Interleukin-6-induced Tethering of STAT3 to the LAP/C/EBPβ Promoter Suggests a New Mechanism of Transcriptional Regulation by STAT3

LAP/C/EBPβ is a member of the C/EBP family of transcription factors and contributes to the regulation of the acute phase response in hepatocytes. Here we show that IL-6 controls LAP/C/EBPβ gene transcription and identify an IL-6-responsive element in the LAP/C/EBPβ promoter, which contains no STAT3 DNA binding motif. However, luciferase reporter gene assays showed that STAT3 activation through the gp130 signal transducer molecule is involved in mediating IL-6-dependent LAP/C/EBPβ transcription. Southwestern analysis indicated that IL-6 induces binding of a 68-kDa protein to the recently characterized CRE-like elements in the LAP/C/EBPβ promoter. Transfection experiments using promoter constructs with mutated CRE-like elements revealed that these sites confer IL-6 responsiveness. Further analysis using STAT1/STAT3 chimeras identified specific domains of the protein that are required for the IL-6-dependent increase in LAP/C/EBPβ gene transcription. Overexpression of the amino-terminal domain of STAT3 blocked the IL-6-mediated response, suggesting that the STAT3 amino terminus has an important function in IL-6-mediated transcription of the LAP/C/EBPβ gene. These data lead to a model of how tethering STAT3 to a DNA-bound complex contributes to IL-6-dependent LAP/C/EBPβ gene transcription. Our analysis describes a new mechanism by which STAT3 controls gene transcription and which has direct implication for the acute phase response in liver cells.

CAAT/enhancer-binding proteins (C/EBPs) are a family of leucine zipper transcription factors currently comprising six members involved in the regulation of various aspects of cellular differentiation and function in multiple tissues. Three closely related members of the C/EBP family, C/EBPα, LAP/C/EBPβ, and C/EBPδ, are known to contribute to liver-specific gene transcription. Specific functions of these transcription factors have been described in the regulation of the acute phase response and during liver regeneration, where they seem to be involved in the process of hepatocyte proliferation and differentiation (reviewed in Ref. 1).

Several inflammatory signals, including lipopolysaccharides (LPS), interleukin 6 (IL-6), interleukin 1 (IL-1), tumor necrosis factor α, and interferon gamma (IFN-γ) contribute to the regulation of the acute phase response in hepatocytes. The activities of the C/EBP family members are differentially modulated in response to the various inflammatory stimuli on the mRNA and protein level. Upon induction of the acute phase response C/EBPα mRNA levels decrease in the liver, whereas LAP/C/EBPβ and C/EBPγ gene transcription is enhanced. For the regulation of LAP/C/EBPβ, however, post-translational mechanisms were described in addition to transcriptional activation (reviewed in Ref. 2). Several phosphorylation sites in the LAP/C/EBPβ protein have been shown to be functionally relevant (3–6). Phosphorylation occurs in vitro in response to an intracellular Ca2+ increase, through activation of the protein kinase C pathway and through activation of the mitogen-activated protein (MAP) kinase following induction of the Ras pathway. Although all these events lead to increased transactivation of LAP/C/EBPβ-dependent genes, only the MAP kinase pathway can be linked to the acute phase response. Moreover, stimulation with lipopolysaccharides, IL-6, IL-1, dexamethasone, and glucagon strongly induces LAP/C/EBPβ expression (7–10). Initially, LAP/C/EBPβ has also been cloned because it binds to IL-6-responsive elements in the promoters of acute phase response genes (11, 12) and thus was originally named NF-IL6 (nuclear factor involved in the IL-6 gene expression).

Of the numerous cytokines and growth factors that are involved in regulating the acute phase response, IL-6 plays a major role (reviewed in Ref. 13). Binding of IL-6 to its receptor induces homodimerization of the signal-transducing component gp130 (14), which results in the activation of constitutive associated JAK kinases and phosphorylation of gp130 at different tyrosine residues (15, 16). Tyrosine phosphorylation creates specific docking sites for signaling molecules containing SH2 domains (17). In the case of gp130 at least three signaling cascades, the STAT1, the STAT3, and the MAP kinase pathway are activated (18–21). Activation of STAT3 is the most prominent pathway during this process (15, 22, 23). Phosphorylation of STAT3 through JAK kinases results in its homo- or hetero-

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Monika Niehof‡§, Konrad Streetz‡, Tim Rakemann‡, Stephan C. Bischoff‡, Michael P. Manns‡, Friedemann Horn, and Christian Trautwein‡‡

From the ‡Department of Gastroenterology and Hepatology, Medizinische Hochschule Hannover, 30625 Hannover and the §Institute of Clinical Immunology, Universität Leipzig, 04129 Leipzig, Germany

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erodimerization and nuclear translocation. STAT3 then binds to target sequences in different promoters and enhances gene transcription. Binding sites for STAT3 are located in most promoters of acute phase genes, and originally STAT3 was identified as acute phase response factor (24–26).

Analysis of the C/EBPβ promoter revealed that STAT3 contributes to higher gene transcription after IL-6 stimulation (27, 28). Besides DNA binding, cooperative interaction of STAT3 with Sp1 is essential for the regulation of the C/EBPβ promoter. In contrast, no information is available about the molecular mechanisms involved in the IL-6-dependent increase of LAP/C/EBPβ gene transcription. Therefore, we were interested in investigating how IL-6 controls the activation of this gene.

