments were purified and analyzed by radiolabeled primer extension and resolved on acrylamide gels. STAT1α and β gave similar
footprints on the chromatin template, with all three STAT1 binding sites (indicated by lines) protected at the protein:DNA ratio used in this experiment (Fig. 6). Thus, the failure of STAT1β to stimulate transcription on chromatin templates was not caused by a failure to bind to cognate sites. 

**IFN-γ-induced Association of STATs and p300 with the Promoter of a Chromosomal IFN-γ-responsive Gene**—We next tested whether STAT1α and -β could bind to natural chromosomal loci in vivo. U3 cells, which lack STAT1 (13), were stably transfected with either STAT1α or STAT1β (Fig. 7). STAT1α, but not STAT1β, restored the IFN-γ-induced response of the endogenous IRF-1 gene (Fig. 7A). To test for association of STAT1α or STAT1β with the endogenous IRF-1 promoter, STAT1α- or STAT1β-expressing cells were treated with IFN-γ, and samples were taken at intervals for ChIP (after crosslinking and sonication) with an antibody that recognizes a common epitope in STAT1α and STAT1β. There was an interferon-dependent increase of IRF-1 promoter DNA in the chromatin immunoprecipitates of extracts from both STAT1α- and STAT1β-expressing cell lines (Fig. 7B), showing that both proteins (1α and 1β) bind to natural sites within chromatin in vivo. Next, chromatin immunoprecipitation using p300 antibody was carried out to see whether both STAT1α and STAT1β would lead to accumulation of p300 on STAT1α within chromatin. Significantly, and despite equal binding of STAT1α and STAT1β to chromatin sites (Fig. 7B), only STAT1α recruited p300 to these sites (Fig. 7C). As a control in the chromatin immunoprecipitation experiments, an upstream region 3 kb from the start site was tested and not found to be precipitated by either STAT or p300 antibodies (data not shown).

**DISCUSSION**

The *in vitro* experiments described in this paper establish that STAT1 molecules bound to known DNA recognition sites for STAT1 can effect recruitment of the transcriptional machinery and stimulate transcriptional initiation on both naked DNA and chromatin templates. The demonstrated ability of STAT1α, but not STAT1β, to stimulate *in vitro* transcription of a chromatin template is in accord with earlier mutagenesis, protein-protein association and transfection experiments that demonstrated a stimulatory role of the STAT1-specific carboxyl terminus in STAT-induced transcription (16, 33). However, the observation that STAT1β, as well as STAT1α, stimulates transcription of naked DNA revealed a heretofore unrecognized transcriptional activation function common to STAT1α and STAT1β and further suggested that STAT1 (α and β) could also interact with proteins other than those (such as p300/CBP) involved in chromatin remodeling. Using a partially purified transcription system, we have shown that one such factor involved in STAT1-dependent transcriptional activation is the TRAP/Mediator complex. Given that several transcription activators have been shown to act in part through direct interactions with TRAP/Mediator (37), we have made extensive, but thus far unsuccessful, efforts to show physical interactions of STAT1 (either free or DNA-bound and with or without phosphorylation) with the TRAP/Mediator complex. Thus, there may be other intermediary proteins linking STAT1 with TRAP/Mediator or, alternatively, the putative direct association may be weak and dependent upon cooperative interactions that are manifested only in the presence of the general transcription machinery (42). In this regard, STAT1 contains a relatively weak activation domain by comparison to other activators or to STAT2 (43). Moreover, the recent demonstration (44) of physical and functional interactions of STAT2 with the TRAP170/vitamin D receptor-interacting protein 150 component of Mediator establishes the concept, suggested here only from functional studies, of STAT1-Mediator interactions. In addition, as indicated above, the ability of both STAT1α and STAT1β to mediate transcription from DNA templates suggests a secondary activation domain distinct from the C-terminal domain that has been linked thus far only to p300/CBP recruitment.

Recent studies have shown that activator- and p300-dependent transcription from chromatin templates depends upon promoter-localized acetylation of nucleosomal histones (Ref. 41 and references therein). Hence, the present demonstration of *in vitro* transcriptional enhancement on chromatin templates by p300/CBP and acetyl-CoA makes it likely that histone acetylation is required at or near the promoter for maximal STAT1-driven transcription. The ability of STAT1α, but not STAT1β, to activate transcription from a chromatin template in the presence of p300 and acetyl-CoA further suggests (see also below) that STAT1α, presumably through the C-terminal 38 amino acids, can recruit CBP/p300 whereas STAT1β cannot. Thus, if there is a role for the reported p300/CBP binding site in the N terminus of STAT1α and STAT1β (15), it is insufficient for significant independent action. It is also possible that other chromatin remodeling proteins and histone transacetylase may be involved in STAT1-mediated gene activation. For example, STAT2 is known to interact with GCN5 (45). However, the ~200 amino acid carboxyl terminus of STAT2 is very different from that of STAT1.

In strong support of the conclusions of the *in vitro* transcription studies, *in vivo* chromatin immunoprecipitation analyses showed that whereas both STAT1α and STAT1β can accumulate on the IFN-γ-induced IRF-1 promoter *in vivo*, only STAT1α is capable of recruiting CBP/p300 and stimulating transcription. This establishes the primary importance of the earlier demonstrated C-terminal STAT1-CBP/p300 interaction relative to the N-terminal STAT1-CBP/p300 interaction (16) and, most importantly, elucidates the specific step at which this C-terminal activation domain functions.

The *in vitro* system faithfully reproduces the decreased ability of STAT1 mutants to stimulate transcription *in vivo* (16). Thus, STAT1 L724A and S727A are poor activators on chromatin templates compared with wild-type protein, although they do show some activity. The basis for stronger activation by the natural amino acid sequence in the activator domain may include a more avid binding of p300/CBP due to Ser-727 phosphorylation or the presence of the Leu-724 residue. However, the ability of an increased level of CBP to stimulate transcription by both mutants suggests that they bind this co-activator less well than wild-type protein.

The present study of STAT1 function on DNA versus chromatin templates has allowed the resolution of distinct activation domains and corresponding functions with distinct coactivators acting at chromatin remodeling versus post-chromatin remodeling steps. They also open the way for further identification of other factors that may be recruited by activated STAT1 molecules in the course of IFN-γ-stimulated gene transcription. Because all of the STAT proteins are so similar (particularly STAT3, which is 45% identical in amino acid sequence to STAT1), these results also suggest likely success in studies of other STAT molecules where *in vivo* interactions with other transcription factors have been documented (11, 12, 46).

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