

# The Nuclear Factor- $\kappa$ B Engages CBP/p300 and Histone Acetyltransferase Activity for Transcriptional Activation of the Interleukin-6 Gene Promoter\*

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**Expression of the pleiotropic cytokine interleukin (IL)-6 can be stimulated by the proinflammatory cytokine tumor necrosis factor (TNF) and the microbial alkaloid staurosporine (STS). In this report, the transcriptional mechanisms were thoroughly investigated. Whereas transcription factors binding to the activator protein-1-, cAMP-responsive element-, and CAAT enhancer-binding protein-responsive sequences are necessary for gene activation by STS, nuclear factor (NF)- $\kappa$ B alone is responsible and sufficient for inducibility by TNF, which reveals distinct signaling pathways for both compounds. At the cofactor level, cAMP-responsive element-binding protein-binding protein (CBP) or p300 potentiate basal and induced IL-6 promoter activation via multiple protein-protein interactions with all transcription factors bound to the promoter DNA. However, the strongest promoter activation relies on the p65 NF- $\kappa$ B subunit, which specifically engages CBP/p300 for maximal transcriptional stimulation by its histone acetyltransferase activity. Moreover, treatment of chromatin-integrated promoter constructions with the histone deacetylase inhibitor trichostatin A exclusively potentiates TNF-dependent (*i.e.* NF- $\kappa$ B-mediated) gene activation, while basal or STS-stimulated IL-6 promoter activity remains completely unchanged. Similar observations were recorded with other natural NF- $\kappa$ B-driven promoters, namely IL-8 and endothelial leukocyte adhesion molecule (ELAM). We conclude that, within an "enhanceosome-like" structure, NF- $\kappa$ B is the central mediator of TNF-induced IL-6 gene expression, involving CBP/p300 and requiring histone acetyltransferase activity.**

cal and pathophysiological processes. Among its many functions, IL-6 plays an active role in immunological responses, bone metabolism, reproduction, inflammation, neoplasia, and aging. Overexpression of IL-6 has been implicated in the pathology of a number of diseases including multiple myeloma, rheumatoid arthritis, Castleman's disease, psoriasis, Alzheimer's disease, and postmenopausal osteoporosis. The cellular and molecular biology of IL-6 has been explored by a variety of approaches (1). In view of its pleiotropic functions, studies on the regulation of IL-6 gene expression may be of prior importance. The characterization of the IL-6 promoter revealed a complex control region that can be triggered by multiple activation pathways (2, 3). In the case of tumor necrosis factor (TNF), the main transcriptional activator for IL-6 gene induction is the nuclear factor NF- $\kappa$ B, which is typically a dimer between p50 and the transactivating subunit p65 (RelA). In unstimulated cells, NF- $\kappa$ B resides in the cytoplasm, bound to its inhibitor I $\kappa$ B. After cell stimulation, NF- $\kappa$ B is released from I $\kappa$ B and migrates into the nucleus, where it induces gene expression after DNA binding (4). Access of NF- $\kappa$ B complexes is partially blocked by the constitutive occupancy of the IL-6- $\kappa$ B site by the recombination signal sequence-binding protein (RBP)-J $\kappa$ , which is involved in the constitutive repression of the IL-6 gene under normal physiological conditions (5). Activation of IL-6 gene expression by NF- $\kappa$ B is probably the most important pathway. For maximum response, additional factors are also required, the most important being activator protein (AP)-1, cyclic AMP-responsive element-binding protein (CREB) and CAAT enhancer-binding protein (C/EBP) (6, 7). AP-1 is formed by the dimerization of Fos and Jun family members through a leucine zipper structural motif and becomes activated by mitogenic stimuli, oncoproteins, cytokines, and UV light. Another dimeric transcription factor is CREB, which binds to cAMP-responsive elements and is involved in cAMP-signaling pathways. The C/EBP family of transcription factors is involved in the expression of both acute phase cytokine genes and cytokine-inducible acute-phase proteins.