In this analysis we show that IL-6 activates LAP/C/EBPβ gene transcription using the two already characterized CRE-like sites in the LAP/C/EBPβ promoter (29). STAT3 activation through the gp130 signal transducer is essential for increased LAP/C/EBPβ transcription despite the lack of sequence-specific STAT DNA binding sites in this promoter region. Enhanced IL-6-dependent transcription of the LAP/C/EBPβ gene correlates with the binding of a new IL-6-inducible factor with a molecular mass of 68 kDa to the CRE-like sites in the LAP/C/EBPβ promoter. Functional disruption of the gp130 signal transducer molecule and the use of STAT1/STAT3 chimeras further demonstrated the essential role of STAT3 and identified specific regions of the protein that are required for the IL-6-dependent increase of LAP/C/EBPβ gene transcription. These results lead to a model of how tethering STAT3 to the promoter region contributes to IL-6-dependent gene transcription without direct DNA binding and thus indicate a new mechanism for STAT3-dependent gene transcription.

MATERIALS AND METHODS

Stimulation of Mice and Preparation of Total RNA and of Liver Nuclear Extracts—C57Bl/6 mice were stimulated intraperitoneally with 40 μg of human recombinant IL-6. At different time points after injection, the livers were removed and preparation of RNA and of liver nuclear extracts was performed. At each time point at least three animals were used in parallel. RNA was isolated by the guanidinium isothiocyanate method (30). For preparation of nuclear extracts the pooled livers were rinsed in ice-cold phosphate-buffered saline, and liver nuclear proteins were prepared by the modified Dignam C method (5). 48 h after transfection, cells were washed twice with ice-cold phosphate-buffered saline, and liver nuclear proteins were prepared as described previously (30). All steps were performed at 4 °C. Nuclear proteins were aliquoted and frozen immediately in liquid nitrogen.

Northern Blot Analysis—Northern blot analysis was performed as described before, according to standard procedures (30). 30 μg of total RNA was analyzed through a 1% agarose formaldehyde gel followed by transfer to Hybond N+ membranes (Amersham Pharmacia Biotech, Braunschweig, Germany). The LAP/C/EBPβ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes were labeled with [γ-32P]ATP according to the instructions for Rediprime (Amersham Pharmacia Biotech). The hybridization procedure was performed as described previously (30). Blots were exposed for autoradiography and exposed to an imaging plate (Fuji) for quantification. LAP/C/EBPβ signals were normalized to the GAPDH signals and set to 1 for untreated animals. The values for IL-6 treatment were shown as -fold stimulation. All transfections contained 0.1 μg of the β-galactosidase reporter pCMVβGal as an internal standard. For stimulation experiments, cells were starved with 1% fetal calf serum for 24 h before transfection. Stimulation was performed with human recombinant IL-6 (Strathmann Biotech, Hannover, Germany) in the amounts and for the time points indicated or with 7 units/ml human recombinant erythropoietin (Epo) (Roche Molecular Biochemicals, Mannheim, Germany) for 4 h. 1 unit of IL-6 is equivalent to 0.005 ng (Strathmann Biotech).

To measure luciferase activity, cells were washed twice with phosphate-buffered saline and lysed by adding 350 μl of extraction buffer (25 mM Tris-HCl, pH 7.8, 2 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, and 2 mM DTT) for 10 min. The lysates were cleared by centrifugation. 50 μl of the supernatant was added by assay of 300 μl of measuring buffer (25 mM glycylglycine, 15 mM MgSO4, and 5 mM ATP). The light emission was measured in duplicate for 10 s with a Lumat LB 9501 (Berthold) by injecting 100 μl of 250 μM luciferin. Data are represented as the mean ± S.D. of triplicate experiments and are representative for three independent experiments.

Preparation of Nuclear Extracts—HepG2 nuclear extracts were prepared by the modified Dignam C method (5). 48 h after transfection, cells were washed twice with ice-cold phosphate-buffered saline, and liver nuclear proteins were prepared as described previously (30). 30 μg of total RNA was analyzed through a 1% agarose formaldehyde gel followed by transfer to Hybond N+ membranes (Amersham Pharmacia Biotech, Braunschweig, Germany). The LAP/C/EBPβ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes were labeled with [γ-32P]ATP according to the instructions for Rediprime (Amersham Pharmacia Biotech). The hybridization procedure was performed as described previously (30). Blots were exposed for autoradiography and exposed to an imaging plate (Fuji) for quantification. LAP/C/EBPβ signals were normalized to the GAPDH signals and set to 1 for untreated animals. The values for IL-6 treatment were shown as -fold stimulation.
oligonucleotide B to −102 to −75 (CCG ACC CGG CCG GGG AGG AGG C), oligonucleotide C to −90 to −68 (CGG GCC GGG GGG AGG GCC CCC GGC GTG AGC CAG CCC GTT GCC AGC GCC GcC), oligonucleotide D to −75 to −43 (GGC CCC GCC GTG AGC CAG CCC GTT GCC AGC GCC GcC), oligonucleotide E to −123 to −107 (GGG GCC GGG CAA TGA CG), oligonucleotide F to −110 to −87 (GAC GGC CAC CGA CCC GCC GGG GGG), and oligonucleotide M to −123 to −99, mutated in −109 to −107 (GCG GCC GGG CAA TGG TTC GCA). The SIE consensus oligonucleotide corresponds to the sequence: 5′-GTG CAT TTC CCG TAA ATC TTG TCT ACA-3′. Supershift experiments were performed with antibodies against STAT3 or CREB1 (Santa Cruz Biotechnology, Santa Cruz, CA). Nuclear extracts were modified in Fig. 4 as indicated. Prior to the incubation with the 32P-labeled probe, nuclear extracts were incubated with 0.08% desoxycholic acid (DOC) for 20 min at 4°C, then 0.15% Nonidet P-40 was added. For the depletion assay 2.5 μl of antibody to CREB1 (Santa Cruz Biotechnology) was coupled to 10 μl of protein A-Sepharose, saturated with 1 μg/μl bovine serum albumin, in TNE buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 2 mM DTT, 2 mM Pefabloc, 1% Nonidet P-40) for 4 h at 4°C, washed twice in TNE buffer, and twice in gel shift sample buffer. Incubation with 3 μg of nuclear extract was performed at 4°C overnight. Protein A-Sepharose without antibody was treated under the same conditions and served as control. The supernatants were analyzed in gel shift experiments.

