Autoregulation of the Stat3 Gene through Cooperation with a cAMP-responsive Element-binding Protein*

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STAT3 (signal transducer and activator of transcription 3) is a key transcription factor mediating the signals for a variety of cytokines, including interleukin-6 (IL-6). The Stat3 gene itself is activated by IL-6 signals. We show that the region of the signal-transducing subunit, gp130, essential for STAT3 activation, is also required for activation of the Stat3 gene. To elucidate the mechanisms activating the Stat3 gene, we identified an IL-6 response element (IL-6RE) in the Stat3 gene promoter containing both a low affinity STAT3-binding element and a cAMP-responsive element (CRE). Electrophoretic mobility shift assays showed that IL-6 induced a slowly migrating complex on the IL-6RE containing a STAT3 homodimer and an unidentified CRE-binding protein. With the combination of transient transfection assays using mutant Stat3 promoter-reporter constructs and electrophoretic mobility shift assays, we found that the formation of a slowly migrating complex was required for full activation of the Stat3 gene. Thus, STAT3 activates the Stat3 gene in cooperation with an unidentified CRE-binding protein. This regulatory mechanism is similar to that of the junB gene, which is activated by IL-6 through the junB IL-6RE, which contains a low affinity STAT3-binding site and a CRE-like site.

STAT (signal transducer and activator of transcription) proteins have been shown to play pivotal roles in cytokine signaling pathways, which are involved in regulating cell growth and differentiation in systems ranging from Drosophila to mammals (1–3). In mammals, the STAT family consists of at least six members, designated STAT1–6 (3). Different STAT family members are activated by a variety of cytokines, including interferon-α/β, interferon-γ, the interleukin-6 (IL-6)† family, growth hormone, erythropoietin, and leptin in a manner dependent on the Janus kinase family of tyrosine kinases (4–12). Furthermore, several growth factors (such as epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor) and non-receptor tyrosine kinases (including c-Src, c-Abl, and their v-oncogene products) have been shown to activate STAT proteins (13–19). Thus, three different types of tyrosine kinases, Janus kinases, receptor-type tyrosine kinases, and certain Src family tyrosine kinases, can all activate STAT proteins, probably by directly phosphorylating the tyrosine residue critical for causing the STAT molecules to form homo- or heterodimers through their SH2 domains (20–25).

Tyrosine-phosphorylated STAT dimers enter the nucleus and activate the target genes by binding to their specific target DNA sequences (26). The cis-acting DNA sequences recognized by STAT dimers, the STAT-binding element (SBE), have the general structure TTN5AA (1, 27). The sequence and the size of the spacer region affect the binding of the respective STAT dimers (27). In certain cases, STAT proteins bind to the target DNA in combination with other DNA-binding proteins. For instance, the STAT3 homodimer forms a complex with p36 CRE-binding protein for an IL-6 response element in the junB gene (JRE-IL6) (28) or with c-Jun for the a2-macroglobulin APRE (29). The STAT1 homodimer has been shown to make a complex with Sp1 for the interferon-γ response element of the intercellular adhesion molecule-1 gene (30). Stocklin et al. (31) showed that the glucocorticoid receptor can act as a coactivator and enhance STAT5-dependent transcription by making complexes with STAT5. STAT dimers even form complexes with other STAT dimers through their amino-terminal regions on the repeated low affinity SBEs in the interferon-γ gene intron (32). Therefore, in addition to the sequence of a SBE itself, the sequence outside the SBE is also critical for determining the target gene specificity because the complex formation with other proteins or other STAT dimers sometimes changes the binding specificity or increases the affinity of the complex for the DNA (21, 28, 32). Other important determinants for STAT function might be the intensity and duration of the STAT activity and the amounts of STAT proteins ready to be activated in the cells. All of these factors eventually determine the range of target genes and the duration and intensity of target gene activation and thereby often determine the outcome, such as differentiation, growth, and cell survival or death.

