Synergistic Activity of STAT3 and c-Jun at a Specific Array of DNA Elements in the α2-Macroglobulin Promoter*

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The transcriptional activity of natural promoters is sensitive to the precise spatial arrangement of DNA elements and their incorporation into higher order DNA-protein complexes. STAT3 and c-Jun form a specific ternary complex in vitro with a synthetic DNA element containing AP1 and SIE sites. These associations are critical for synergistic activation of transcription from a synthetic promoter by STAT3 and c-Jun. Expression of the acute phase protein α2-macroglobulin is induced in vivo by interleukin-6 (IL-6)-related cytokines; we demonstrate that coordinate interactions among STAT3, c-Jun, and a specific array of DNA elements contribute to activation of the α2-macroglobulin promoter in response to IL-6 family members. At least five promoter elements are involved in activation: two AP1 sites at −113 to −107 and −152 to −140, an acute phase response element (APRE (SIE)) at −171 to −163, and two AT-rich regions at −143 to −138 and −128 to −123. Synergism between STAT3 or STAT3-C and c-Jun is impeded by mutation of the APRE (SIE) or either AP1 site, as well as by mutations that alter the AT-rich regions or their phasing. Mutations of STAT3 previously shown to disrupt physical and functional interactions with c-Jun do not disrupt synergy between STAT3-C and c-Jun at the α2-macroglobulin promoter in HepG2 cells, suggesting that STAT3-C and STAT3 differ with respect to their precise contacts with c-Jun.

Signal transducers and activators of transcription (STATs) play central roles in the induction of gene expression by a diverse variety of signaling pathways involving cytokines, growth factors, and peptide hormones. Cytokine receptor-associated protein-tyrosine kinases of the JAK family phosphorylate STAT proteins upon binding of cytokines to their cognate receptors (1–3). The phosphorylated STATs then form dimers via their SH2 domains and rapidly translocate from the cytoplasm to the nucleus, where they bind regulatory DNA elements of target genes (4–7). STAT proteins thereby exert effects on a number of fundamental biological processes, including cell proliferation, differentiation, apoptosis, and development (8, 9).

STAT3 was originally identified in mouse liver as an acute phase response factor that binds an interleukin-6 (IL-6)-responsive element in the α2-macroglobulin gene (10, 11). IL-6 is a multifunctional cytokine that positively regulates T cell proliferation; differentiation of B cells, macrophages, and megakaryocytes; production of acute phase proteins by hepatocytes; and bone resorption (12–16). The IL-6 receptor consists of a ligand-binding α chain (IL-6Ra) and gp130, a signal transducer shared by related cytokine receptors, including those for ciliary neutrophic factor, oncostatin M (OSM), leukemia inhibitory factor (LIF), cardiotoxine-1, and IL-11 (1, 3). STAT3 is also phosphorylated on tyrosine in response to epidermal growth factor (EGF), platelet derived growth factor, hepatocyte growth factor, granulocyte colony stimulating factor, thrombopoietin, leptin, and IL-10 (17, 18–22).

In mammals, the acute phase response (APR) is invoked in response to tissue injury, trauma, or infection (16, 22). During the early stages of inflammation serum concentrations of several acute phase response proteins increase as much as 1000-fold. These increases are triggered by glucocorticoids or by cytokines such as IL-1, IL-6, tumor necrosis factor α, and interferon γ (IFN-γ) (23, 24). A number of APR genes contain both IL-1- and IL-6-responsive elements, suggesting that these signaling pathways act in synergy to induce APR gene transcription (25, 26). Glucocorticoids exert a synergistic effect on the IL-6-mediated inflammatory response through a direct interaction between IL-6-activated STAT3 and ligand-bound glucocorticoid receptor (27–30).

Physiologic enhancers serve as substrates for the assembly of multicomponent complexes containing transcriptional regulators and proteins that modify DNA structure. Such DNA-protein complexes, termed enhanceosomes, exhibit specific patterns of spatial organization. Enhanceosomes in the genes encoding the T-cell receptor α-chain and interferon β (IFN-β) have been studied extensively (31–35). Two pairs of AT-rich sequences positioned in-phase on the IFN-β enhancer are required for cooperative binding of the IFN-β enhancer are required for cooperative binding of the HMG-I(Y) and enhancement of transcriptional activity in vivo (34). Formation of a stable ternary complex of transcription factors on the T-cell receptor α-chain enhancer and enhancer activity in nonlymphoid cells requires the lymphoid-specific, HMG domain-containing protein LEF-1, which facilitates interactions between proteins bound at nonadjacent sites (33). Similarly, STAT3 and Smad1, when bridged by the transcriptional coactivator p300, are reported to exert a synergistic effect on transcription from the glial fibrillary acidic protein promoter, thereby inducing astro-

