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Nucleic Acids Research, 1995, Vol. 23, No. 16 3283–3289

STAT protein complexes activated by interferon-γ and gp130 signaling molecules differ in their sequence preferences and transcriptional induction properties

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Received April 10, 1995; Revised and Accepted July 10, 1995

ABSTRACT

Activation of members of the STAT (signal transducers and activators of transcription) family of latent transcription factors is an early event following the binding of many cytokines to their cognate receptors. Although the patterns of STATs activated by different cytokines are well described, the consequences of differential STAT activation are less well studied. We show by mutational analysis that STAT binding elements (SBEs) exist that discriminate between STAT complexes containing STAT1α, STAT3 or both, and that these elements show altered cytokine responsiveness. We also show that in the context of a minimal promoter, single and multiple SBEs exhibit strikingly different patterns of transcriptional activation in response to IFN-γ, IL-6, OSM or LIF. These differences in transcriptional activation are correlated with the differential ability of these cytokines to activate STAT1α, STAT3 or both. Our results show that the pattern of STATs activated by a cytokine and the arrangement and sequence of the SBEs in the responding promoter have a profound effect on the ability of the cytokine to elicit a transcriptional response.

INTRODUCTION

Cytokines are a large family of soluble proteins that play a major role in the growth, differentiation and function of cells. Many of these effects can be ascribed to changes in gene expression triggered by the binding of cytokines to cell-surface receptors. The link between cytokine receptors at the cell membrane and changes in gene transcription in the nucleus is mediated, at least in part, by a novel family of latent transcription factors termed STATs (signal transducers and activators of transcription) (1–3). These proteins were first described in the context of interferon signaling. Treatment of cells with interferon-γ (IFN-γ) causes the rapid phosphorylation of STAT1α at a specific tyrosyl residue, causing it to assemble into a homodimeric complex and translocate to the nucleus, where it binds to IFN-γ response elements known as GASs (gamma activation sequences) (1,4–6). Binding of STAT1α proteins to GAS elements in the promoters of responsive genes stimulates their transcription (7–9). Considerable evidence demonstrates that two members of a specific subfamily of tyrosine kinases, known as JAK1 and JAK2, are required for STAT1α phosphorylation in response to IFN-γ (1,10–13). These kinases appear to associate with the cytoplasmic domain of the IFN-γ receptor, and are activated by the binding of IFN-γ (10–15).

Interleukin-6 (IL-6), leukemia inhibitory factor (LIF) and oncostatin M (OSM) are structurally related cytokines that bind to receptors that share a common subunit required for signal transduction, termed gp130 (16). Binding of these cytokines to their receptors induces homo or hetero dimerization of the gp130 subunit, which initiates downstream signaling. As with IFN-γ, this signaling process involves activation of members of the JAK family, the specific pattern of JAKs activated being dependent on cell type (17,18). Treatment of cells with IL-6, LIF or OSM also induces the rapid tyrosine phosphorylation of a recently characterized member of the STAT family, STAT3 (also called acute phase response factor, APRF) which is homologous to STAT1α and binds to elements related to GAS sequences found in the promoters of some acute phase response genes (19–25).

Activation of STAT proteins is an early event following the binding of many cytokines to their receptors, resulting in the formation of complexes with GAS-binding activity (1–3, 26–30). In recognition of this fact, we will refer to GAS elements as STAT-binding elements (SBEs) throughout this paper. The ability of multiple cytokines to activate STAT complexes that bind to SBEs of similar sequence raises the question of how selective regulation of gene expression is achieved. In order to address this question, we have undertaken a thorough mutagenesis of a SBE to determine whether SBEs with selective STAT binding properties exist. We find that the SBE has a palindromic structure and identify SBEs which discriminate between the binding of STAT1α and STAT3. We also find that IFN-γ, IL-6, LIF or OSM have strikingly different abilities to activate transcription from reporter plasmids carrying single or multiple SBEs, and that these differences are related to the ability of these cytokines to activate STAT1α, STAT3 or both. These results indicate that regulation of gene expression by cytokines can be influenced by both the

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precise sequence and arrangement of SBEs in the promoter of a potentially inducible gene, and by the composition of the STAT complex(es) activated by the inducing cytokine.

MATERIALS AND METHODS

Reagents

Human recombinant IFN-γ, anti-STAT1 and anti-STAT3 sera and expression vectors for STAT1α and STAT3 were the gift of Dr J. Darnell. Recombinant human IL-6, LIF and OSM were obtained from R&D Systems. The anti-phosphotyrosine monoclonal antibody 4G10 was obtained from Upstate Biotechnology Inc. Protein A agarose, protease inhibitors and poly d(I-C) poly d(I-C) were from Boehringer Mannheim.