**Southwestern Analysis**—Southwestern analysis was performed as described with some modifications (36, 37). 20 μg of liver nuclear extracts prepared before and 1 h after stimulation of C3H mice with 40 μg of IL-6 was heated for 30 min at 60°C in SDS sample buffer and separated on a 10% SDS-polyacrylamide gel. After electrophoresis the gel was incubated in renaturing buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 50 mM NaCl, 1 mM EDTA, 4 μl urea, adjusted to pH 7.5) for 4 h at room temperature and then blotted onto a nitrocellulose membrane (Millipore, Bedford, MA) in a solution containing 25 mM Tris and 192 mM glycine (adjusted to pH 8.3) at 4°C for 2 h at a constant current of 200 mA. The membranes were prehybridized in binding buffer (10 mM Tris, pH 7.5, 0.1 mM EDTA, 1 mM DTT, 50 mM NaCl, 2× Denhardt’s solution, adjusted to pH 7.2) at 4°C overnight and then incubated with 2.5 × 10⁶ cpm 3²P-labeled oligonucleotide A for 1 h at room temperature. After incubation the membranes were washed several times in binding buffer and exposures were made to x-ray films.

**Western Blot Analysis**—Nuclear extracts were separated on a 10% SDS-polyacrylamide gel (38) and blotted onto nitrocellulose membrane (Millipore) in 1% SDS, 20% methanol, 400 mM glycine, 50 mM Tris, pH 8.3, at 4°C for 2 h at 200 mA. The antibody directed against Gal4-(1–147) was purchased from Santa Cruz Biotechnology, the antibody directed against phospho-STAT3 was purchased from New England Biolabs (Beverly, MA). The antigen-antibody complexes were visualized using the ECL detection system as recommended by the manufacturer (Amersham Pharmacia Biotech, Braunschweig, Germany).

### RESULTS

**IL-6-induced LAP/C/EBPβ mRNA Expression in the Liver Correlates with STAT3 Activation**—LAP/C/EBPβ was originally identified, because an increase in DNA binding was evident after IL-6 treatment in human hepatoma cells (11, 12). IL-6 stimulates LAP/C/EBPβ at the transcriptional and post-translational level (2, 7). However, the molecular mechanisms responsible for IL-6-mediated LAP/C/EBPβ gene transcription are unknown. In an initial experiment, C3H mice were stimulated with IL-6 and LAP/C/EBPβ mRNA expression was studied by Northern blot analysis (Fig. 1A). LAP/C/EBPβ signals were normalized to the GAPDH signals and set to 1 for untreated animals. Quantification revealed an increase in LAP/C/EBPβ mRNA expression already after 30 min while maximum levels (3.8-fold) were found 6 h after stimulation (Fig. 1A). At later time points LAP/C/EBPβ mRNA expression decreased again. After binding of IL-6 to its receptor, several pathways are activated. However, STAT3 activation has been shown to stimulate transcription of many acute-phase genes. Therefore, activation of STAT3, as already described before (39), was studied. IL-6 induced an increase in nuclear expression of tyrosine-phosphorylated STAT3 30 min after injection as shown by Western blot analysis (Fig. 1B). The nuclear expression of phospho-STAT3 remained high for the first 4 h and was no more detectable after 12 h. Gel shift experiments with an SIE element as STAT3 target sequence showed activation of DNA binding 30 min after IL-6 injection (Fig. 1C). DNA binding remained high for up to 3 h and then subsequently decreased. No complex formation was found after 12 h. The specificity of STAT3 in the new appearing complex was verified by

**Fig. 1.** IL-6-induced LAP/C/EBPβ mRNA expression in the liver correlates with STAT3 activation. A, C3H mice were stimulated with 40 μg of IL-6, total RNA was isolated from the liver before and at different time points after stimulation (as indicated), and Northern blot analysis with a 3²P-labeled DNA for LAP/C/EBPβ (upper panel) and for GAPDH were performed. The ratio (lower panel) between the LAP/C/EBPβ signal and the GAPDH signal was calculated and set to 1 for the untreated probe. Changes after IL-6 treatment are shown as -fold stimulation. B, Western blot analysis was performed with 10 μg of liver nuclear extract prepared before and at different time points after stimulation (as indicated) of C3H mice with 40 μg of IL-6. Nuclear expression of tyrosine-phosphorylated STAT3 (P-STAT3) was detected with an antibody directed against tyrosine-phosphorylated STAT3. C, gel shift experiments were performed with 3 μg of liver nuclear extract prepared before and at different time points after stimulation (as indicated) of C3H mice with 40 μg of IL-6. Complex formation was performed with the 3²P-labeled STAT3-specific oligonucleotide comprising the SIE site (Santa Cruz Biotechnology).
supershift experiments (data not shown) and has been shown before using a STAT3 DNA target sequence derived from the α2-macroglobulin promoter (39). Thus, these experiments show a close correlation between an increase in LAP/C/EBPβ mRNA expression and STAT3 activation after IL-6 stimulation in the liver in vivo.