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The abbreviations used are: STAT, signal transducers and activators of transcription; IL-6, interleukin-6; IL-6Ra, IL-6 receptor with a ligand-binding α chain; OSM, oncostatin M; LIF, leukemia inhibitory factor; EGF, epidermal growth factor; APR, acute phase response; IFN, interferon; GST, glutathione S-transferase; α2-MG, α2-macroglobulin; APRE, acute phase response element; TK, thymidine kinase; bp, base pair(s); EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; TKR, T346A,K348A,R550A triple mutant; HMG, high mobility group; HMG-I(Y), high mobility group protein-I(Y).
cytotoxicity of primary fetal neural progenitor cells (36).

Direct interactions between the c-Jun and STAT3 proteins have been detected using the yeast two-hybrid system or an in vitro GST precipitation assay (37, 38). Our laboratory has reported that c-Jun and a carboxyl-terminal-truncated form of STAT3 (STAT3β) show synergistic activation of the IL-6-responsive α2-macroglobulin (α2-MG) promoter in transfected F9 and EGF-stimulated COS-7 cells (37). The α2-MG promoter contains binding sites for STAT3 (acute phase response element [APRE]; TTCTGGGAAA) and AP1 (TGAACCTC) (39). These are separated by 49 base pairs (39–41). Synergistic activation of the α2-MG promoter by the same transcription factors has also been reported upon IL-6 stimulation of HepG2 cells (38). Here we present evidence that sequence-specific DNA and protein interactions at a specific array of DNA elements in the α2-MG promoter are critical for cooperative transcriptional activation of the α2-MG transcription by STAT3 and c-Jun. Our observations suggest that members of the IL-6 cytokine family induce formation of an α2-MG enhancerosome containing activated transcription factors in a specific spatial arrangement.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Human HepG2 cells were maintained in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum (HyClone). Mouse fibroblast NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells were plated at 2 × 10^5 cells per well in 24-well plates. On the day following, the cells were transfected with DNA (up to 1.5 μg per well) and LipoFectAMINE (Life Technologies, Inc.) in triplicate. The plasmid pRL-TK (Promega) was cotransfected to assess transfection efficiency. Twenty hours after transfection, cells were transferred to medium containing 0.5% fetal bovine serum and incubated overnight. Cells were then stimulated by addition of IL-6 (10 ng/ml), LIF (10 ng/ml), or OSM (10 ng/ml) in the presence of 0.5% fetal bovine serum for 16 h as indicated. IL-6, LIF, and OSM were purchased from R&D Systems. Luciferase was assayed according to the manufacturer’s directions (Promega).

Plasmids and Mutagenesis—Plasmids encoding STAT3 and c-Jun in the mammalian expression vector pYN3218 have been described previously (37, 39). Expression plasmids for STAT3C, STAT3C-TKR, STAT3C-L148A, and STAT3C-V151A were generated in pYN3218 by previously (37, 39). Expression plasmids for STAT3, STAT3C, STAT3C-TKR, the mammalian expression vector pYN3218 have been described previously (37, 39). Expression plasmids for STAT3, STAT3C, STAT3C-TKR, the mammalian expression vector pYN3218 have been described previously (37, 39).

To understand better the mechanism of cooperation between STAT3 and c-Jun, we first examined the binding of STAT3 and c-Jun in vitro to an oligonucleotide containing binding sites for both proteins. Polyhisidine-tagged STAT3β and GST-fused c-Jun proteins were expressed separately in Sf9 cells and COS-7 cells, respectively. STAT3β was activated by co-infection with baculovirus-encoded Jak1 or Jak2. STAT3β or GST-fused c-Jun protein each exhibited specific binding to an AP1/SIE-containing DNA probe