Transcription is a multistep process with many potential levels of control. Recent data indicate that numerous transcription factors mediate their effects via recruitment of cofactors (8, 9). The cofactors CREB-binding protein (CBP), p300, and steroid receptor coactivator (SRC)-1 have indeed received much attention due to their promiscuous interactions with a wide range of transcription factors, such as AP-1, CREB, C/EBP, and NF- $\kappa$ B (10–15). In addition to their bridging function between upstream DNA-binding proteins and the basal transcription

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<sup>1</sup> The abbreviations used are: IL, interleukin; AP, activator protein; CBP, cyclic AMP-responsive element-binding protein-binding protein; C/EBP, CAAT enhancer-binding protein; CREB, cyclic AMP-responsive element-binding protein; HAT, histone acetyltransferase; HDAC, histone deacetylase; RBP, recombination signal sequence-binding protein; SRC, steroid receptor coactivator; STS, staurosporine; TNF, tumor ne-

crosis factor; TSA, trichostatin A; wt, wild-type; BSA, bovine serum albumin; bp, base pair(s); EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; NF, nuclear factor.

complex, some cofactors also appear to play a role in chromatin remodeling via their intrinsic histone acetyltransferase (HAT) or deacetylase (HDAC) activity (16, 17).

We have already reported on the essential role of NF- $\kappa$ B to trigger IL-6 gene activation in response to TNF in the mouse fibrosarcoma cell line L929sA (3). In addition, we showed that staurosporine (STS) sensitizes tumor cells to TNF cytotoxicity and markedly potentiates TNF-induced IL-6 production (18). In the present paper, we further studied the transcriptional mechanisms of TNF- and STS-mediated IL-6 gene activation. We focused on the functional interaction between TNF- or STS-responsive DNA-bound factors and the cofactor CBP/p300 in the IL-6 promoter context. The relation between cofactor-dependent HAT activity and IL-6 promoter stimulation was further explored with a HAT-defective p300 variant and with the potent HDAC inhibitor trichostatin A (TSA). Finally, we also extend our observations to other NF- $\kappa$ B-containing promoters.

#### MATERIALS AND METHODS

**Cell Culture, Cytokines, and Inhibitors**—Murine fibrosarcoma L929sA cells and human embryonic kidney HEK293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum, 5% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. 24 h before induction, cells were seeded in multiwell dishes such that they were confluent at the time of the experiment. Recombinant murine TNF has been characterized previously (3). STS was purchased from Calbiochem-Novabiochem International (San Diego, CA) and was stored as a 2-mM solution in dimethyl sulfoxide at  $-20^{\circ}\text{C}$ . TSA was purchased from Biomol (Plymouth Meeting, PA) and was stored as a 10 mM solution in EtOH at  $-20^{\circ}\text{C}$ . Control experiments showed that the final quantities of organic solvent used did not interfere with any of the assays. Secreted IL-6 levels were determined in a biological 7TD1 assay (19).

**Plasmids**—The IL-6 promoter-containing plasmids p1168hu.IL6P-luc+, p234hu.IL6P-luc+, p110hu.IL6P-luc+, p50hu.IL6P-luc+, and p(IL6 $\kappa$ B)<sub>3</sub>50hu.IL6P-luc+ were described previously (3, 5). p1481.IL8P-luc+, containing an IL-8 promoter fragment of 1481 bp (20), was kindly provided by Dr. N. Mukaida (Cancer Research Institute, Kanazawa, Japan). pELAMP-luc+, containing the ELAM promoter, was a gift from Dr. D. V. Goeddel (Tularik, San Francisco, CA) (21). The synthetic reporter gene constructs pCRE-luc+ and pAP1-luc+, containing multimerized responsive elements in front of a minimal promoter, were purchased from Stratagene Cloning Systems (La Jolla, CA). pPGK $\beta$ GeobpA, constitutively expressing a neomycin-resistant  $\beta$ -galactosidase fusion protein under control of the 3-phosphoglycerate kinase promoter from the mouse housekeeping 3-phosphoglycerate kinase enzyme (22), was a gift of Dr. P. Soriano (Fred Hutchinson Cancer Research Center, Seattle, WA). pCMV-CBP or pCMV-p300, expressing full-length CBP or p300, were provided by Dr. R. Eckner (Institute for Molecular Biology, Zurich, Switzerland) (23). The expression vectors containing wild-type (wt) p300 (pCI.p300) and its HAT deletion derivative (pCI.p300HATA1472-1522) were a gift of Dr. J. Boyes (MRC Clinical Sciences Center, London, UK) (24). The mammalian expression plasmid for full-length SRC-1 was provided by Dr. B. W. O'Malley (25). pcDNA3 was purchased from Invitrogen (San Diego, CA). NF- $\kappa$ B p50 and p65 expression plasmids were provided by Dr. G. Manfioletti (University of Trieste, Trieste, Italy) (26). The c-Jun expression plasmid was cloned by inserting a full-length EcoRI-flanked cDNA fragment of murine c-Jun in pRSV-cDNA3.