**Fig. 2. IL-6-induced LAP/C/EBPβ transcription is mediated by a region in proximity to the TATA box.** A, increasing deletions were introduced in the 5′-flanking region of the LAP/C/EBPβ gene. LAPPRO 1 corresponds to the whole 1.4-kb 5′-region located between the SfiI and AvaII sites. The AvaII site is located 16 bp downstream of the start site of transcription in the LAP/C/EBPβ gene. ○ potential STAT3 binding sites. Restriction sites in the 1.4-kb fragment were used to create increasing 5′-deletions as indicated (LAPPRO 2, 3, 7, 8, and 9). All fragments were linked to a luciferase reporter gene. HepG2 cells were cotransfected with 2 μg of the respective LAPPRO luciferase reporter constructs and with 50 ng of a STAT3 expression plasmid. Stimulation was performed for 4 h after transfection cells were starved with 1% fetal calf serum, and then stimulation was performed with 1000 units of IL-6/ml for 4 h. The relative luciferase activity of the respective LAPPRO luciferase reporter construct without stimulation was set to 1, and the changes after IL-6 stimulation are shown as -fold stimulation. B and C, HepG2 cells were cotransfected with 2 μg of the LAPPRO 8 luciferase reporter construct and without or with 50 ng of a STAT3 expression plasmid. Stimulation was performed for 4 h with increasing units IL-6/ml as indicated (B) or with 1000 units of IL-6/ml for different time points as indicated (C). The relative luciferase activity without stimulation was set to 1, and the changes after IL-6 treatment are shown as -fold stimulation.

**IL-6-induced LAP/C/EBPβ Transcription Is Mediated by a Region in Proximity to the TATA Box**—As LAP/C/EBPβ mRNA expression increased after IL-6 stimulation in vivo, we performed experiments to identify regulatory sequences in the LAP/C/EBPβ promoter that mediate IL-6 induction. In transfection experiments luciferase reporter constructs with increasing deletions in the 5′-flanking region, located upstream of the start site of transcription in the LAP/C/EBPβ gene, were analyzed in HepG2 cells. The constructs were cotransfected with 50 ng of STAT3 expression vector, and cells were treated with 1000 units/ml IL-6 for 4 h (Fig. 2A). The relative luciferase activity of the respective LAPPRO reporter construct without stimulation was set to 1, and the changes after IL-6 treatment were shown as -fold stimulation. Strong induction (up to 20-fold) of the luciferase reporter gene was observed when the whole 1.4-kb 5′-region of the promoter was used (LAPPRO 1). IL-6-dependent stimulation of the 5′-flanking region was still found when deletions up to nucleotide −121 were analyzed. No increase in reporter gene activity after IL-6 treatment was evident with the LAPPRO 9 construct (truncated to position −71, see Fig. 2A). These data indicate that the region located between nucleotides −121 and −71 in close proximity to the TATA box is involved in mediating IL-6-dependent transcription of the LAP/C/EBPβ gene. The IL-6-dependent effect on the LAP/C/EBPβ promoter was further investigated by dose and time-kinetic experiments (Fig. 2, B and C). This analysis showed that a maximal effect on LAP/C/EBPβ promoter activity was found with 1000 units/ml IL-6 for 4 h.

**STAT3 Activation Is Essential for IL-6-mediated LAP/C/EBPβ Transcription**—Our next interest was to analyze the relevance of the STAT3 signaling cascade for IL-6-mediated LAP/C/EBPβ transcription despite the finding that there is no STAT consensus sequence located in the LAP/C/EBPβ promoter region, which confers IL-6 inducibility. Therefore cotransfection experiments were performed with the LAPPRO 8 reporter construct and an empty control vector or expression vectors for STAT1 or STAT3. These studies showed that IL-6 stimulated the promoter construct 2-fold, whereas cotransfection of the STAT3 expression vector enhanced this effect (Fig. 3A). In contrast, cotransfection of a STAT1 expression vector had no influence on the IL-6 inducibility compared with cotransfection of the empty control vector (Fig. 3A). In further experiments, the STAT3-specific inhibitor PIAS3 (protein inhibitor of activated STAT3) (14) was used to characterize the STAT3-dependent mechanism. Cotransfection experiments of the LAPPRO 8 constructs and increasing amounts of an expression vector for PIAS3 were performed with or without an expression vector for STAT3. Under both conditions PIAS3 inhibited the IL-6-dependent increase in LAP/C/EBPβ gene transcription in a concentration-dependent manner (Fig. 3B).

After binding of IL-6 to gp130 at least three intracellular signaling cascades, STAT1, STAT3, and the Map kinase path-
way (reviewed in Ref. 40), are activated. Participation of these signaling pathways in IL-6-dependent LAP/C/EBPβ transcription was further analyzed by using chimeric receptors. These chimeras consisted of the extracellular domain of the erythropoietin receptor (EpoR) fused to the transmembrane domain and different cytoplasmic tyrosine modules specifically either
activating STAT3 (gp130 Y759, EpoR/STAT3 site), the MAP kinase pathway (gp130 Tyr-759, EpoR/Map site), or STAT1 (IFN γR Tyr-440/EpoR/STAT1 site) (Fig. 3C). Stimulation of HepG2 cells with erythropoietin (Epo) had no effect on LAP/C/EBP-PRO 8 activity (data not shown). Epo stimulation after cotransfection of HepG2 cells with the LAPPRO 8 construct and the EpoR/Map site or the EpoR/STAT1 site fusion receptors also resulted in no increase of reporter gene activity (Fig. 3D). However, when the EpoR/STAT3 site chimera was used, a 3-fold induction of LAP/C/EBPβ gene transcription was found. Therefore these data indicate that of the three pathways studied only STAT3 can mediate IL-6-dependent transcription of the LAP/C/EBPβ gene.

IL-6 Induces Binding of a 68-kDa Protein to the CRE-like Sites in the LAP/C/EBPβ Promoter.—The cotransfection experiments indicated that STAT3 is involved in mediating IL-6-dependent LAP/C/EBPβ transcription. However, even computer-assisted analysis showed no potential STAT DNA binding site (41) between nucleotide 121 and the TATA box in the LAP/C/EBPβ promoter (for the whole sequence see Ref. 29). To exclude the possibility that STAT3 may bind to a yet unknown target sequence in this region, gel shift experiments were performed using mouse liver nuclear extracts prepared before and 2 h after IL-6 treatment when STAT3 binding to its consensus sequence occurred. Overlapping oligonucleotides derived from the LAP/C/EBPβ promoter (A–M, Fig. 4A) were used as 32P-labeled probes. As shown in Fig. 4B, complex formation of two complexes was only found with oligonucleotide A and D containing the two CRE-like sites of the LAP/C/EBPβ promoter. Complex formation was blocked when the mutant oligonucleotide M (mutation of the first CRE-like site) was used as a 32P-labeled probe. In contrast to unstimulated extracts, a third, new complex was detected in IL-6-stimulated liver nuclear extracts, when the intact CRE-like site was included in the oligonucleotides.