RESULTS

DNA Binding Activity in Vitro—STAT3β, a truncated isoform of STAT3 lacking the carboxyl-terminal 55 amino acid residues, cooperates with c-Jun in transcriptional activation of the α2-MG promoter in EGF-stimulated COS-7 cells (37). To understand better the mechanism of cooperation between STAT3 and c-Jun, we first examined the binding of STAT3 and c-Jun in vitro to an oligonucleotide containing binding sites for both proteins. Polyhisidine-tagged STAT3β and GST-fused c-Jun proteins were expressed separately in Sf9 cells and COS-7 cells, respectively. STAT3β was activated by co-infection with baculovirus-encoding Jak1 or Jak2. STAT3β or GST-fused c-Jun protein each exhibited specific binding to an AP1/SIE-containing DNA probe...
when assayed by EMSA (Fig. 1A, lanes 1–3). In addition, a second, more slowly migrating species was observed when STAT3β and GST-c-Jun were combined with the AP1/SIE probe (Fig. 1A, lanes 6 and 7). This slower species was not seen in control reactions containing STAT3β and GST protein (Fig. 1A, lanes 4 and 5). Mutation of either the STAT3 binding site (AP1/SIE(−)) or c-Jun binding site (AP1(−)/SIE) abolished this effect (Fig. 1A, lanes 8–21), whereas mutation of both sites (AP1(−)/SIE(−)) eliminated all specific binding (Fig. 1A, lanes 22–28). Antibodies against STAT3, GST, or c-Jun all decreased the yield of the slow mobility complex while inducing the appearance of supershifted species (Fig. 1B, compare lanes 5–7 with lane 4). In comparison, a DNA-protein complex containing c-Jun alone was disrupted by the antibody against c-Jun but not by the anti-STAT3 antibody (data not shown). These results indicated that both STAT3β and GST-c-Jun were present in the slow mobility complex. We next examined STAT3α and c-Jun proteins for cooperative DNA binding activity in vitro, using the AP1/SIE oligonucleotide as a probe. As was seen for STAT3β, incubation of the AP1/SIE probe with STAT3α and c-Jun was associated with the appearance of a slowly migrating complex (Fig. 1C, compare lanes 2 and 3 with lane 4). Mutation of either DNA binding site abolished formation of the slower complex (Fig. 1C, lanes 5–12), and mutation of both binding sites abolished specific binding altogether (Fig. 1C, lanes 13–16). Taken together, these results indicate that the formation in vitro of DNA-protein complexes containing STAT3 and c-Jun requires specific DNA binding by both proteins.

AP1 and SIE Elements Synergistically Support Cytokine-induced Transcription from Artificial Promoters—We next attempted to correlate the results of in vitro DNA binding experiments with the transcriptional activities of endogenous STAT3 and c-Jun proteins in cytokine-stimulated NIH3T3 or HepG2 cell lines. For this purpose, we assembled a luciferase reporter construct (pGL3-AP1/SIE-luc) in which transcription is driven by an artificial promoter containing an SIE (TTCCCGTAA) and an AP1 site (TGACTCA), separated by 11 bp. Additional reporter constructs contained mutations at the AP1 site (pGL3-AP1(−)/SIE-luc), the SIE (pGL3-AP1/SIE(−)) luc, or both (pGL3-AP1(−)/SIE(−)-luc). Wild-type or mutant constructs were transfected into NIH3T3 or HepG2 cells, and luciferase activity was assayed in response to stimulation with IL-6, LIF, or OSM (Fig. 2A, A and B).

Treatment of NIH3T3 cells with IL-6 resulted in a 5-fold induction of luciferase expression from the standard construct, whereas LIF or OSM induced increases of 10- to 18-fold (Fig. 2A, AP1/SIE-luc). Mutation of the c-Jun binding site (AP1(−)/SIE-luc) reduced the response to IL-6, LIF, or OSM to about 15% that seen for pGL3-AP1/SIE-luc (Fig. 2A, AP1(−)/SIE-luc); the residual inducible response may reflect the binding of active, endogenous STAT3 to the intact SIE site. Mutation of the STAT3 binding site (AP1/SIE(−)-luc) reduced induction of luciferase activity by IL-6, LIF, or OSM to about 30% that seen for pGL3-AP1/SIE-luc (Fig. 2A, AP1/SIE(−)-luc); the residual induction may in part result from endogenous protein binding to the AP1 site. Mutation of both the AP1 site and the SIE resulted in a near complete loss of responsiveness to cytokines (Fig. 2A, AP1(−)/SIE(−)-luc). These observations indicate that the SIE and AP1 sites interact synergistically in
Synergy between STAT3 and c-Jun

the induction of transcription from pGL3-AP1/SIE-luc by IL-6, LIF, or OSM in NIH3T3 cells.