**Site-directed Mutagenesis**—The IL-6 promoter was mutated as described previously (3). To create the double point-mutated IL-6 promoter variant CRE-C/EBP-mut 1168hu.IL6P-luc+, the following mutator oligonucleotide, containing specific restriction sites, was used (altered nucleotides are italicized): CRE-C/EBP-mut 5'-GCGATGCTA-AAGGGATCCACAGATATCAATCTTAATAAGG-3'. Mutant clones were screened for the presence of newly created restriction sites and confirmed by sequence analysis. To obtain the double mutant AP-1-CRE-mut 1168hu.IL6P-luc+, a *NheI-HindIII* IL-6 promoter fragment from the CRE point mutant plasmid 1168hu.IL6P-luc+, containing a 234-bp proximal promoter sequence including the CRE mutation, was used to replace the corresponding sequence in the AP-1 point-mutated IL-6 promoter variant 1168hu.IL6P-luc+. The single IL-6 promoter mutant IL6 $\kappa$ B>Ig $\kappa$ B-mut, which does no further bind RBP-J $\kappa$ , was described previously (5). All designed mutations have been described to abolish respective transcription factor binding (2) and were confirmed by sequence analysis.

**Transfection Procedures**—Stable transfection of L929sA cells was described previously (3). Transient transfection of HEK293T cells was performed with Fugene (Roche Molecular Biochemicals) according to the manufacturer's instructions. Briefly, approximately  $5 \times 10^4$  exponentially growing HEK293T cells were seeded in 24-well plates 24 h before transfection. After appropriate mixing of Fugene with the DNA plasmids of interest, transfection mixtures (containing a total amount of 500 ng of DNA) were added to each well and left on the cells for 24 h, after which medium was refreshed and cells were further used in induction experiments 60 h after transfection. Total amounts of expression vectors were kept constant in all set-ups by using an empty vector DNA. All experiments were carried out at least in triplicate.

**Electrophoretic Mobility Shift Assay (EMSA)**—L929sA cells were seeded in dishes at  $5 \times 10^5$  cells/dish at day  $-1$ . After appropriate induction, cells were washed with ice-cold PBS, harvested with a rubber policeman and pelleted in 15 ml PBS by centrifugation for 5 min at  $1100 \times g$ . Lysate preparation and EMSAs were performed essentially as described previously (5). The binding sequences for appropriate EMSAs comprised the oligonucleotides 5'-CGCTTGATGACTCAGCCG-GAA-3' (AP-1), 5'-AGAGATTGCTGACGTCAGAGAGCTAG-3' (CREB), 5'-TGCAGATTGCGCAATCGCA-3' (C/EBP), and 5'-AGCTA-TGTGGGATTTCCCATGAGC-3' (NF- $\kappa$ B). For competition assays, extracts were incubated with a 100-fold excess of unlabeled over labeled oligonucleotide. For supershift analysis, anti-p50, anti-p65, anti-CREB, anti-C/EBP $\beta$ , anti-C/EBP $\delta$ , anti-c-Fos, and anti-c-Jun antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the extracts 15 min before addition of the probe.