Cells and cell culture

HepG2 cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine and 100 U/ml of penicillin and streptomycin, and were treated with cytokines at 50–75% confluency. Cytokines were used at the following concentrations: IFN-γ, 5 ng/ml, IL-6, 10 ng/ml, LIF, 10 ng/ml and OSM 10 ng/ml.

Oligonucleotides.

Oligonucleotides were obtained from either National Biosciences or Integrated DNA Technologies. The sequences of the SBEs are as follows (the antisense strand was synthesized so that when annealed to the sense strand both strands would have a GATC overhang):

Ly6E/A (7)	5′-GATCATATTCCTGTAAGTG-3′
IRF-1 (8)	5′-GATCGATTTCCCCGAAATG-3′
Antichymotrypsin (31)	5′-GATCATATTACCAGAAATG-3′
Haptoglobin (31)	5′-GATCATTTTCCAGTAACAG-3′

These sequences derive from the human promoters, except for the Ly6E/A sequence which is mouse. The Ly6E/A point mutants 3C, 3G, 5A, 7C and 8C are based on the above sequence with a single base pair change as indicated in Table 1. All the other point mutants lack the GATC overhangs.

Preparation of nuclear extracts and gel retardation assays

Nuclear extracts were prepared and gel retardation assays run as described (28). Protein concentrations were measured using the Bradford dye binding assay.

Oligonucleotide competition was performed by preincubating nuclear extract with either 0.5, 5 or 50 ng of the competitor oligonucleotide and poly d(I-C) poly d(I-C) for 10 min at room temperature prior to the addition of 0.2 ng of labeled probe. The ability of each oligonucleotide to compete for the STAT1α homodimer or STAT3 homodimer was assessed according to the following scale: 0 = no competition, 1 = <50% competition at 50 ng, 2 = >50% competition at 50 ng, 3 = <50% competition at 5 ng, 4 = >50% competition at 5 ng, 5 = <50% competition at 0.5 ng and 6 = >50% competition at 0.5 ng.

Antibody supershift experiments were performed by incubating nuclear extract and antibody for 10 min at room temperature prior to the addition of radiolabeled GAS probe.

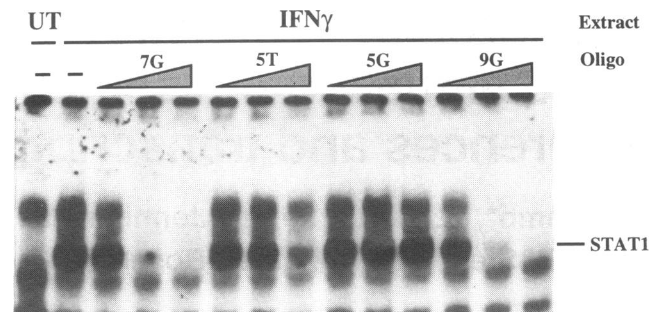

Figure 1. Competition experiment with Ly6E/A mutant oligonucleotides. Increasing amounts of the indicated Ly6E/A mutant oligonucleotides were incubated with extracts from IFN-γ treated cells. Radiolabeled Ly6E/A oligonucleotide was then added, and the reactions resolved on a non-denaturing polyacrylamide gel. The autoradiograph of the gel is shown. The position of the IFN-γ induced STAT1 complex is indicated with an arrow.

Cell extracts, immunoprecipitation and immunoblotting

Cell extracts for immunoprecipitation were prepared by lysing cells in 0.1% NP-40, 0.25% sodium deoxycholate and clarified by centrifugation. Lysates were precleared by incubation with normal rabbit serum at 1:100 and 50 μl of a 50% (v/v) protein-A agarose slurry for 1 h at 4°C. Antigens were then precipitated by incubation with specific sera at 1:100 for 4 h at 4°C followed by the addition of 25 μl of a 50% (v/v) protein-A agarose slurry and further incubation for 30 min. Immune complexes were collected by centrifugation, boiled in SDS-sample buffer and fractionated on 8% polyacrylamide–SDS gels. Proteins were transferred to nitrocellulose and antigen detected by enhanced chemiluminescence (Amersham).

Transient transfection assays

Reporter plasmids were constructed by cloning test SBEs into a modified pZLUC plasmid that contains the HSV TK promoter from –35 to +10 (5). HepG2 cells were seeded at 2×10^5/ml the day before transfection. Cells were exposed to a calcium phosphate precipitate containing 15 μg/ml reporter and 5 μg/ml of the β-galactosidase expressing plasmid pCH110 for 5 h. When included, STAT1α (5), STAT3 (25) or empty expression vectors were present at 10 μg/ml. The medium was then changed and the cells allowed to recover for 16 h. Recombinant cytokines were then added directly to the medium and the cells harvested 5 h later. Cells were lysed and luciferase and β-galactosidase activities determined using standard techniques. For each sample the normalized response was determined by dividing relative light units obtained from the luciferase assay with the β-galactosidase activity in the same lysate as determined using a chromogenic substrate. Each point represents the average normalized response from three transfections. In each case the standard deviation is shown as a line extending above the bar.