Treatment of HepG2 cells with OSM or IL-6 resulted in a 2- to 3-fold induction of AP1/SIE-luc reporter expression, whereas LIF had little effect on reporter activity (Fig. 2B, AP1/SIE-luc). The IL-6 and OSM induction ratios were lower than those seen in NIH3T3 cells; this difference may be due in part to the higher basal activity of the AP1/SIE-luc reporter in HepG2 cells. Mutation of the c-Jun or STAT3 binding sites reduced induction to between 10 to 25% that seen with the AP1/SIE-luc plasmid (Fig. 2B, AP1(−)/SIE-luc and AP1/SIE(−)/SIE-luc). Cyto-

tine-induced transcription from the artificial AP1/SIE promoter was completely abolished by mutations of both STAT3 and AP1 binding sites in HepG2 cells (Fig. 2B, AP1(−)/SIE(−)/SIE-luc). These results suggested that cooperative binding of STAT3 and c-Jun might underlie the synergism between SIE and AP1 elements in supporting induction of AP1/SIE-luc transcription by cytokines.

STAT3 and c-Jun Binding Sites Cooperate in IL-6-dependent Activation of the α2-MG Promoter—The rat α2-MG promoter contains an IL-6-responsive APRE sequence and an AP1 site, both of which are required for IL-6-inducible transcription (39, 41, 45). To examine STAT3 and c-Jun interactions at this physiological promoter, we constructed a reporter plasmid (pGL3-α2MG-luc) that contains the luciferase coding region under control of the rat α2-MG promoter sequence from −100 to −100 (Fig. 3A). The pGL3-α2MG-luc plasmid was transfected into HepG2 cells, and luciferase expression was assayed after stimulation with IL-6 or OSM. Transcriptional activity was markedly increased upon treatment with IL-6 (6-fold) or OSM (9-fold) (Fig. 3B, α2MG-luc). No induction was seen with the control plasmid pGL3-TKm-luc, which lacks α2-MG promoter elements (Fig. 3B, pGL3-TKm).

Mutation of the APRE site of the α2-MG promoter (Fig. 3A, SIEm) abolished cytokine induction of luciferase expression (Fig. 3B, α2MG-SIEm-luc), consistent with the interpretation that STAT3 binding to the APRE site is essential for activation of the α2-MG promoter by IL-6 or OSM. To determine whether an intact APRE could support IL-6-mediated α2-MG transcription in the absence of an AP1 site, the c-Jun binding site in the α2-MG promoter was mutated (Fig. 3A, AP1m). Despite the presence of an intact STAT3 binding site, transcription of the AP1 mutant reporter was decreased to 30% of the levels supported by the intact α2-MG sequence in the presence of IL-6 or OSM (Fig. 3B, α2MG-AP1m-luc). Transcription of the reporter construct containing intact AP1 and SIE sites was 3-fold greater than the additive effects of the AP1 and SIE sites alone, suggesting that STAT3 and AP1 binding sites cooperate in activation of the α2-MG promoter by IL-6 or OSM.

Upon inspection, the −190 to −100 region of the α2-MG promoter was found to contain an additional potential AP1 site (AP1*, at −152 to −140) and two AT-rich regions (AT1 and AT2, at −143 to −138 and −128 to −123, respectively) (Fig. 3A). A duplex oligonucleotide corresponding to the AP1* site and flanking sequences from the α2-MG promoter was found by EMSA to bind purified c-Jun protein (Fig. 3C, lanes 2 and 4 versus lane 1, no protein added). Binding is specific, because it was abolished in the presence of excess unlabeled, wild-type AP1* oligonucleotide, but not by a mutant (AP1*m) oligonucleotide (Fig. 3C, lanes 3 and 5). A number of mammalian proteins, including those of the high-mobility group (HMG), are known to bind AT-rich regions (44). A duplex oligonucleotide spanning both AT-rich sites of the α2-MG promoter was specifically bound by one or more proteins from nuclear extracts of HepG2 cells maintained in serum with or without OSM (Fig. 3D, lanes 2 and 3 versus lane 1, in which no protein was added). This binding was abolished by excess unlabeled, wild-type oligonucleotide but not by an oligonucleotide carrying mutant AT-rich sites (Fig. 3D, lanes 4 and 5).