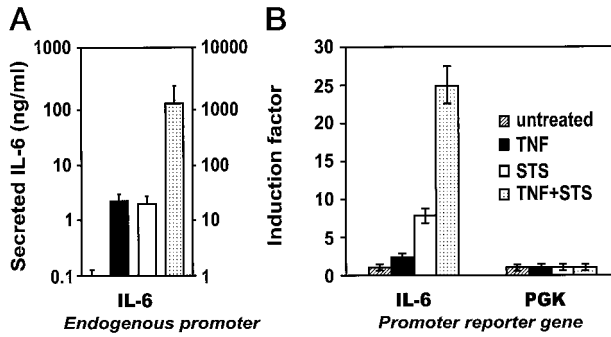
**Coimmunoprecipitation and Western Analysis**—The immunoprecipitation conditions were essentially as described previously (10).  $3 \times 10^5$  cells were seeded in six-well plates on day 1. 40 h after transfection of the various expression plasmids, 150  $\mu$ g of protein from cell lysate was incubated with 5  $\mu$ g/ml anti-p65 antibody or an irrelevant antibody as control (anti-X-press; Invitrogen) in a final reaction volume of 800  $\mu$ l. After immunoprecipitation, 20  $\mu$ g of protein was supplemented with Laemmli buffer and loaded on a 6% denaturing polyacrylamide gel for electrophoresis. Following blotting onto nitrocellulose membranes, samples were subjected to Western analysis with anti-CBP antibody and, after stripping, with anti-p65 according to the manufacturer's instructions.

**Immunofluorescence Assay Revealing Histone-4 Acetylation**—Cells were grown on coverslips for 48 h. Serum-deprived cells (24 h in 0.5% serum) were stimulated for 2 h, after which cells were washed with pBSA and fixed for 1 min in 95% methanol, 5% acetic acid at room temperature. Following fixation, cells were washed twice in PBS and incubated for 1 h in PBS + 8% BSA. Incubations with primary antisera started overnight at  $4^{\circ}\text{C}$ , using rabbit polyclonal anti-acetylated histone-4 (Upstate Biotechnology, Lake Placid, NY) diluted up to 10  $\mu$ g/ml in pBSA + 1% BSA. Coverslips were washed twice in pBSA for 5 min, after which cells were incubated with secondary antibody (goat anti-rabbit IgG biotin-conjugated antibody at 1/100 in PBS, 1% BSA) for 1 h at room temperature. After washing with pBSA, marker IgG (streptavidin-fluorescein isothiocyanate 1/100 dilution in pBSA + 10% BSA) was added for 1 h. Coverslips were finally washed three times for 15 min, after which cells were examined under a fluorescent microscope. Images were recorded using a Zeiss Axiophot fluorescence microscope coupled to a CCD video camera. Captured images were processed by MacProbe 3.4 video software.

**Reporter Gene Analysis**—Cell lysates were assayed for luciferase and galactosidase reporter gene activities as described previously (3). All promoter activities are expressed as "induction factor," *i.e.* the ratio of expression levels recorded either under induced and noninduced conditions, or under transfected and mock-transfected conditions.

#### RESULTS

**STS Potentiates TNF-induced IL-6 Production**—The supernatant of L929sA cells was tested for the presence of secreted IL-6 in response to TNF or STS or to their combination. Elevated levels of IL-6 protein were detected after TNF or STS treatment; a synergistic effect was observed after TNF + STS treatment (Fig. 1A). To verify whether the increase in IL-6 protein levels was due to transcriptional regulation at the promoter level, the IL-6 promoter reporter gene p1168hu.IL6P-luc+ and the internal control plasmid pPGK $\beta$ GeobpA were stably transfected in L929sA cells. The resulting stable cell pool was identically treated, and the lysates were assayed for corresponding reporter gene activity (Fig. 1B). Enhanced lucif-