RESULTS

In order to gain an understanding of the base pairs important for the binding of STAT1α and STAT3 proteins to DNA, we

Table 1. *In vitro* binding of STAT1α or STAT3 homodimers to mutant Ly6E SBEs

	SEQUENCE	STAT1α	STAT3
WT	TATTCCTGTAAGT	4	4
2G	TGTTCCTGTAAGT	0	1
2C	TCTTCCTGTAAGT	4	3
2T	TTTTCCTGTAAGT	0	0
3A	TAATCCTGTAAGT	1	1
3G	TAGTCCTGTAAGT	1	0
3C	TACTCCTGTAAGT	1	0
4A	TATACCTGTAAGT	3	1
4G	TATGCCTGTAAGT	3	1
4C	TATCCCTGTAAGT	0	0
5A	TATTACTGTAAGT	1	2
5T	TATTTCTGTAAGT	1	1
5G	TATTGCTGTAAGT	0	0
6A	TATTCATGTAAGT	1	0
6T	TATTCTTGTAAGT	3	2
6G	TATTCGTGTAAGT	1	0
7A	TATTCCAGTAAGT	0	0
7G	TATTCCGGTAAGT	4	5
7C	TATTCCCGTAAGT	5	5
8A	TATTCCTATAAGT	4	2
8T	TATTCCTTTAAGT	3	0
8C	TATTCCTCTAAGT	3	1
9A	TATTCCTGAAAGT	0	1
9G	TATTCCTGGAAGT	5	5
9C	TATTCCTGCAAGT	3	1
10G	TATTCCTGTGAGT	1	0
10T	TATTCCTGTTAGT	3	3
10C	TATTCCTGTCAGT	3	3
11G	TATTCCTGTAGGT	2	1
11T	TATTCCTGTATGT	0	0
11C	TATTCCTGTACGT	3	1

The ability of each mutant Ly6E SBE to compete for either STAT1α or STAT3 binding to the labeled wild type Ly6E SBE was assessed on a scale from 0 (no binding) to 6 (strongest binding) as described in Materials and Methods.

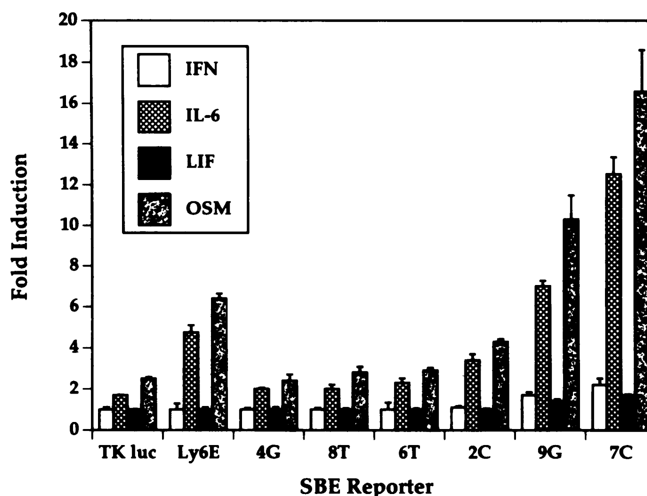

Figure 2. Transcriptional induction mediated by single Ly6E/A wild type and mutant SBEs in response to cytokines. Cells were transfected with reporter plasmids containing a single copy of mutant or wild type Ly6E/A SBEs driving expression of luciferase. Cells were treated with either IFN-γ, IL-6, LIF or OSM as indicated, and induction of transcription over untreated cells determined as described in Materials and Methods. The TK luc reporter lacks an SBE.