These sequence elements were tested for involvement in IL-6- or OSM-mediated transcription of α2-MG promoter. Mutation of the AP1* site (Fig. 3A, AP1*m), like mutation of the canonical AP1 site, reduced transcriptional activity to about 30% that of the unmutated reporter construct (Fig. 3B, α2MG-AP1*m-luc), indicating that AP1* sites also contribute to α2-MG promoter activation by IL-6 or OSM. Moreover, the effect of either single AP1 mutation was equivalent to ablation of both AP1 sites: double mutation of the AP1 and AP1* sites impaired transcriptional activity no more than did the single site mutations (Fig. 3B, compare α2MG-AP1mAP1*m-luc with α2MG-AP1m- or α2MG-AP1*m-luc). Thus, the presence of both AP1 sites is essential for full promoter activity. Surprisingly, mutation of either AT-rich region (Fig. 3A, AT1m and AT2m) had a significant debilitating effect, reducing transcription to between 20 and 60% that of the unmutated control (Fig. 3B, α2MG-AT1m-luc and α2MG-AT2m-luc); mutation of both AT-rich regions nearly abolished induction of transcription by IL-6 or OSM (Fig. 3B, α2MG-AT1/2m-luc).

We then asked whether the AT-rich regions could cooperate with AP1 or SIE elements in formation of DNA-protein complexes on the α2-MG promoter. Four prominent protein complexes (I–IV) were formed with a 105-base pair DNA fragment.
containing the minimal αα-MG promoter (−190 to −100) in nuclear extracts of OSM-treated, HepG2 cells (Fig. 3E, lane 1). Formation of complex I was dependent on the AT-rich elements, as this species was not observed with probes containing mutated AT-rich regions (Fig. 3E, lanes 3 and 4), consistent with formation of protein-DNA complexes involving the AP1 and AT-rich elements of the αα-MG promoter. The yield of complex IV was somewhat diminished by mutation of the SIE site (Fig. 3E, lane 2), possibly because the effects of transfected c-Jun were blunted by endogenous STAT3 or c-Jun.

STAT3-C, a highly active form of STAT3 that constitutively dimerizes, binds to DNA, and activates transcription (42). As previously reported (42), overexpressed STAT3-C exhibited constitutive transcriptional activity when assayed using an artificial SIE-containing promoter (data not shown). We proceeded to ask whether overexpressed STAT3-C acts synergistically with c-Jun in activating transcription from the αα-MG promoter in HepG2 cells. In the absence of cytokine stimulation, STAT3-C was unable to stimulate transcription from the

**Fig. 3.** Cooperativity between STAT3 and c-Jun DNA binding sites in transcription from the αα-MG promoter. A, nucleotide sequence of the αα-MG promoter in the interval ranging from −190 to −100. AP1 and APRE sites, an additional potential AP1 site (AP1*) and two AT-rich regions (AT1 and AT2) are marked in boldface and underligned. The nucleotide sequences of mutated sites (SIEm, AP1*m, AT1m, AT2m, and AP1m) are given in lowercase letters. B, HepG2 cells were transfected in 24-well plates with 1 μg of each reporter plasmid, as indicated, and 50 ng of pRL-TK. After 24-h serum starvation, cells were left untreated (Unstim.) or treated for 9 h with 10 ng/ml IL-6 or OSM. Luciferase activity was determined; means and standard deviations (n = 3) are indicated. Results are representative of five independent experiments. C, binding of purified c-Jun to the AP1* site. End-labeled oligonucleotides spanning the AP1* site were incubated in the absence of protein (lane 1) or with 1 μg of c-Jun (lanes 2–5). Where indicated, 100-fold excess unlabeled wild-type (AP1*, lane 3) or mutant (AP1*m, lane 5) oligonucleotide probe was added. DNA-protein complexes were separated on a 4% polyacrylamide gel. D, a radiolabeled oligonucleotide probe spanning the AT-rich sites of the αα-MG promoter was incubated with nuclear extract (10 μg of total protein) from HepG2 cells maintained in serum without OSM stimulation (lane 2) or stimulated with 10 ng/ml OSM (lanes 3–5). A control incubation was carried out in the absence of protein (lane 1). Where indicated, 100-fold excess unlabeled wild-type (AT, lane 4) or mutant (ATm, lane 5) oligonucleotide probe was added. DNA-protein complexes were separated on an 8% polyacrylamide gel. E, nuclear extracts (10 μg) from OSM-stimulated HepG2 cells were mixed with a 105-bp, end-labeled DNA duplex, including residues −190 through −100 of the αα-MG promoter. Lane 1, wild-type; lane 2, mutant SIE; lane 3, mutant AP1 and AP1*; lane 4, mutant AT1 and AT2. DNA-protein complexes were resolved on a 6% polyacrylamide gel.
Synergism between STAT3-C and c-Jun at the α2-MG promoter.