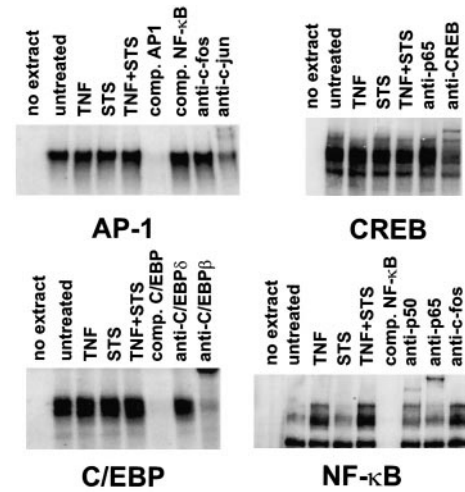
**FIG. 1. TNF- and STS-dependent gene regulation.** Confluent L929sA cell monolayers of a stable pool of promoter reporter gene constructs were untreated, treated with 2500 IU/ml TNF for 6 h, or treated with 60 nM STS, starting at -2 h. At the end of induction, supernatants were pooled for testing the secreted IL-6 levels in a biological 7TD1 assay (A), or cell lysates were assayed for reporter gene activities (B).

erase expression levels were measured in response to TNF or STS alone; they increased synergistically after a combined treatment, thus mimicking endogenous IL-6 gene regulation. This shows that the necessary and appropriate regulatory elements for IL-6 promoter activation are present in the 1168-bp promoter fragment used. However, the stronger TNF response at the IL-6 protein level as compared with that obtained with the IL-6 promoter-driven reporter gene may be explained by a combination of transcriptional effects (3) and posttranscriptional events (27). The specificity of the observed regulatory effects is further demonstrated by the internal control, which remained unaffected by the different stimulating agents used.

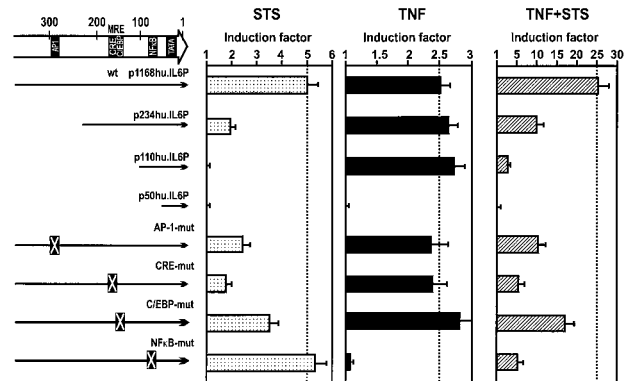
**STS Does Not Affect AP-1, C/EBP, CREB, or NF-κB Binding**—EMSA with L929sA cell extracts showed constitutive binding complexes for AP-1-, CRE-, and C/EBP-specific probes, irrespective of any stimulation with TNF or STS. The specificity of these complexes was further demonstrated by competition and/or supershift analysis (Fig. 2). On the other hand, TNF activated a specific doublet of NF-κB complexes, but the amount of constitutively binding factor RBP- $\kappa$  remained unchanged. However, no quantitative alterations of the complexes involved were observed after treatment with STS. Similar results were obtained in HEK293T cells (data not shown).

**IL-6 Gene Activation by TNF and STS Can Be Discriminated**—Since (reversible) phosphorylation of the transcription factors may have important implications in functionality and transactivation capacity (28), we further focused on reporter gene analysis. After transfection of various IL-6 promoter mutants in L929sA cells, the resulting stable pools were assayed for their responsiveness to TNF and STS (Fig. 3). After truncation of the IL-6 promoter from the 5' end, a fairly decreased inducibility by STS is observed in the 234-bp promoter lacking the AP-1 site, as compared with the full-size fragment; moreover, the inducibility by STS is completely lost in the 110- and 50-bp IL-6 promoter variants, in which the three upstream sequence elements for AP-1, CREB and C/EBP are lacking (Fig. 3, STS). The inducibility by TNF, however, remains fairly similar for the different deletion variants, except for p50hu.IL6P-luc+, in which the NF-κB motif is absent (Fig. 3, TNF). These results clearly point at different activation mechanisms after stimulation of the IL-6 promoter by TNF or STS.