In time course experiments, formation of the IL-6-stimulated complex was studied. Oligonucleotide A (see Fig. 4A) represents the sequence between nucleotide −123 and −95 of the LAP/C/EBPβ promoter that was used as the 32P-labeled probe. After IL-6 stimulation the intensity of the two complexes found already in untreated animals did not change (Fig. 4C). Formation of the IL-6-inducible third complex was found 1 h after IL-6 injection. Maximal DNA binding was evident after 2 h. At later time points complex formation decreased again (Fig. 4C). Supershift experiments were performed to study whether STAT3 binds to the CRE-like sequence in the LAP/C/EBPβ promoter after IL-6 stimulation (Fig. 4D). Antibodies directed against CREB and STAT3 were used as described before (30, 39). However, DNA binding of the IL-6-dependent complex was not changed by using a STAT3 antibody, whereas DNA binding of one of the two unstimulated complexes could be inhibited with a CREB antibody (Fig. 4D). The protein of the second unstimulated complex is not known (38).

Because binding of this IL-6-inducible factor occurred at the CRE-like site in the LAP/C/EBPβ promoter, we were interested in examining whether binding of the IL-6-induced factor to the CRE-like binding site occurs only in the presence of CREB or if the IL-6-induced factor is able to bind independently of CREB. Therefore, gel shift experiments with modified liver nuclear extracts as described under “Experimental Procedures” were performed. We used detergent incubation with desoxycholic acid and Nonidet P-40 (DOC) to inhibit protein-protein interaction. However, DNA binding of the IL-6-induced factor was unchanged after DOC treatment (Fig. 4E). Complete depletion of liver nuclear extracts with an anti-CREB antibody coupled to protein A-Sepharose (ProtA/CREB) also had no effect on DNA binding of the IL-6-induced factor (Fig. 4E). Thus, these data indicate that the IL-6-induced factor binds to the CRE-like site in the LAP/C/EBPβ promoter independently of CREB.

Next, Southwestern analysis was performed to characterize the molecular weight of the IL-6-induced factor (Fig. 4F). In control as well as in IL-6-stimulated liver nuclear extracts we found p30, corresponding to the lower gel shift band, and p43, corresponding to CREB. An additional p68 protein corresponding to the upper band was only found in IL-6-stimulated extracts (Fig. 4F). In parallel UV cross-linking experiments showing very weak signals (data not shown) confirmed our results indicating a molecular mass of 68 kDa for the IL-6-inducible factor binding to both CRE-like sites in the LAP/C/EBPβ promoter. The Southwestern analysis revealed weak binding of an additional IL-6-stimulated factor with a molecular mass of ~45 kDa.

Both CRE-like Sites in the LAP/C/EBPβ Promoter Are Essential for IL-6-dependent Induction.—To further characterize the significance of the two CRE-like sites for IL-6 induction, we analyzed mutant LAP/C/EBPβ promoter constructs in HepG2 cells (Fig. 5A). Mutations in both CRE-like sites (LAPPRO 8 MUT I+II) led to a strong decrease in IL-6-dependent transcription (Fig. 5B). No stimulation was observed after transfection of the LAPPRO 9 construct that contains only one CRE-like site. Recently Cantwell et al. (27) described the cooperative function of STAT3 and Sp1 in IL-6-mediated C/EBPβ induction. We therefore analyzed the deletion construct LAPPRO 8 WT Δ, which carries a deletion of 27 nucleotides between the two CRE-like sites in the GC-rich sequence with possible Sp1 binding sites. This deletion caused a marked decrease in IL-6-dependent transcription (Fig. 5B). The data show that both CRE-like sites contribute to IL-6-mediated LAP/C/EBPβ induction and indicate that the spacer sequence between them might be of functional relevance.

The Amino-terminal Domain of STAT3 Is Essential for IL-6-mediated LAP/C/EBPβ Transcription.—Our results indicate that STAT3 contributes to IL-6-dependent LAP/C/EBPβ transcription without direct DNA binding to the promoter region. To better understand the role of different domains of STAT3 in this context, cotransfection experiments were performed with the LAPPRO8 reporter construct and expression vectors for different STAT3/STAT1 domain swap mutants and analyzed after IL-6 stimulation (Fig. 6). The expression level and the characterization of the domain swap mutants have been shown before (15).