A. Synergism between STAT3 and c-Jun. HepG2 cells were transfected in 24-well plates with 0.5 μg of α2-MG-luc, 50 ng of pRL-TK, and 0.3 μg of STAT3, 0.3 μg of c-Jun or both, as indicated. After 24-h serum starvation, cells were left untreated (Unstim.) or treated with 10 ng/ml OSM for 16 h and assayed for luciferase activity. B. Synergism between STAT3-C and c-Jun. HepG2 cells were transfected in 24-well plates with 0.5 μg of α2-MG-luc, 50 ng of pRL-TK, the c-Jun expression vector (0–0.5 μg), and 0.5 μg of STAT3-C or control vector. After 24-h serum starvation, cells were left untreated (Unstim.) or treated with 10 ng/ml OSM for 16 h. C. Synergism between STAT3-C and c-Jun was dependent on the presence of their specific DNA binding sites. HepG2 cells were transfected in 24-well plates with 0.5 μg of the indicated reporter plasmid, 50 ng of pRL-TK, and the c-Jun expression vector (0–0.5 μg), with or without 0.5 μg of the STAT3-C expression vector. The total amount of DNA per well was adjusted to 1.5 μg with empty vector. After 24-h serum starvation, cells were treated for 16 h with 10 ng/ml OSM. Means and standard deviations (n = 3) are indicated. Results are representative of two independent experiments.

α2-MG promoter (−190 to −100) (Fig. 4B, Unstim.). This stands in contrast to a report that STAT3-C constitutively activates transcription from the α2-MG promoter (−1151 to +54) in 293T cells (42); the difference may reflect the presence of additional regulatory elements outside of the −190 to −100 interval. Increasing amounts of c-Jun had no effect on transcription of α2-MG-luc in the presence or absence of STAT3-C in unstimulated HepG2 cells (Fig. 4B, Unstim.). Transcription of pGL3-α2M-luc was induced 6-fold by OSM stimulation, and this was unaffected by increasing amounts of c-Jun expression (Fig. 4B, +OSM, vector). Expression of STAT3-C alone was associated with a 20% increase in the level of OSM-induced transcription; this was stimulated further upon coexpression of increasing amounts of c-Jun, indicating transcriptional synergism between c-Jun and STAT3-C (Fig. 4B, +OSM, STAT3-C). Similar synergism was observed in HepG2 cells stimulated with IL-6 (data not shown).

We next asked whether the synergism between STAT3-C and c-Jun was dependent on the presence of their specific binding sites in the α2-MG promoter. When either c-Jun binding site was mutated singly, transcriptional activity was coordinately reduced to about 15–20% of that of the intact promoter (Fig. 4C, compare α2M-AP1m-luc to α2M-AP1m-luc with α2M-luc). Neither mutation, however, abolished synergism between STAT3-C and c-Jun. This indicates that a single intact AP1 site is sufficient to support a functional, cooperative interaction between c-Jun and STAT3-C. Nonetheless, the presence of both AP1 sites is essential for full promoter activity (Figs. 3B and 4C). As expected, when both AP1 sites were mutated, synergism between STAT3-C and c-Jun was abolished, although weak STAT3-C-dependent, c-Jun-independent promoter activity remained (Fig. 4C, α2M-AP1mAP1m-luc). As observed above (Fig. 3B), mutation of the APRE site nearly abolished transcriptional activity, although cotransfection of STAT3-C and increasing amounts of c-Jun was associated with a slight increase in expression (Fig. 4C, α2M-SIEm-luc). The latter result could reflect possible recruitment of STAT3-C into the α2-MG regulatory complex through direct protein-protein interactions. Upon mutation of the SIE and either AP1 site, no cooperative transcriptional activation was observed (Fig. 4C, α2M-AP1mSIEm-luc and α2M-AP1mSIEm-luc). The dependence on specific cis-acting elements observed for STAT3-C was confirmed with the wild-type protein. Synergism between wild-type STAT3 and c-Jun in OSM-treated HepG2 cells exhibited a similar pattern of dependence on the AP1 sites and the APRE (Fig. 5D, compare α2M-luc with α2M-SIEm-luc, α2M-AP1mSIEm-luc, and α2M-AP1mSIEm-luc).