We have previously shown that Stat3 is critical for IL-6-induced gene regulation, including the repression of c-myc and c-myc, the induction of junB and IRF1, and IL-6-induced growth arrest and terminal macrophage differentiation in M1 leukemic cells (33, 34). Moreover, Stat3 activity is critical for the gp130-mediated anti-apoptotic signal in murine pro-B BaF/33 cells (35). To understand how Stat3-mediated signals affect growth, differentiation, and cell death or survival in different types of cells, it is important to study the regulatory mechanisms of Stat3 gene expression itself. In this study, we characterize the signals required for Stat3 gene activation and show that Stat3 activates the Stat3 gene promoter through a
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novel IL-6 response element containing two DNA motifs, a low affinity SBE and a CRE. This is similar to the mechanism for JRE-IL6, a previously characterized IL-6 response element in the junB promoter (36).

EXPERIMENTAL PROCEDURES

Cell Lines—M1 murine myeloid leukemic cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO₂. M1 transformants expressing the chimeric receptors containing the extracellular domain of the GHSR and the transmembrane and cytoplasmic domain of gp130 have been described previously (34). Of those, seven clones for each (M1-GHR277, M1-GHR133, M1-GHR108, M1-GHR68, M1-GHR333f2, M1-GHR333f3, and M1-GHR333f23) were used in this study. HepG2 hepatoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies, Inc.). MH60 B cell hybridoma cells (37) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 0.2 ng/ml recombinant human IL-6.

Northern Blot Analysis—Total RNA was extracted using the TRizol reagent (Life Technologies, Inc.) according to the procedures recommended by the manufacturer. Total RNA (15 μg/sample) was separated by agarose-formaldehyde gel and transferred to Hybond N⁺ nylon membranes (Amersham Corp.). Membranes were hybridized with 3²P-labeled cDNA fragments overnight, washed three times with 0.1× SSC and 0.1% SDS at 56 °C, and subjected to autoradiography. The amount of loaded RNA was verified by ethidium bromide staining or by measuring the expression level of CHO-B mRNA. The probes used were the 2.5-kb Sall/BamHI fragment of pBSA-STAT3 containing a full-length STAT3 cDNA and the 0.6-kb EcoRf/BamHI fragment of CHO-B cDNA (a gift from J. E. Darnell, Jr.).

Isolation of the 5′-Flanking Region of the Mouse Stat3 Gene and Plasmid Construction—A mouse Stat3 5′-flanking region was obtained by PCR using the Promoter Finder DNA Walking kit (CLONTECH). Two gene-specific primers, 5′-CTCAGCAGCTGGTACGGGTTCTGCT-3′ (GSP1) and 5′-CAGGTTCCCTCCTGTCTGATAGTCTGAC (GSP2) (Fig. 2A), were made based on the sequence of the mouse Stat3 cDNA reported by Akira et al. (38). Primary PCR and secondary nested PCR were done using the GSP1 and GSP2 primers, the primers (AP1 and AP2) corresponding to the adaptor sequences and adaptor-ligated genomic libraries. The resulting 2.2-kb PCR fragment was subcloned using the TA cloning vector pCR2.1 (Invitrogen). DNA sequencing was carried out with 5′-AGTGCGTGATGACAAGACACTTTGAATGCCCT-3′ and 5′-GTGTCTTGACGTCACGCACTGCCAGGTCCT-3′ as transferred to Hybond N⁺ nylon membranes (Amersham Corp.). Membranes were hybridized with 3²P-labeled cDNA fragments overnight, washed three times with 0.1× SSC and 0.1% SDS at 56 °C, and subjected to autoradiography.

The oligonucleotides used as probes or constructs were as follows: for mSTAT, 5′-AGTGCGTGATGACAAGACACTTTGAATGCCCT-3′; for mCRE, 5′-AGCTGAGGACCTGGCAGTGCGTGACGT-3′; and for the 3′-end deletion mutations of the JRE-IL6, a previously characterized IL-6 response element in the context of the intact Stat3 promoter with the 2.2-kb upstream region by the overlap extension technique using PCR. The primers used for these reactions were as follows: for mSTAT, 5′-ACTGCGAGCTCCTGACGTGAGTGGTTTCAG-3′; and for the 3′-end, 5′-CCAGCTACCCACAGGAGGCGAGCAGGCG. The PCR products were digested with PstI and KpnI and inserted upstream of the minimal junB promoter at the PstI/KpnI sites of pSPBLC1.