undertook a thorough mutational analysis of a natural response element that binds both proteins. We chose to study the SBE from the promoter of the murine Ly6E/A gene. This gene encodes a B-cell surface antigen that is up-regulated by IFN-γ. The region of the promoter required for this regulation has been mapped, and includes a SBE that binds to STAT1α in IFN-γ treated cells (7). We have previously shown that this SBE will bind to STATs activated by several cytokines, including STAT3 activated by IL-6 (28). This element has a moderate affinity for both STAT1α and STAT3 *in vitro*, giving us the opportunity to score both up and down mutants for binding. Double stranded oligonucleotides containing individual point mutations with respect to the wild-type sequence were used in a gel shift competition analyses using extracts derived from HepG2 cells treated with IFN-γ or IL-6. IFN-γ induces STAT 1α and IL-6 induces STAT1α and STAT3 in these cells (see below). An example of one such competition experiment is shown in Figure 1. The ability of each oligonucleotide to compete for binding to STAT1α or STAT3 was assessed as described in Materials and Methods and is summarized in Table 1 [the data for mutants 2G/T/C, 5G/T, 7G/A, 8T and 9G/A/C/T have been published elsewhere and are included for completeness (28)]. The core of the Ly6E/A gene promoter SBE is an imperfect inverted repeat with the sequence TTCC-GTAA. Mutations in the first half of the repeat (positions 3, 4, 5 and 6) are less well tolerated than those in the second half (positions 8, 9, 10 and 11), suggesting that the first half is more important for STAT binding than the second half. Mutation of position 9 to a G, which yields the perfect inverted repeat TTCC-GGAA, shows increased binding to STAT1α and STAT3, whereas mutation 5A, which gives the perfect inverted repeat TTAC-GTAA shows reduced binding to both STATs. These data indicate that the half site TTCC is preferred over GTAA. Mutations at position 7, at the center of the repeat, show a preference for C or G over A or T. Several mutations, particularly those at positions 4 and 8, yield elements that preferentially bind STAT1α and show negligible affinity for STAT3. None of the sequences show a marked preference for binding STAT3 versus STAT1α. In general, STAT3 is more sensitive to mutations in this element than STAT1α.

We evaluated the ability of the wild type Ly6E/A gene promoter SBE as well as selected variants to confer transcriptional regulation on a reporter gene in response to IFN-γ, IL-6, LIF or OSM in HepG2 cells. Single copies of test SBEs were cloned immediately upstream of the HSV TK gene TATA sequence such that they direct expression of a luciferase reporter gene. These constructs were transfected into HepG2 cells, together with a plasmid expressing β-galactosidase as a transfection control, and the cells treated with either IFN-γ, IL-6, LIF or OSM. Cell lysates were made 5 h later, and fold inductions calculated by dividing the luciferase values (normalized to β-galactosidase values in the same samples) from cytokine treated cells by those from untreated controls. The results are shown in Figure 2. In all cases, IFN-γ or LIF treatment of transfected cells gave no induction of luciferase activity. However, both IL-6 and OSM treatment resulted in induction of luciferase activity over background for several of the constructs. The degree of induction by IL-6 or OSM correlated with the ability of the SBE to bind to STAT proteins. The construct containing the 7C SBE, which binds to STATs1α and 3 well, gave the largest inductions, whereas constructs containing SBEs that bind STATs less well, such as 2C and 6T, gave lower inductions. We also tested the ability of single copies of SBEs from the promoters of the IRF-1 gene (8), the anti-chymotrypsin (ACT) gene and the haptoglobin gene (31) to

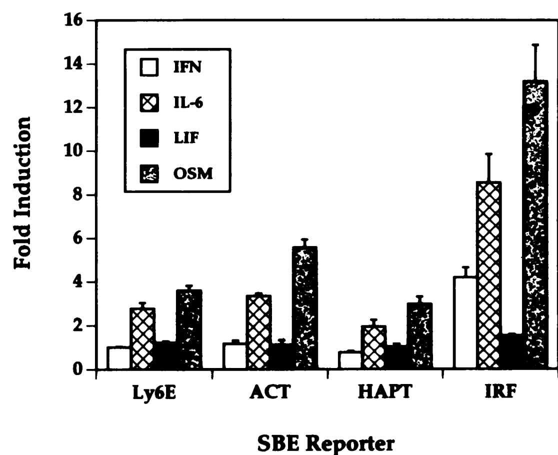

Figure 3. Transcriptional induction mediated by single SBEs from the Ly6E/A, antichymotrypsin (ACT), haptoglobin (Hapt) and IRF-1 gene promoters in response to cytokines. Cells were transfected and treated with the indicated cytokines, and fold inductions calculated as described in Materials and Methods.