Synergism between STAT3-C and c-Jun at the α2-MG Promoter Requires Specific Phasing of AT-rich Regions—Functional interactions between transcriptional regulatory proteins can be exquisitely sensitive to alterations in their spatial orientation. Because AT-rich regions of DNA exhibit intrinsic curvature (46–48) and are often binding sites for DNA bending proteins (49, 50), we asked whether the AT-rich regions of the α2-MG promoter contribute to the synergy between STAT3-C and c-Jun (Fig. 5A). Mutation of the first AT-rich region (AT1m) reduced overall transcription levels to about 10–15% of those of the intact construct, although synergism between STAT3-C and c-Jun was maintained (Fig. 5A). Mutation of the second AT-rich region (AT2m) had less of an inhibitory effect than mutation of AT1 (reduction to about 30% of pGL3-α2M-24-24-MG-luc activity); cooperativity between STAT3-C and c-Jun was preserved (Fig. 5A, compare α2M-AT1m-luc and α2M-AT2m-luc). Mutation of both AT-rich regions, however, abolished synergy between STAT3-C and c-Jun as well as basal transcriptional activity (Fig. 5A, compare α2M-AT12m-luc).

The center-to-center distance between the two AT-rich regions in the α2-MG promoter is 15 bp, placing them on opposite sides of the DNA helix. To determine whether the helical phasing of these AT-rich regions affects the functional interaction between STAT3-C and c-Jun, we constructed a variant α2-MG promoter in which the center-to-center distance between the
two AT-rich regions is 10 bp, placing them on the same side of the DNA helix. To maintain the overall integrity of the α2-MG promoter, the first AT-rich region was shifted 5 bp to the right (Fig. 5B). This alteration resulted in a 5-fold inhibition of luciferase activity, relative to the intact pGL3-a2MG promoter in HepG2 cells; moreover, synergy between STAT3-C and c-Jun was impaired (Fig. 5C). Dependence on the presence and phasing of the AT-rich sites was also observed in OSM-treated HepG2 cells expressing wild-type STAT3 and c-Jun (Fig. 5D). These results suggest that the phasing of the AT-rich regions of the α2-MG promoter as well as AT-rich DNA sequences are critical for the maintenance of transcriptional synergism between STAT3 and c-Jun in the induction of transcription from the α2-MG promoter by IL-6 in vivo (38).

Mutations in the c-Jun Interaction Regions of STAT3-C Do Not Impair Synergism at the α2-MG Promoter—Putative c-Jun interaction domains have been mapped to two regions of STAT3-C (38). Point mutations within these interacting regions (L148A and V151A in region 1 and T346A,K348A,R350A (TKR) in region 2) inhibited physical interactions between STAT3 and c-Jun in vitro and impaired cooperativity between STAT3 and c-Jun in the induction of transcription from the α2-MG promoter by IL-6 in vivo (38). To test whether these putative interaction regions are required for synergism between STAT3-C and c-Jun in OSM-inducible transcription from the α2-MG promoter, we introduced identical mutations into STAT3-C (STAT3-C (TKR), STAT3-C (L148A), and STAT3-C (V151A); Fig. 6A). Surprisingly, the STAT3-C mutants retained the ability to synergize with increasing amounts of c-Jun in the induction of α2-MG transcription by OSM. In contrast, transcriptional synergism between STAT3 and c-Jun was impaired by the TKR mutation (Fig. 6B), as previously described (38). These data indicate that the interaction regions disrupted by the L148A, V151, and TKR mutations, although essential for cooperative interaction of STAT3 with c-Jun, are dispensable for synergistic activation of the α2-MG promoter by STAT3-C and c-Jun in OSM-stimulated HepG2 cells.

**DISCUSSION**

In this report we have demonstrated that coordinate interactions among STAT3, c-Jun, and a specific array of DNA elements contribute to cytokine-mediated activation of the minimal α2-MG promoter. Three classes of DNA sequence ele-
ments are essential for synergism between STAT3 and c-Jun in the −90 to −100 region of the α2-MG promoter: a single APRE site at −171 to −163, a pair of AP1 sites at −152 to −140 and −113 to −107, and a pair of AT-rich sites at −143 to −138 and −128 to −123. Significantly, mutations of STAT3 previously shown to disrupt a physical interaction with c-Jun do not impair transcriptional synergism between STAT3-C and c-Jun at the α2-MG promoter.