Transient Transfection Assay—For transfection experiments, HepG2 cells were transfected with DNA using the calcium phosphate coprecipitation method (36). Typically, 1.2 μg of one of the reporter plasmids containing the firefly luciferase gene by the transfection reagent containing a luciferase gene encoding β-galactosidase as an internal control for transfection efficiency, were used. Three μg of pCAGGS-Neo (an expression vector without an insert; control) or pCAGGS-NeoHA-STAT1F or pCAGGS-NeoHA-STAT3F (expression vectors containing a cDNA encoding either HA-STAT1F or HA-STAT3F (38)) were transfected in some experiments. Cells were incubated with DNA precipitates for 16 h, washed with phosphate-buffered saline, fed Dulbecco's modified Eagle's medium containing 0.1% fetal calf serum for 20–24 h, and stimulated with 100 ng/ml IL-6 for the last 16 h. Approximately 40–45 h after transfection, cells were collected in 120 μl of lysis buffer and subjected to assays for luciferase and β-galactosidase activities as described (28).

Electrophoretic Mobility Shift Assay—This was performed according to the procedure published previously (28). Briefly, nuclear extracts (10 μg) were incubated in a final volume of 10 μl of 10 mM HEPS, pH 7.9, 80 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 1 mM EDTA, and 100 μg/ml poly(dI-dC):poly(dI-dC) with each 10⁶-pmol labeled probe (10,000 cpm, 0.5–1 ng) for 20 min at room temperature. The protein-DNA complexes were resolved on an 0.4% non-denaturing agarose gel. To confirm the identity of the probe-DNA complexes, the gel was stained with 0.1% crystal violet and 0.01% Coomassie blue R-250.

RESULTS

Increased Stat3 Gene Expression in Response to IL-6—Akira et al. (38) reported that treatment with IL-6 increases the level of Stat3 mRNA severalfold in liver. Also, we showed that IL-6 treatment of M1 cells increases the protein level of STAT3 and that the increased level is sustained for >48 h (33). To begin a deeper analysis of STAT3 regulation, we first determined a detailed time course for the IL-6-induced mRNA expression of the Stat3 gene in M1, HepG2, and IL-6-dependent MH60 B cell hybridoma cells. In M1 cells, the level of Stat3 mRNA started to increase at 1 h in response to IL-6 and reached its maximum level at 5 h. After reaching its maximum, Stat3 mRNA levels showed a slight transient decrease at 6 h and then increased again for over 48 h (Fig. 1A, lanes 1–6). The induction of Stat3 mRNA was also tested in the presence of cycloheximide, a protein synthesis inhibitor. Treatment of M1 cells with cycloheximide failed to inhibit the IL-6-induced activation of the Stat3 gene (Fig. 1A, lanes 7–9), indicating that the induction of Stat3 by IL-6 did not require new protein synthesis. The rapid induction of Stat3 mRNA by IL-6 was also observed with HepG2 cells (Fig. 1B) and MH60 cells (data not shown).

The Cytoplasmic Region from Amino Acids 108 to 133, Especially the Third Tyrosine (Tyr-3) of gp130, Is Required for
Regulating the Expression of Stat3—We next analyzed which part of gp130 was necessary for the induction of Stat3 gene expression. Using M1 transformants expressing chimeric receptors consisting of the extracellular domain of the GHR and the transmembrane and cytoplasmic domains of gp130 with progressive C-terminal truncations and point mutations at the tyrosine residues (34), we proceeded to identify the cytoplasmic region of gp130 required for Stat3 gene transactivation. Growth hormone enhanced Stat3 gene expression in the transformants expressing the chimeric receptors containing at least 133 amino acid residues of the gp130 cytoplasmic domain (Fig. 2, lanes 1–6). Chimeric receptors with further truncation of gp130 to 108 or 68 amino acid residues (GHR108 and GHR68) were unable to induce Stat3 gene expression (Fig. 2, lanes 7–12). GHR133F2, which contains a Tyr-to-Phe mutation at the second tyrosine (Tyr-2), could activate the Stat3 gene (Fig. 2, lanes 13–15), but GHR133F3, which has a Tyr-to-Phe mutation at the third tyrosine (Tyr-3), and GHR133F2/3, which has Tyr-to-Phe mutations at both Tyr-2 and Tyr-3, did not activate the Stat3 gene (lanes 16–21). These results show that the cytoplasmic region of gp130 from amino acids 108 to 133, with an intact YXXQ motif at Tyr-3, is required for activating the Stat3 gene. As this region corresponds to the region required for activating the Stat3 protein, it is likely that STAT3 participates in the transriptional activation of its own promoter.