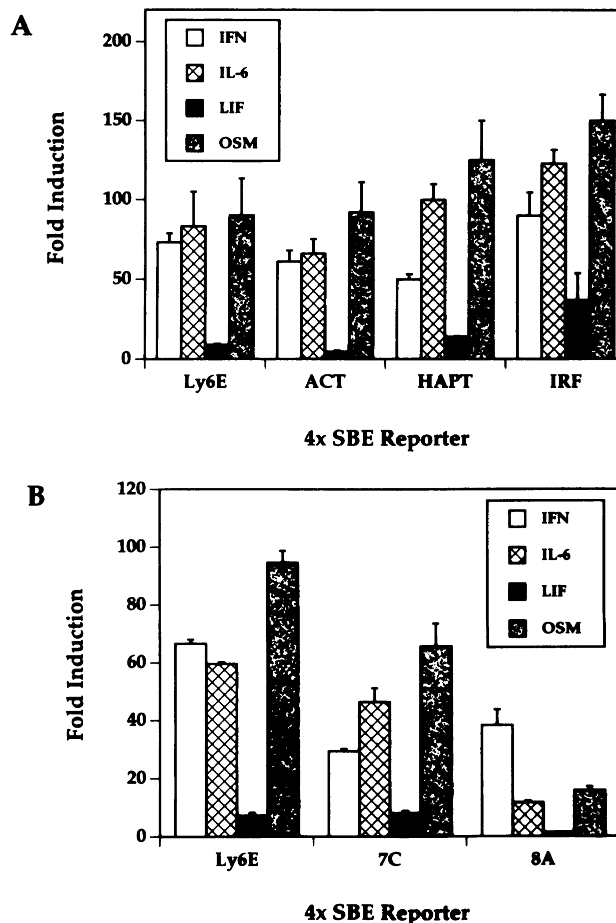

Figure 4. Transcriptional induction mediated by multiple SBEs in response to cytokines. (**A**) Cells were transfected with reporter plasmids containing four copies of the SBEs from either the Ly6E/A, antichymotrypsin (ACT), haptoglobin (Hapt) and IRF-1 gene promoter and treated with the indicated cytokines as in Figure 2. (**B**) Cells were transfected with reporter plasmids containing four copies of either the Ly6E SBE or the Ly6E mutant SBEs 7C or 8A, treated with the indicated cytokines and fold inductions calculated as described in Materials and Methods.

confer cytokine responsiveness to a luciferase reporter. Although these SBEs are related both to each other and the Ly6E/A-derived SBE, their sequences differ at multiple positions. All three elements conferred induction of luciferase activity in response to IL-6 and OSM, the IRF-1 element being the strongest (Fig. 3). As with the Ly6E/A-derived sequences, the fold inductions correlated with the ability of the elements to bind STAT1α and STAT3 *in vitro* (data not shown). The ACT and haptoglobin elements did not respond to IFN-γ; however, the IRF-1 element gave a reproducible induction in response to IFN-γ, consistent with previous reports (8) . None of the elements gave inductions in response to LIF. We conclude from these experiments that IL-6 and OSM, but not IFN-γ or LIF, can efficiently activate transcription from a single SBE in these cells.

We next tested the effect of multimerizing selected SBEs. Reporter constructs containing four copies of SBEs 7C, 8A, Ly6E/A, IRF-1, ACT and haptoglobin were assayed for responsiveness to the four test cytokines (Fig. 4). All constructs were responsive to IL-6 and OSM, and gave much larger inductions than the constructs with a single SBE. The degree of induction is not always related to the ability of the SBE to bind to STATs *in vitro*, however. Thus the 4× 7C SBE gives lower inductions than the wild type 4× Ly6E/A SBE, despite binding STATs1α and 3 better *in vitro*. This is the result of increases in the level of transcription in the absence of cytokine that are larger than the increases in the presence of cytokine, which results in a lowered fold induction. In contrast to the results with single elements, the constructs also gave sizable inductions in response to IFN-γ. Only the 4× IRF-1 construct showed an appreciable induction in response to LIF. We also observed differences between the 4× 8A construct and the other constructs with respect to the ratio of IFN-γ to IL-6 or OSM induction. The 4× 8A construct gave reproducibly larger inductions in response to IFN-γ than to IL-6 or OSM, in contrast to the other constructs tested.

The differential ability of IFN-γ, IL-6, OSM and LIF to activate transcription from SBE-containing promoters prompted us to characterize the STATs induced by these cytokines in HepG2 cells. Cells were treated with either IFN-γ, IL-6, OSM or LIF, and lysates were immunoprecipitated with either anti-STAT1 or anti-STAT3 sera. The immunoprecipitates were fractionated on SDS–polyacrylamide gels, blotted and proteins detected using a phosphotyrosine antibody. As controls, aliquots of the same immunoprecipitations were blotted and detected with either anti-STAT1 or anti-STAT3 sera. The results are shown in Figure 5A. IFN-γ induces rapid tyrosine phosphorylation of STAT1α but no detectable phosphorylation of STAT3. In contrast, LIF induces tyrosine phosphorylation primarily of STAT3, with very low amounts of STAT1α becoming phosphorylated. IL-6 and OSM induce tyrosine phosphorylation of both STAT1α and STAT3, OSM inducing slightly more phosphorylation of both STATs than IL-6. These patterns of STAT activation were confirmed by gel retardation and antibody supershift experiments, shown in Figure 5B. IFN-γ induces a complex that supershifts with anti-STAT1α but not anti-STAT3 sera; LIF induces a complex that supershifts with the anti-STAT3 but not anti-STAT1α sera; IL-6 and OSM