Synergism between STAT3 and c-Jun at Synthetic and Natural Promoters—Using a synthetic promoter containing adjacent AP1 and SIE sites, we observed formation of a specific ternary complex containing STAT3, c-Jun, and DNA. DNA sequence-specific binding of STAT3 and c-Jun to this synthetic promoter was associated with synergistic activation of transcription in NIH3T3 and HepG2 cells in response to the related cytokines IL-6, OSM, and LIF. Synergism between STAT3 and c-Jun was also observed at the natural, α2-MG promoter, although the relative contribution of STAT3 to transcriptional activation, as revealed by mutational analysis of cis-acting elements, was somewhat greater than seen with the synthetic promoter. The differential contributions of STAT3 and c-Jun in the activation of synthetic and natural promoters within the same cell line may in part reflect differences in the intrinsic affinities of these promoters for STAT3 and c-Jun. Moreover, the α2-MG promoter responded differently to cytokines in fibroblast-derived NIH3T3 cells versus hepatocyte-derived HepG2 cells. In NIH3T3 cells, transcription from the α2-MG promoter was poorly inducible in comparison to the synthetic promoter (data not shown), suggesting that the α2-MG promoter may be more sensitive than the synthetic promoter to cell-to-cell variation in the amount of active STAT3 and c-Jun or in the amounts of undefined costimulatory factors.

**Cis-regulatory Elements of the α2-MG Promoter Mediate Transcriptional Activation by IL-6 or OSM**—Activation of the α2-MG promoter by IL-6 is principally dependent on DNA sequence within 209 bp upstream of the transcription start site (40, 45). This region contains a sequence motif (5′-TTCCTGG-GAA-3′; −171 to −163) that is conserved among various APR genes (40, 51). At least five regulatory elements in the minimal α2-MG promoter are essential for induction of transcription by IL-6 or OSM, including the following: 1) Two AP1 sites and an APRE site. Our observations suggest that an array of proteins bound at AP1 and APRE sites is essential for full activation of the α2-MG promoter. When this array was compromised by mutation, synergy between c-Jun and STAT3 was eliminated and transcriptional activity was substantially impaired. 2) Two AT-rich sites. Intrinsic DNA bends at AT-rich sites and transcription start sites (e.g. the AP1 sites and the APRE) that is essential for transcriptional synergy. It is possible that repositioning of an AT-rich site interferes directly with a productive interaction between STAT3 and c-Jun at the α2-MG promoter, perhaps through steric hindrance by a protein bound to the AT-rich site.

Alternatively or in addition, proteins bound to the AT-rich sites in the α2-MG promoter may make contacts that are sensitive to their phasing on the DNA helix. An antibody specific for HMG-I(Y) failed to react with the protein complex bound to AT-rich regions of α2-MG promoter (data not shown). It remains possible that a member of the HMG protein family other than HMG-I(Y) is bound to these AT-rich sites.

**Formation of a Multicomponent Transcription Complex at the α2-MG Promoter**—Transcriptional activation at natural enhancers is exquisitely sensitive to the precise arrangement of DNA elements and proteins within them. For example, upon viral infection, three transcription factors (an ATF2/c-Jun heterodimer, an IRF family member, and the p50/p65 NF-kB heterodimer) bind to the four distinct regions of the β-interferon promoter. This array of factors, together with HMG-I(Y) bound at three AT-rich sequences, synergistically activates transcription of the β-interferon gene. Such multicomponent complexes have been termed enhancerosomes (reviewed in Ref. 52). In response to IL-6-related cytokines, the α2-MG promoter is also activated through synergistic interactions of discrete transcrip-
Several mutations in STAT3 were found to disrupt binding to transcriptional proteins that contribute to cytokine-induced activation of STAT3-C. These mutations may reflect a structural alteration in STAT3-C that is involved in the complex formation in response to cytokine stimulation. It will be important to identify additional transcriptional proteins that contribute to cytokine-induced activation of this promoter and the elements with which they interact. A major challenge will be to define the spatial organization of this higher order complex and its assembly in response to cytokine stimulation.

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Synergistic Activity of STAT3 and c-Jun at a Specific Array of DNA Elements in the α2-Macroglobulin Promoter

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