The −478/-229 Region of the Stat3 Promoter Shows IL-6 Responsiveness in a Manner Dependent on STAT3—To elucidate the molecular mechanisms activating the Stat3 gene promoter, we obtained a 2.2-kb fragment of the Stat3 gene 5′-flanking region by genomic PCR. The sequence of the proximal region up to −478 bp with the known DNA motifs is shown in Fig. 3A. The sequence published by Shi et al. (39) is missing a 0.25-kb region between positions −424 and −169. We first constructed a reporter gene containing the 2.2-kb Stat3 gene 5′-flanking region linked to the luciferase reporter gene and tested whether this 2.2-kb fragment showed IL-6 responsiveness by transfecting the reporter gene construct into HepG2 cells, followed by IL-6 stimulation for 16 h. Dominant-negative STAT3 and STAT1 expression vectors were also cotransfected with the promoter-reporter construct to test whether STAT3 is involved in the IL-6 activation of the Stat3 gene promoter. As shown in Fig. 3B, IL-6 increased the promoter activity of the 2.2-kb 5′-flanking region by ∼5-fold, and this induction was effectively inhibited by dominant-negative STAT3 (DN-STAT3), but not by dominant-negative STAT1 (DN-STAT1), indicating that the IL-6 activation of the 2.2-kb Stat3 gene promoter was dependent on STAT3. Next, to localize the region containing the IL-6 response element, a series of 5′-deletion mutations of the Stat3 gene promoter constructs were made and tested for IL-6 responsiveness as described above. The promoter regions retained a similar level of IL-6 responsiveness when the promoter contained up to position −478 (Fig. 3C). However, deletion up to position −229 substantially decreased the IL-6 responsiveness, suggesting that an IL-6 response element(s) resides in the −478/-229 region (Fig. 3C).

Both STAT3 and CRE-binding Proteins Are Necessary for the IL-6-induced Transcriptional Activation of the Stat3 Gene—To test whether the −478/-229 region alone has the ability to render a heterologous minimal promoter responsive to IL-6, we assayed the IL-6 responsiveness of a luciferase construct containing the −478/-229 DNA fragment inserted upstream of the minimal junB promoter-luciferase gene construct (p478/-229-Luc). As shown in Fig. 4A, IL-6 increased the transcription of the reporter gene driven by the −478/-229 fragment by ∼10-fold. As expected, dominant-negative STAT3 (DN-STAT3), but not STAT1 (DN-STAT1), effectively inhibited the IL-6 responsiveness of the −478/-229 region. These results indicate that STAT3 activates its own transcription through the −478/-229 region. We next searched for STAT3-binding sequence(s) within the −478/-229 DNA region. Although we could not find a typical STAT3-binding sequence (a TT-AA motif with a spacing of 5 bp) within the −478/-229 DNA region, instead we found an atypical SBE (TGCCCTGGAA) and a CRE (TGACGTTCTA) with a 5-bp spacing between the motifs, which is very similar to the junB response element for IL-6 (JRE-IL6).
A, nucleotide sequence of the 478-bp upstream region of the murine Stat3 promoter with DNA-binding motifs.

B, STAT3-dependent activation of the 2.2-kb Stat3 promoter region. Luciferase reporter plasmids containing the 2.2-kb mouse Stat3 promoter (p2166-Stat3-Luc; 1.2 μg) were cotransfected into HepG2 cells with 3 μg of expression vector pCAGGS-Neo (control), pCAGGS-NeoHA-STAT3F (DN-Stat3), or pCAGGS-NeoHA-STAT1F (DN-Stat1) and 1 μg of pEF-BOS-lacZ. Sixteen h later, transfected cells were fed Dulbecco’s modified Eagle’s medium containing 0.1% fetal calf serum and further incubated without (black bars) or with (hatched bars) 100 ng/ml IL-6 for 16 h, and then the cell lysates were assayed for luciferase and β-galactosidase activities. The averages of normalized luciferase activities from four independent experiments performed in duplicate are shown. The numbers above the bars indicate the -fold increases in response to IL-6.