After receptor stimulation, the SH2 domain of the STAT proteins interacts with specific phosphorylated tyrosines in the intracellular receptor domain (19, 22). Substitution of the STAT3 by the STAT1 SH2 domain (Stat 3/1 (SH2)) prevented IL-6-mediated LAP/C/EBPβ transcription (Fig. 6). Therefore, the SH2 region of STAT3 is essential for the IL-6 effect, as already implicated by the EpoR transfection experiments (Fig. 3D). The expression of the tyrosine region (Stat 3/1 (Y)), responsible for dimerization of STAT proteins after phosphorylation by members of the JAK kinase family, maintained the IL-6 effect (Fig. 6). The amino-terminal part (NT) of the STAT proteins contributes to several protein-protein interactions (reviewed in Ref. 41). Substitution to STAT1 NT (Stat 3/1 (NT)) abolished the IL-6 effect (Fig. 6). Therefore, our experiments indicate that the amino-terminal region of STAT3 is required for IL-6-mediated LAP/C/EBPβ transcription. The carboxy-terminal part (CT) of STAT proteins mediates the transcriptional activation of target genes (42, 43). The exchange of the carboxy-terminal region (Stat 3/1 (CT)) or a mutation of serine 727 to alanine (Stat 3S1/A) maintained IL-6 stimulation (Fig. 6). However, no IL-6-dependent transcription was found.
FIG. 4. IL-6 induces binding of a 68-kDa protein to the CRE-like sites in the LAP/C/EBPβ promoter. A, oligonucleotides A, B, C, D, E, F, and M derived from the LAP/C/EBPβ promoter and used for gel shift experiments are shown. The two CRE-like binding sites in the LAP/C/EBPβ promoter (TGACGC, 11 to 104 and TGACGCAG, 65 to 58) are marked as circles. B, gel shift experiments were performed with 3 μg of liver nuclear extract prepared before (−) and 2 h after stimulation (+) of C3H mice with 40 μg of IL-6. Complex formation was performed with the respective oligonucleotide as 32P-labeled probe. IL-6-induced complex formation is marked with an arrowhead. C, gel shift experiments were performed with 3 μg of liver nuclear extracts prepared before and at different time points after stimulation (+) of C3H mice with 40 μg of IL-6. Complex formation was performed with oligonucleotide A (123 to 95) derived from the LAP/C/EBPβ promoter as 32P-labeled probe. IL-6-induced complex formation is marked with an arrowhead. D, for supershift/binding inhibition analysis antibodies against STAT3 or CREB were added to the 2 h value after IL-6 stimulation (w/o = without antibody). IL-6-induced complex formation is marked with an arrowhead. E, gel shift experiments were performed with 3 μg of liver nuclear extract prepared before (−) and 2 h after stimulation (+) of C3H mice with 40 μg of IL-6 and oligonucleotide A (123 to 95) as 32P-labeled probe. Before complex formation was performed, the extracts were modified as follows: Doc = incubation with detergent (desoxycholic acid and Nonidet P-40), Prot. A = incubation with protein A-Sepharose beads, Prot. A/CREB =
when cotransfection experiments were performed with carboxyl-terminal deletion mutants (Stat3Δ57, Stat3Δ711). A mutation in the DNA binding region (Stat 3D) as well as substitution of the STAT3 DNA binding region to STAT1 (Stat 3/1 (D,Linker)) abolished IL-6 induction (Fig. 6).

These data indicate that interaction with the basal transcriptional machinery and, thereby, activation of LAP/C/EBPβ transcription is mediated by the STAT3 carboxyl-terminal region and that this function is exchangeable toward STAT1. In contrast, the amino-terminal part and the DNA binding region of STAT3 are absolutely required to mediate IL-6 induction, which shows that in addition to the SH2 site these two domains of STAT3 mediate specificity during this process.

**Overexpression of the Amino-terminal Domain of STAT3 Prevents IL-6-mediated Transcription of the LAP/C/EBPβ Promoter**—The experiments using the chimeric STAT3/STAT1 constructs suggested that interactions at the amino terminus of STAT3 are crucial to mediate IL-6-dependent transcription of the LAP/C/EBPβ promoter. To confirm this hypothesis we fused the amino-terminal part of STAT3 or STAT1 to the Gal4 DNA binding domain to provide this region with a nuclear translocation signal and investigated whether overexpression of the STAT3 construct might act as a dominant-negative inhibitor of IL-6-dependent LAP/C/EBPβ induction (Fig. 7A). Fusion to the Gal4 DNA binding domain was performed with the amino-terminal (5’Gal4-Stat1NT, 5’Gal4-Stat3NT) as well as with the carboxyl-terminal domain (Stat1NT-3’Gal4, Stat3NT-3’Gal4) of the respective STAT amino terminus (Fig. 7A). Both approaches were chosen, because it would minimize the possible risk of misfolding in the tertiary structure.

Comparable expression of the respective fusion proteins after transfection of HepG2 cells was found by Western blot analysis of nuclear extracts (Fig. 7B). In luciferase reporter gene experiments cotransfection of 5’Gal4-STAT3NT with LAPPRO 8 inhibited the IL-6-mediated LAP/C/EBPβ induction in a concentration-dependent manner, whereas the 5’Gal4-STAT1NT and the Gal4 alone showed no effect (Fig. 7C). The same results were found with the 3’ fusion constructs. STAT3NT-3’Gal4 prevented IL-6 induction, whereas STAT1NT-3’Gal4 had no influence (Fig. 7D). However, the STAT3NT-3’Gal4 constructs were significantly more effective (concentration range 10–100 ng) compared with the 5’Gal4-STAT3NT constructs (concentration range 100–1000 ng) (Fig. 7, C and D). These experiments clearly show that the amino-terminal domain of STAT3 is able to act as a dominant-negative inhibitor, and therefore, these results suggest that the STAT3 amino terminus has an important function in IL-6-mediated transcription of the LAP/C/EBPβ gene.

**DISCUSSION**

Most of the acute phase genes are activated on a transcriptional level through IL-6-responsive elements in their promoter regions. The best studied example for a transcription factor involved in this mechanism is STAT3 (23, 25); however, C/EBP