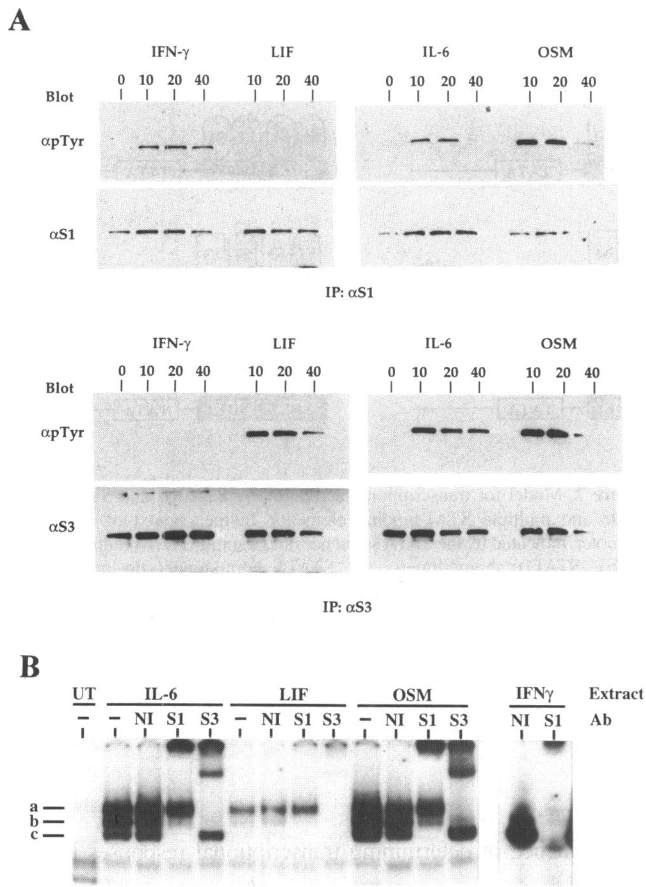

Figure 5. (**A**) Tyrosine phosphorylation of STAT1α and STAT3 in response to cytokines. Cells were treated with either IFN-γ, LIF, IL-6 or OSM for the indicated time, and lysates prepared. Lysates were immunoprecipitated with either a STAT1 antisera (top panels) or a STAT3 antisera (bottom panels). After resolution on SDS–polyacrylamide gels and blotting, proteins were detected with antisera directed against either phosphotyrosine, STAT1 or STAT3 as indicated at the left of the figure. (**B**) Alternately, nuclear extracts were prepared from the cytokine-treated cells and used in a gel retardation analysis. Extracts were incubated with a radiolabeled GAS probe in the presence either no antisera (–), non-immune sera (NI), anti-STAT1 sera (S1) or anti-STAT3 sera (S3). Protein–DNA complexes were then resolved on non-denaturing polyacrylamide gels.

induce three complexes, the fastest migrating of which supershifts with anti-STAT1α sera, the slowest migrating supershifts with anti-STAT3 sera and the complex of intermediate mobility supershifts with both sera. Consistent with previous data, we interpret these results as meaning that IFN-γ activates a homodimer of STAT1α (complex c, Fig. 5B), LIF induces primarily a homodimer of STAT3 (complex a, Fig. 5B) and IL-6 and OSM induce homodimers of STAT1α and STAT3 as well as heterodimers of STAT1α and STAT3 (complex b, Fig. 5B). In these experiments we do not detect activation of STATs other than STATs 1α and 3. The differences in the ability of IFN-γ, IL-6, OSM and LIF to activate transcription from SBE containing reporters is therefore accompanied by differences in their ability to activate STAT1α, STAT 3, or both.

In a final set of experiments we tested the effect of overexpressing either STAT1α, STAT3 or both, on the ability of a single IRF-1 SBE to respond to IFN-γ, OSM or LIF (Fig. 6).

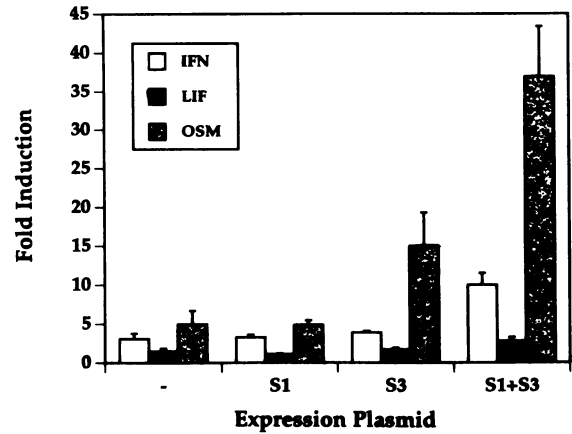

Figure 6. Effect of STAT overexpression on transcriptional induction mediated by a single SBE. Cells were transfected with IRF-1 TK luc together with either empty expression vector (–), a STAT1α expression vector (S1), a STAT3 expression vector (S3) or both STAT1α and STAT3 expression vectors (S1+S3). Cells were then treated with the indicated cytokine and fold inductions calculated as described in Materials and Methods.