In order to further characterize the contribution of defined sequence elements, different point-mutated IL-6 promoter variants were also tested for their responsiveness to TNF or STS. As far as TNF treatment is concerned, only the specific mutation affecting the NF-κB motif abolished TNF inducibility (Fig. 3, TNF); however, the same mutation had no effect at all on



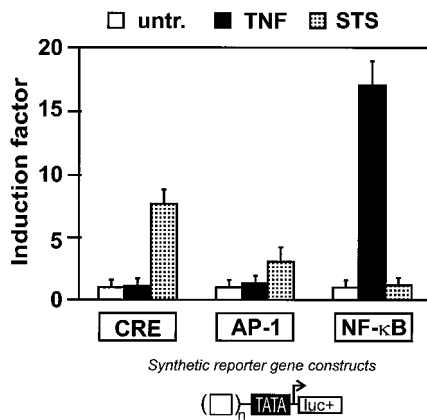
**FIG. 2. Effect of TNF and/or STS on DNA binding by AP-1, C/EBP, CREB, and NF-κB complexes.** Confluent L929sA cell monolayers were assayed for binding activity with specific oligonucleotides containing respective binding sequences. Equivalent amounts of total cellular extracts were analyzed by EMSA. Cells were treated either with 2500 IU/ml TNF for 2 h and/or with 60 nM STS, starting at -2 h. TNF-treated extracts were used for supershift analysis with antibodies or for competition (comp.) assays with cold oligonucleotide.



**FIG. 3. Localization of TNF- and STS-responsive elements in the IL-6 promoter.** Various IL-6 promoter-derived recombinant reporter gene constructs were used in induction experiments (black boxes, transcription factor-binding sites; crossed boxes, mutations of the transcription factor-binding sites yielding the point-mutated versions of p1168hu.IL6P-luc+). Stable cell pools of the promoter reporter gene constructs were left untreated or were induced with 2500 IU/ml TNF for 6 h, with 60 nM STS for 8 h, or added 2 h prior to TNF in a combined treatment.

activation by STS (Fig. 3, STS). It appears that regulatory elements other than the NF-κB motif are involved in the responsiveness to STS, since single mutations of the AP-1, CREB, or C/EBP elements partially affect the inducibility by STS and point at a redundancy in the activation mechanism. Treatment with TNF + STS confirmed the observations made with TNF and STS alone, and resulted in a superposition of both separate profiles; mutations in the AP-1, CRE, C/EBP, or NF-κB motif all drastically affect the synergistic effect of TNF + STS (Fig. 3, TNF + STS).

Additional evidence for the specific role of NF-κB in TNF inducibility and of AP-1 or CREB in responsiveness to STS was obtained from reporter gene variants containing multimerized synthetic responsive sequences for AP-1, CREB, or NF-κB in front of an unresponsive minimal promoter. Whereas the NF-κB reporter construct exclusively responds to TNF and not to STS, the CRE and AP-1 reporter constructs respond to STS but not to TNF (Fig. 4).

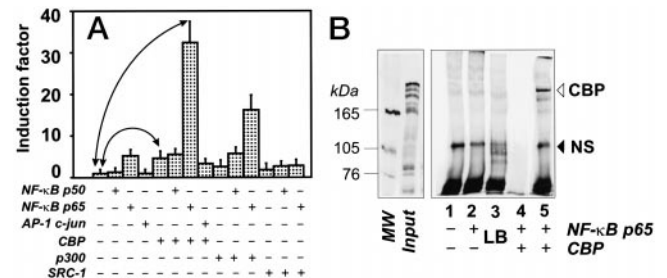


**FIG. 4. TNF- and STS-dependent gene activation can be discriminated.** Stable cell pools of reporter gene constructs were left untreated or were induced with 2500 IU/ml TNF for 6 h, with 60 nM STS for 8 h, or added 2 h prior to TNF. Lysates were assayed for reporter gene expression and normalized for protein concentration.