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*CREB = no modification, supershift reaction with CREB antibody. IL-6-induced complex formation is marked with an arrowhead. F, Southwestern analysis. 20 μg of liver nuclear extract prepared before (−) and 1 h after stimulation (+) of C3H mice with 40 μg of IL-6 were separated by SDS-polyacrylamide gel electrophoresis, blotted on nitrocellulose, incubated with 3P-labeled oligonucleotide A (−123 to −95), and exposed to autoradiography. The arrows mark the proteins in the stimulated extracts (p68, p65, p30) corresponding to the three gel shift bands. Molecular mass markers are shown in kDa on the left.*
family members also contribute to this regulation (2, 24). Induction of the acute phase response in hepatocytes enhances the expression of LAP/C/EBPβ and C/EBPδ, which then activate transcription of several acute phase response genes. Earlier results showed that the C/EBPδ promoter contains two Sp1 binding sites, an STAT3 binding site, and a CRE-like binding site in close proximity to the TATA-box. For the IL-6-dependent transcription, the STAT3 and the Sp1 d gene (2, 44). "The STAT3 Signaling Cascade Is Involved in IL-6-mediated LAP/C/EBPβ Transcription—Our in vivo and in vitro experiments demonstrated that the IL6/gp130/STAT3 pathway induces higher LAP/C/EBPβ gene transcription. An increase in LAP/C/EBPβ gene transcription was also reported after lipopolysaccharide (LPS) stimulation and hepatocyte growth factor treatment. This is mediated by an autoregulatory loop in which phosphorylation of LAP/C/EBPβ increases transactivation and thus higher LAP/C/EBPβ gene transcription. The LAP/C/EBPβ binding site(s), which contribute to this regulation, were mapped outside the IL-6-responsive region characterized in our study (45, 46). IL-6 also stimulates the MAP kinase pathway, which has been shown before to lead to post-translational activation of LAP/C/EBPβ by its phosphorylation (4). This mechanism could be further excluded, because the EpoR/Map site chimera was unable to induce LAP/C/EBPβ transcription, indicating that an autoregulatory loop does not explain our observations. The IL-6-responsive Element in the LAP/C/EBPβ Promoter Contains No STAT3 Consensus Sequence and Is Regulated through Two CRE-like Binding Sites—Analysis of the whole 1.4-kb LAP/C/EBPβ promoter by transfection experiments revealed that the three potential STAT DNA binding sites are not involved in mediating the increase in IL6-dependent gene transcription. In contrast, the recently described CRE-like sites (−111 to −104 and −65 to −58) in the promoter control this effect, and IL-6 stimulates DNA binding of a 68-kDa protein to these sequences. Induction of CRE-like site binding proteins after IL-6 stimulation was already reported for the STAT3 (47) and JunB promoter (48). However, these proteins were not further characterized. Meanwhile, the ATF/CREB family comprises a growing number of proteins that recognize the CREconsensus or CRE-like binding sites (reviewed in Ref. 49). The mechanism for the induction of the DNA binding activity of the CRE-like site binding proteins after IL-6 stimulation is not known.

Mutations in the CRE-like sites of the LAP/C/EBPβ promoter resulted in a lack of p68 binding and in a decrease in IL-6-dependent transcription. Mutation of the first site had a stronger effect on IL-6 inducibility than that of the second site (data not shown), but strongest inhibition was observed with the double mutant construct. Additionally, our results indicate that the spacer region between the two CRE sites is important in mediating IL-6-dependent gene transcription. To exclude the possibility that other transcription factors are involved in this regulation, we subcloned the two CRE sites with the wild type promoter by transfection experiments revealed that the three potential STAT DNA binding sites are not involved in mediating the increase in IL6-dependent gene transcription. In contrast, the recently described CRE-like sites (−111 to −104 and −65 to −58) in the promoter control this effect, and IL-6 stimulates DNA binding of a 68-kDa protein to these sequences. Induction of CRE-like site binding proteins after IL-6 stimulation was already reported for the STAT3 (47) and JunB promoter (48). However, these proteins were not further characterized. Meanwhile, the ATF/CREB family comprises a growing number of proteins that recognize the CRE-consensus or CRE-like binding sites (reviewed in Ref. 49). The mechanism for the induction of the DNA binding activity of the CRE-like site binding proteins after IL-6 stimulation is not known.

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**Fig. 6.** The amino-terminal domain of STAT3 is essential for IL-6-mediated LAP/C/EBPβ transcription. STAT3/STAT1 domain swap mutants combining portions of murine STAT3 and human STAT1α are shown. Open and closed bars represent domains derived from STAT1α and STAT3, respectively. The numbers on top according to the STAT3 amino acid sequence indicates boundaries between the domains. In the chimera nomenclature, parts derived from STAT1α are indicated in brackets: NT = amino terminus, C-C = coiled-coil domain, D = DNA-binding domain, Linker = Linker domain, SH2 = SH2 domain, Y = tyrosine phosphorylation site, CT = carboxy terminus. HepG2 cells were cotransfected with 2 μg of the LAPPRO 8 luciferase reporter construct and 100 ng of the respective STAT domain swap expression vector, starved as described in Fig. 2, and stimulated with 1000 units of IL-6/ml for 4 h. The relative luciferase activity of the respective transfection without stimulation was set to 1, and the changes after treatment were determined as fold stimulation and presented as mean ± S.D. (left column). No stimulation (−) corresponds to the effect without cotransfection of any STAT expression vector, stimulation (+) corresponds to a STAT effect (right column).
binding to this region are crucial to confer IL-6 inducibility of the LAP/C/EBPβ promoter.

A Model for Tethering STAT3 to the LAP/C/EBPβ Promoter—Our results suggested that STAT3 contributes to higher IL-6-dependent LAP/C/EBPβ gene transcription without DNA binding. Further analyses with STAT3/STAT1 domain swap mutants suggested that the amino-terminal part, the SH2 domain, and the DNA binding domain of STAT3 are crucial for the IL-6-dependent increase in LAP/C/EBPβ gene transcription. The essential role of the STAT3 DNA binding domain for this regulation was unexpected. Our mutation analysis in the LAP/C/EBPβ promoter clearly showed that only the CRE-like sites are relevant for IL-6 inducibility. Consequently, STAT3 would have to bind to this region in a non-specific manner. Thus we cannot completely rule out the possibility that STAT3 contributes to p68 DNA binding through a yet undefined mechanism, which is not detected by gel shift experiments. To prove this possibility we also depleted IL-6-induced nuclear extracts from STAT3 as shown for CREB. However, STAT3-depleted extracts had no influence on DNA binding of p68 (data not shown). Therefore we favor the possibility that the DNA binding domain of STAT3 contributes to protein-protein interaction. In a recent report (50), parts of the STAT3 DNA binding domain are shown to be involved in mediating the interaction between STAT3 and c-Jun.