C, IL-6 responsiveness of the various Stat3 gene promoters. Luciferase reporter plasmids bearing a series of 5’-deletion mutations of the mouse Stat3 promoter, shown schematically, were transfected into HepG2 cells. IL-6 responsiveness of the promoter constructs was assayed, and normalized luciferase activities are shown as described above. Values are from four independent experiments. The numbers above the bars indicate the -fold increases in response to IL-6.

Fig. 3. A, nucleotide sequence of the 478-bp upstream region of the murine Stat3 promoter with DNA-binding motifs. B, STAT3-dependent activation of the 2.2-kb Stat3 promoter region. Luciferase reporter plasmids containing the 2.2-kb mouse Stat3 promoter (p2166-Stat3-Luc; 1.2 μg) were cotransfected into HepG2 cells with 3 μg of expression vector pCAGGS-Neo (control), pCAGGS-NeoHA-STAT3F (DN-Stat3), or pCAGGS-NeoHA-STAT1F (DN-Stat1) and 1 μg of pEF-BOS-lacZ. Sixteen h later, transfected cells were fed Dulbecco’s modified Eagle’s medium containing 0.1% fetal calf serum and further incubated without (black bars) or with (hatched bars) 100 ng/ml IL-6 for 16 h, and then the cell lysates were assayed for luciferase and β-galactosidase activities. The averages of normalized luciferase activities from four independent experiments performed in duplicate are shown. The numbers above the bars indicate the -fold increases in response to IL-6. C, IL-6 responsiveness of the various Stat3 gene promoters. Luciferase reporter plasmids bearing a series of 5’-deletion mutations of the mouse Stat3 promoter, shown schematically, were transfected into HepG2 cells. IL-6 responsiveness of the promoter constructs was assayed, and normalized luciferase activities are shown as described above. Values are from four independent experiments. The numbers above the bars indicate the -fold increases in response to IL-6.
(Fig. 3A) (28). To investigate whether both the SBE and CRE were responsible for IL-6 responsiveness, we made two different p478/229-Luc mutants containing mutations at the SBE (p478/229-Luc-mSBE) or the CRE (p478/229-Luc-mCRE), as illustrated in Fig. 4B, and tested them for IL-6 responsiveness. Mutations at the putative SBE reduced the IL-6 responsiveness by >90%, and mutations at the CRE reduced it by >80% (Fig. 4B). These results indicated that both DNA motifs were required for IL-6 responsiveness. To assess the role of the two DNA motifs in the intact 2.2-kb Stat3 gene promoter, we introduced the same mutations as described above into the Stat3 IL-6RE in the intact 2.2-kb Stat3 promoter (p2166-Stat3-Luc-mSBE and p2166-Stat3-Luc-mCRE). As shown in Fig. 4C, mutations at the putative Stat3-binding site in the 2.2-kb Stat3 promoter (p2166-Stat3-Luc-mSBE) severely reduced the IL-6 responsiveness of the Stat3 promoter by >80%, and mutations at the CRE reduced the IL-6 responsiveness of the promoter by ~60%. The weaker inhibition by the mutations at the CRE in the otherwise intact promoter compared with that by mutations at the CRE in the context of the ~478/~229 region alone may be due to the existence of unidentified region(s) cooperatively working with Stat3 outside the ~478/~229 region. These results, in any case, indicate that the two DNA motifs in the Stat3 IL-6RE are the major determinants in the 2.2-kb Stat3 promoter for IL-6 responsiveness.