**Cofactor-dependent Regulation of NF- $\kappa$ B Mainly Relies on the p65 Subunit**—Since interaction of CBP, p300, and SRC-1 with various IL-6 promoter-binding factors has already been demonstrated, we tested the functional implication of the p50 and p65 NF- $\kappa$ B subunits together with CBP, p300, or SRC-1 in activating 1168hu.IL6P-luc+ (Fig. 5A). To avoid interference of signaling cascades with the expression levels of transgenes controlled by cytomegalovirus- or Rous sarcoma virus-driven promoters (29), experiments were performed in the absence of TNF or STS. As a matter of fact, transcription factor interactions with CBP can be achieved in the absence of appropriate stimuli, since transient transfection by itself enhances constitutive kinase activities (30). While basal IL-6 promoter activity is clearly induced after overexpression of p65, overexpression of p50 does not affect the promoter activity. This is in complete agreement with the presence and absence of transactivation domains in the p65 and p50 subunits, respectively. Furthermore, we detected a synergistic promoter activation of 20–30-fold after cotransfection of p65 with p300 or CBP, but a very weak activation only with p50. On the other hand, SRC-1 cotransfection marginally enhanced basal IL-6 promoter activity with p50, but not with p65. It may be noted that IL-6 promoter stimulation with p300 or CBP alone, *i.e.* in the absence of p65, also enhances reporter gene activity (3–5-fold), since AP-1, CREB, and C/EBP are constitutively bound to their corresponding responsive elements and have been shown to also interact with CBP/p300. Extra supply of the AP-1 subunit c-Jun (in addition to the endogenous amounts of c-Jun present) under transfection conditions identical to those used for p65 does not further enhance basal or CBP-stimulated IL-6 promoter activity. This experiment demonstrates a potent and specific role for p65 in coactivator effects with CBP/p300 within the IL-6 promoter context and to their strong cooperative activity in transcriptional stimulation.

To assess the physical interaction between p65 and CBP, cells transfected with pRSV-p65 and/or pCMV-CBP were subjected to coimmunoprecipitation analysis with anti-p65 antibody, followed by Western analysis with anti-CBP (Fig. 5B). p65 is able to trap CBP from the cell lysate (lane 5), which suggests a strong and distinct interaction between these two factors. Membranes were subsequently stripped and reprobed with anti-p65 antibody to verify equal amounts of immunoprecipitated p65 protein (data not shown).

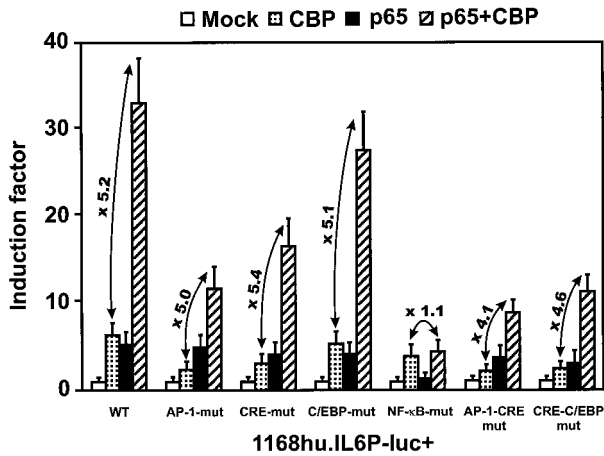
**CBP Potentiates Basal and Inducible IL-6 Gene Expression**—We further analyzed the relative contribution of CBP



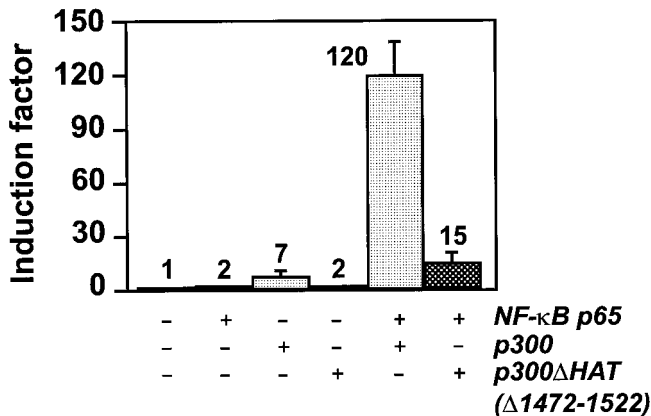
**FIG. 5. Cofactor recruitment by NF- $\kappa$ B mainly depends on the p65 subunit.** A, HEK293T cells were transiently transfected with p1168hu.IL6P-luc+ in combination with various expression plasmids (+). The quantity of DNA per 24-well amounts to 80 ng (IL-6 promoter construct), 80 ng (p65, p50, or c-Jun expression vector), and 350 ng (CBP, p300 or SRC-1 expression plasmid). The total amount of expression vectors was kept constant in all set-ups by using empty-vector DNA. Cells were lysed 60 h after transfection, and the luciferase expression levels in the lysates were normalized for protein concentration. B, HEK293T cells were transiently transfected with two expression plasmids (+). Lanes 1, 2, and 5 contain lysates of mock-transfected cells and of cells transfected with pRSV-p65 and pRSV-p65 + pCMV-CBP, respectively, immunoprecipitated with anti-p65 antibody and revealed with anti-CBP antibody after Western blot analysis. Control set-ups (lanes 3 and 4) contain immunoprecipitates of lysis buffer (LB) alone and an irrelevant antibody, respectively, revealed by anti-CBP antibody in a Western blot. The input lane represents one third of cell lysate used for the assay. Black arrowhead, nonspecific band; white arrowhead, 265-kDa band corresponding with coimmunoprecipitated CBP.