Cotransfection of the expression vector alone reduced the fold-activation in response to OSM slightly (compared with Fig. 3), but did not alter the pattern of activation. Overexpressing STAT1α had no effect on induction by any cytokine. Overexpressing STAT3 slightly increased the response to OSM. Overexpressing both STATs together resulted in a modest increase in the response to IFN-γ [perhaps as a consequence of the ability of IFNγ to activate STAT3 in some circumstances, (22)], but a substantial increase in the response to OSM (from 5- to 37-fold over untreated cells). These increases in fold induction were due to an increase in the induced levels of luciferase activity, not to a decrease in the uninduced level. Similar results were obtained using the Ly6E/A7C reporter (data not shown). Overexpression of STATs did not alter the response to LIF, which remained negligible. These results show that increasing the levels of both STAT1α and STAT3 results in an increased induction of single SBEs in response to a cytokine that activates both STATs, but does not effect induction in response to cytokines that activate predominately one STAT. The levels of both STATs must be elevated to produce this effect fully; increasing the level of either STAT alone had a minimal effect on induction.

DISCUSSION

Analysis of the Ly6E/A SBE

The SBE in the Ly6E/A gene promoter lies in a region of the promoter required for induction of the gene by IFN-γ and binds to STAT1α in response to IFN-γ treatment of cells (7). We tested the sequences required for the binding of both STAT1α, induced by IFN-γ, and STAT3, induced by IL-6, to this element. Our mutational analysis revealed the existence of both up and down mutants with respect to STAT binding, as well as mutations that differentially affected binding of STAT1α and STAT3. The palindromic structure of the element, observed in many SBEs identified to date (1,8,9,24,32,33) is important for efficient binding to STAT proteins since many mutations that impair the palindrome also reduce binding of STATs 1α and 3. In contrast,

mutations that improve the palindrome increase STAT binding. However, STAT3 has a greater dependence on an intact palindromic structure than STAT1α, since several mutations exist in both half sites which severely curtail STAT3 binding but still allow STAT1α binding (though at reduced levels). This observation demonstrates that selectivity at the level of STAT binding can be achieved by subtle changes in the sequence of a SBE. These results suggest that specificity with respect to cytokine induction of gene expression can be influenced by the exact sequence of the SBE(s) in the control regions of the induced genes. Differences in the ability of SBEs from the promoters of several genes to bind to STAT1α and STAT3 are evident in some previous studies, although the basis for these differences was not established (24,32,33).

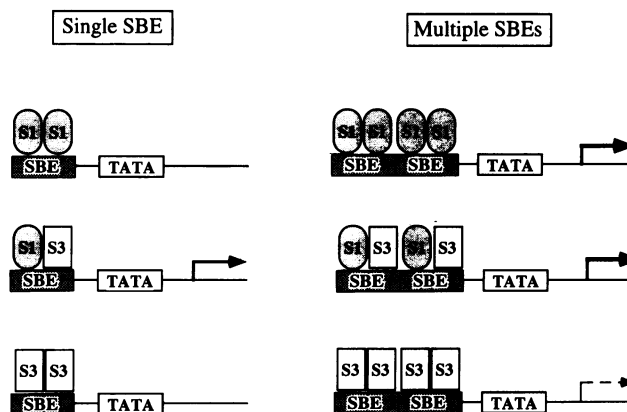

Figure 7. Model for transcriptional activation by STAT1α and STAT3 from single and multiple STAT-binding elements. In the context of a minimal promoter, indicated by the TATA sequence, and a single STAT-binding element (SBE), STAT1α homodimers and STAT3 homodimers do not activate transcription. However, STAT1α/STAT3 heterodimers do activate transcription, indicated by the arrow. In the context of a minimal promoter and multiple SBEs, both STAT1α homodimers and STAT1α/STAT3 heterodimers now activate transcription efficiently, shown by the bold arrow. Other arrangements, such as a homodimer of STAT1α adjacent to a homodimer of STAT3 may also contribute to transcriptional activation. STAT3 homodimers activate transcription weakly, depicted by the dashed arrow.