Characteristics of Stat3 IL-6RE-binding Proteins—We next examined the nature of the Stat3 IL-6RE-binding complexes in the nuclear extracts from IL-6-stimulated M1, HepG2, and MH60 cells by electrophoretic mobility shift assays using an oligonucleotide containing the Stat3 IL-6RE as a probe. We also used another oligonucleotide containing the α2-macroglobulin APRE (40) as a control for STAT binding. Nuclear extracts from IL-6-stimulated MH60 cells showed a prominent IL-6-inducible Stat3 IL-6RE-binding complex on the Stat3 IL-6RE probe (indicated as Stat3IL6RE-BC), which appeared rapidly at 15 min, declined transiently at 3 h, and returned to the level of 15 min at 6 h (Fig. 5A, lanes 2–5). The mobility of the IL-6-induced Stat3 IL-6RE-binding complex is slower than that of the APRE-binding complex containing a STAT3 homodimer, called APRE (40) (Fig. 5A, lane 7). More important, the level of the IL-6-inducible complex is equivalent to that of APRE (Fig. 5A, compare lanes 2 and 7). This IL-6-inducible complex could be seen more clearly in MH60 nuclear extracts than in M1 and HepG2 nuclear extracts, although similar IL-6-inducible complexes were present in the nuclear extracts from all three cell lines (data not shown). Therefore, only results with MH60 nuclear extracts are shown. To investigate the binding sites and binding specificities of the Stat3 IL-6RE-binding complex, we used two mutant Stat3 IL-6RE oligonucleotides containing mutations either at the CRE site (Stat3 IL-6RE-mCRE) or at the Stat3-binding element (Stat3 IL-6RE-mSBE) as probes and other oligonucleotides as competitors, including the APRE, JRE-IL6, and somatostatin CRE. The Stat3 IL-6RE-mCRE probe did not show any IL-6-inducible Stat3 IL-6RE-binding complexes (Fig. 5A, lanes 8 and 9). On the other hand, the Stat3 IL-6RE-mSBE probe showed an IL-6-inducible complex with the same mobility as that of APRE (Fig. 5A, lanes 10 and 11), and this complex was likely to be a STAT3 homodimer since anti-STAT3 antibody shifted the complex (data not shown). Both the APRE and JRE-IL6 oligonucleotides effectively competed with the Stat3 IL-6RE probe for forming the IL-6-inducible Stat3 IL-6RE-binding complex (Fig. 5B, lanes 2–5), whereas the somatostatin CRE oligonucleotides competed for all of the CRE-binding complexes and the Stat3 IL-6RE-binding complex, but made the appearance of a fast migrating IL-6RE-binding complex (lanes 6 and 7). The complex is likely to be a STAT3 homodimer since the complex migrated as APRE on the APRE probe, and anti-STAT3 antibody shifted the complex (data not shown). This STAT3 homodimer could barely be seen on the wild-type Stat3 IL-6RE probe (Fig. 5A). These results indicate that formation of the Stat3 IL-6RE-binding complex requires both the STAT-binding element and the CRE and suggest that the STAT-binding element has a low affinity for STAT3 homodimers alone.
Next, to test whether the IL-6-inducible Stat3 IL-6RE-binding complex contained STAT3, STAT1, or other known CRE-binding proteins, we included the appropriate antibody against such molecules in the electrophoretic mobility shift assay (Fig. 5C). Anti-STAT3 antibody, not anti-STAT1 antibody, shifted the inducible complex, indicating that the IL-6-inducible Stat3 IL-6RE-binding complex contained STAT3 as a constituent. None of the other antibodies against transcription factors that are known to bind the CRE sites, such as c-Jun, CREB, ATF2, ATF3, and ATF4, shifted or decreased the IL-6-inducible Stat3 IL-6RE-binding complex (Fig. 5C). These results indicated that the Stat3 IL-6RE-binding complex contained both a STAT3 homodimer and unidentified CRE site-binding protein(s) distinct from CREB, ATF1, ATF2, ATF3, or ATF4.

DISCUSSION

In this study, we showed that STAT3 rapidly induced transcriptional activation of the Stat3 gene through an IL-6RE located at positions −335 to −314 in the Stat3 gene promoter. This IL-6RE was shown to consist of a low affinity SBE (TGCCAGGAA) and a CRE (TGACGTCGA). The functional role of the two DNA motifs in the Stat3 IL-6RE was confirmed in two ways. First, mutations at either the SBE or CRE effectively inhibited the IL-6 responsiveness of the −478/−229 fragment linked to a heterologous promoter. Second, the same mutations at the DNA motifs in the otherwise intact 2.2-kb Stat3 promoter also effectively inhibited the IL-6 responsiveness of the promoter. The functional roles of the two DNA motifs were further confirmed by the demonstration that IL-6 rapidly induced the formation of complexes with the Stat3 IL-6RE. A slowly migrating complex with the IL-6RE required both the SBE and CRE. We showed that this complex contained a STAT3 dimer and unidentified CRE-binding protein(s) distinct from CREB, ATF1, ATF2/CRE-BP1, ATF3, ATF4, or the c-Jun/AP-1 family of proteins. Ternary complex formation on other IL-6 response elements containing a SBE and CRE has been shown by us (28), including the IL-6 response elements seen in the junB promoter, JRE-IL6 (36), and the IRF1 promoter, IRF/IRF1 (41). Table I compares the sequences of such IL-6 response elements with that of the Stat3 IL-6RE. The size of the spacing between the two DNA motifs also varies from 1 to 5 bp. The combination of the DNA motifs varies: a low affinity CRE (TGACGTCGA) and a CRE (TGCCAGGAA). The functional role of the DNA motifs varies: a low affinity CRE (TGACGTCGA) and a CRE (TGCCAGGAA). STAT3-mediated Activation of the Stat3 gene