The STAT3/STAT1 amino terminus (NT) domain swap mutants and the Gal4-STAT3NT fusion constructs indicated that the amino-terminal part of STAT3 is very crucial for mediating the link between the basal machinery and the two CRE-like sites. This observation implies that the amino terminus most likely does not bind to a more general factor, which would be a part of the general RNA polymerase machinery, but to one of the proteins, e.g. p68, binding to the CRE-like sites in the LAP/C/EBPβ promoter. Thus this interaction seems to mediate specificity during the regulation. The Gal4 part was fused to both the carboxyl-terminal and the amino-terminal end of the STAT3 amino terminus to minimize steric effects. Fusion to the carboxyl-terminal end (STAT3NT-39Gal4) resulted in a 10-fold more potent dominant-negative inhibitor than fusion to the amino-terminal end (59Gal4-STAT3NT). The difference be-
between the two constructs might be best explained by the localization of the interacting region.

In recent reports there is evidence that several transcription factors activate transcription without DNA binding. In specific situations a pre-existing complex of transcription factors bound to DNA allows the association with an additional transcription factor without DNA binding. This mechanism has also been called tethering, and meanwhile several examples are known. ATF6 can be tethered to the c-Fos and to the atrial natriuretic factor promoter by serum response factor bound to the serum response element, without direct binding of ATF6 to the serum response element (51, 52). Ubeda et al. (53) reported the re-recruitment of CHOP to an AP1-DNA complex without DNA binding of CHOP. Glucocorticoid receptors can also act as transcriptional coactivators for STAT5 without binding to a glucocorticoid response element (54, 55). The involvement of STAT1 in tumor necrosis factor-α-mediated apoptosis, where SH2 mutants can restore wild-type function in STAT1 knockout cells, lead to the model that STAT1 might be recruited to a promoter through protein-protein interaction with a DNA-bound partner (56, 57). STAT3 has also been reported as a transcriptional coactivator without association with its DNA binding motif. IL-6-activated STAT3 associates with ligand-bound glucocorticoid receptor to form a transactivating complex bound to a glucocorticoid response element (58). Besides the model of the tethered coactivator function for STAT3, there is evidence for its role during other mechanisms not related to DNA binding. An adapter function for STAT3 has been implicated in the recruitment of phosphatidylinositol 3-kinase to the IFN-α receptor (59).

The close correlation between DNA binding of p68 to the CRE-like elements and the role of STAT3 for the increase in LAP/C/EBPα transcription indicates that these mechanisms could be directly linked to each other. Therefore, based on our results we would like to propose a hypothetical model explaining the role of STAT3 during IL-6-dependent activation of LAP/C/EBPα gene transcription. We suggest that IL-6 activates STAT3 and DNA binding of p68. STAT3 is tethered to the p68-containing complex at the CRE-like site in the LAP/C/EBPα promoter. In this regulation, STAT3 would act as transcriptional coactivator whereby the carboxyl-terminal region interacts with the basal transcription machinery and activates LAP/C/EBPα transcription while the amino terminus may interact with p68 or other related factors. It is conceivable that certain steric conditions are necessary for tethering of STAT3 by p68 bound to both CRE-like sites. This would explain the critical distance between the two sites for IL-6 induction. DNA binding can affect the quaternary structure of transcriptional regulators and thereby determine heterodimerization partners (60). This conformational change would allow tethering of STAT3 to p68 after binding to the CRE-like site in the LAP/C/EBPα promoter.

Importance for an Interplay of Different Regulatory Sites and for Interaction of Different Activators with the Same Sites in the LAP/C/EBPα Promoter—Among mouse, rat, and humans the two CRE-like sites and the GC-rich linker sequence are highly conserved regulatory elements in the LAP/C/EBPα promoter (61). Recently, we have reported that basal and protein kinase A-inducible LAP/C/EBPα promoter activity in cells of hepatic and neuronal origin requires the synergistic activity of the two CRE-like sites (29). Since then additional examples for the relevance of a cAMP-dependent regulation of LAP/C/EBPα through these sites were described during memory formation in hippocampal neurons (62), in human endometrial stroma cells as part of prolactin induction (63), in sertoli cells (64), for the gonadotropin response in granulosa cells (65, 66), and during adipocyte differentiation (67).

Besides the DNA binding elements in the promoter region other more cell type-specific mechanisms, e.g. starvation, proliferation, determine gene regulation in a certain cellular background. Analysis of the LAP/C/EBPα promoter in monocytic cells showed the relevance of an interplay between an Sp1 site and the second CRE-like site for basal activity and for PMA or LPS stimulation, whereas the first CRE-like site was not involved in this regulation (61). In hepatocytes both CRE-like sites are required and recognized by different activators and coactivators, which contribute to the regulation of LAP/C/EBPα gene transcription during the acute phase response and during liver regeneration. The increase in promoter activity varied after protein kinase A stimulation (29) and as shown here after IL-6 treatment between 6- and 20-fold. The mechanisms, which contribute to these differences, are currently unknown. However, for example, after IL-6 stimulation, binding of an additional factor p45 as shown by Southwestern analysis could be detected, which might be a possible heterodimerization partner for p68 modulating its activity. Additionally, the proliferative state of the cell may directly modulate the basal activity of the LAP/C/EBPα promoter. Therefore, to better understand all the different transcriptional control mechanisms under various physiological conditions, it might be necessary in the future to further characterize the transcription factors binding to the CRE-like sites and to identify other possible interacting coactivators.

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