Transcriptional activation mediated by natural and mutant SBEs

Our data show that both the sequence and arrangement of SBEs in a promoter effect its inducibility by cytokines. All the single SBEs tested mediated induction in response to IL-6 and OSM in the context of the TK minimal promoter, with the degree of induction closely following the ability of the elements to bind to STATs. Strikingly, none of the elements showed any induction in response to LIF and only one, the SBE from the IRF-1 gene promoter, mediated a modest induction in response to IFN-γ. In contrast, multimerizing the SBEs yielded promoters capable of large inductions in response to IFN-γ, and amplified the responses to IL-6 and OSM. We note that the SBE from the murine ICSBP gene promoter also responds to IFN-γ only when multimerized (34). Only one of the multimerized SBEs tested gave an appreciable response to LIF, the IRF-1 gene promoter-derived SBE. The pattern of cytokine induction was radically changed by changing the number of SBEs from 1 to 4. This shows that the number of SBEs in a gene promoter can influence the cytokine response of the promoter both in terms of its magnitude and its selectivity. Promoters with both single and multiple SBEs have been described (7–9,35) but the role of the arrangement of SBEs in these promoters on cytokine selectivity awaits further study. The inability of an isolated Ly6E/A gene promoter SBE to respond to IFN-γ suggests that in the Ly6E/A promoter, other sequences as well as the recognized SBE form the functional IFN-γ response element. The IFN-γ responsive region of this promoter may be similar to that of the FcγR1 gene, which contains an SBE closely linked to the binding site for a second protein, both of which are required for IFN-γ inducibility (9). This suggests two arrangements that confer IFN-γ inducibility to a promoter; multimerization of an SBE, or a combination of an SBE with bindings sites for other cooperating proteins. In the context of natural promoters, the binding of adjacent transcription factors may therefore exert a considerable influence on the response of a gene to different cytokines.

The data also show that the sequence of the SBE can influence the relative inducibility of a promoter to different cytokines. For example, Ly6E/A mutant 8A responds better to IFN-γ than IL-6 or OSM when multimerized. In contrast, the multimerized wild type Ly6E/A or mutant 7C SBEs respond better to IL-6 and OSM than IFN-γ. This probably reflects the preferential binding of the 8A mutant to STAT1α versus STAT3 containing complexes. Results with a series of synthetic SBEs also demonstrate the importance of SBE sequence in determining transcriptional responses to cytokines (36).

Differential STAT activation by cytokines in HepG2 cells

The ability of cytokines to activate transcription from minimal promoters containing single and multiple SBEs depends on the particular pattern of STAT proteins activated by each cytokine. In our HepG2 subline, IFN-γ activates predominantly STAT1α, LIF activates predominantly STAT3, and IL-6 and OSM activate both STAT1α and STAT3. IFN-γ and LIF do not activate transcription from single SBEs, indicating that activation of STAT1α or STAT3 alone is insufficient to promote transcription from a single SBE in the context of the minimal TK promoter. Only IL-6 and OSM, which efficiently activate both STAT1α and STAT3, activate transcription from a single SBE. Since a single SBE can bind to only a single STAT dimer, we conclude that STAT1α/STAT3 heterodimers formed in response to IL-6 and OSM (19,22,25) treatment are responsible for the activation of transcription via a single SBE. This interpretation is supported by the observation that transfection of both STAT1α and STAT3 expression vectors greatly increases the response of a promoter with a single SBE to OSM, but transfection of either expression vector alone has a minimal effect. This suggests that there may be some synergy or cooperation between the transcriptional activation domains of STAT1α and STAT3 that allows a single heterodimer to activate the general transcriptional machinery more effectively than single homodimers. In contrast, multimerized SBEs direct high levels of reporter expression in response to IFN-γ as well as IL-6 and OSM, but still show poor induction in response to LIF. Thus in the context of a minimal promoter containing multiple SBEs, STAT1α homodimers activate transcription more efficiently than STAT3 homodimers. These results are summarized in Figure 7.

Many cytokines activate STAT complexes that bind to SBEs. However, different cytokines induce the expression of distinct sets of genes, which raises the question of how activation of STAT complexes contributes to differential regulation of gene expression. Our results demonstrate that the identity of the STAT proteins present in these STAT complexes determines both their precise sequence specificity and their ability to activate transcription. These observations suggest that differential activation of STAT proteins can play a significant role in mediating selective regulation of gene expression in response to cytokines.

ACKNOWLEDGEMENTS

We thank Dr J. E. Darnell (Rockefeller University, NY) and Dr D. Levy (NYU Medical Center, NY) for gifts of reagents and for useful discussions. We would also like to thank our colleagues at Ligand, especially Jeff Miner, Peter Tapley and Shin-Shay Tian for helpful discussions.

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