**FIG. 5.** Characteristics of the IL-6-inducible complexes formed on the Stat3 IL-6RE. A, DNA binding activities induced by IL-6 on the Stat3 IL-6RE. Nuclear extracts from MH60 cells either untreated (lanes 1, 6, 8, and 10) or treated with IL-6 at 100 ng/ml for 15 min (lanes 2, 7, 9, and 11) were subjected to an electrophoretic mobility shift assay using Stat3 IL-6RE probes (lanes 1–5), APRE probes (lanes 6 and 7), Stat3 IL-6RE-mSTAT (lanes 8 and 9), or Stat3 IL-6RE-mCRE (lanes 10 and 11). The position of the IL-6-inducible Stat3 IL-6RE-binding complex (Stat3IL6RE-BC) is indicated by an arrow. The APRE-binding complex, APREF (also called SIF-A), is indicated as Stat3 homodimer with an arrow. B, binding specificity of the Stat3 IL-6RE-binding complex. Nuclear extracts prepared from MH60 cells stimulated with IL-6 for 15 min were preincubated with unlabeled oligonucleotide competitors (50- or 250-fold molar excess as indicated) for 5 min, followed by incubation with labeled Stat3 IL-6RE probes. The competitors used were APRE (lanes 2 and 3), JRE-IL6 (lanes 4 and 5), and somatostatin CRE (somCRE; lanes 6 and 7). C, characterization of the protein components of the IL-6-inducible binding complex. Nuclear extracts prepared from the indicated antisera or monoclonal antibody for 30 min on ice prior to the addition of 32P-labeled Stat3 IL-6RE probes and were then subjected to an electrophoretic mobility shift assay. The effectiveness of these antibodies, except for anti-ATF3 and anti-ATF4, was verified using recombinant proteins overexpressed in transfected 293T cells (data not shown).
IL-6-binding complexes shown by Kojima et al. (28), similar CRE-like site-binding protein(s) may make complexes with the STAT3 homodimer on the Stat3 IL-6RE DNA. The identification of CRE-binding protein(s) would help prove such a structure.

The results of the kinetics study of the IL-6 induction of Stat3 mRNA in wild-type M1 cells (Fig. 1A) are fully consistent with the levels of STAT3 activity detected by tyrosine phosphorylation of STAT3 and its DNA binding activity shown previously by us (33), supporting the notion of the STAT3-mediated activation of the Stat3 gene. It is remarkable that three of the IL-6 target genes inducible in M1 cells, junB, IRF1, and Stat3, contain very similar IL-6 response elements in their promoters. Interestingly, these genes can be activated by a low dose of IL-6 (such as 1 ng/ml) in M1 cells.2 In contrast, in another case of

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Table I
Sequence comparison of the Stat3 IL6-RE in the mouse Stat3 promoter, JRE-IL6 in the junB promoter, and the IL-6 response element in the human IRF1 gene promoter

| CRE            | SBE               |
|----------------|-------------------|
| TCTGGACGTCAGGC | GCGCCGAGCATC     |
| TCGTTGATCTCG   | GAGCGGAGGACG      |
| TGCGCTGATTG    | GCGGAGGAGTCC      |

The core SBE and CRE sites are indicated.

Mouse Stat3 IL-6RE
Mouse junB IL-6RE/JRE-IL6
Human IRF1 IL-6RE (IRF1F)

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