interactions with AP-1, C/EBP, CREB, or NF- $\kappa$ B in basal or p65-driven IL-6 gene expression. Mutation of the AP-1- or CREB-responsive elements clearly shows a decreased CBP-mediated effect on basal promoter activity, while mutations in the C/EBP or NF- $\kappa$ B elements are less pronounced (Fig. 6). Consequently, a further reduction of CBP effects is observed in the double mutants AP-1-CRE or CRE-C/EBP. The role of CBP interactions in the activated state of the IL-6 promoter was also analyzed. Mimicking NF- $\kappa$ B activation by overexpression of p65 consistently increased the IL-6 promoter activity, whereas mutation of the NF- $\kappa$ B motif abolished the induction (none of the other mutations affected p65-mediated transactivation). Coexpression of p65 and CBP resulted in a strong synergistic up-regulation of IL-6 promoter activity with the wt promoter and the different promoter variants; this was not the case for the NF- $\kappa$ B mutant, where the synergistic effect is totally absent and where the induction level is similar to the promoter activity in the presence of CBP alone. Since promoter activation after coexpression of CBP and p65 also includes CBP effects with the constitutively bound factors, the various mutations in the AP-1, CRE, and/or C/EBP motifs correspondingly reduce the level of promoter activation; the synergism, however, between p65 and CBP (*i.e.* the activity of cotransfected CBP and p65 *versus* CBP alone) remains constant (5-fold). These data suggest that CBP is settled in the IL-6 promoter complex by multiple protein-protein interactions with different transcription factors bound constitutively or activated by external stimuli.

**HAT Activities Specifically Potentiate NF- $\kappa$ B-driven IL-6 Gene Expression**—Several characterized transcriptional adaptors and cofactors, including CBP/p300, have been recognized to be HATs. Since we showed an important role of CBP/p300 in IL-6 promoter activity, we might expect the HAT capacity of CBP/p300 and/or its associated factors to be an integral part of the activation mechanism for promoter stimulation. We measured the contribution of CBP/p300 HAT function in IL-6 promoter activation after coexpression of p65 with wt p300 or p300HAT( $\Delta$ 1472–1522) (Fig. 7). Remarkably, the strong synergism between p65 and p300 is drastically reduced after deletion

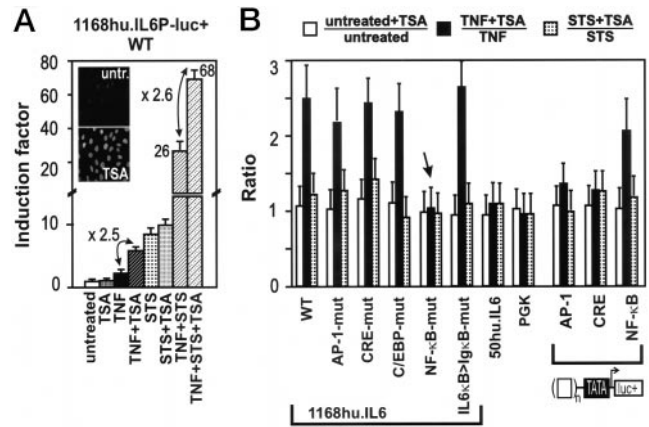


**FIG. 6. CBP overexpression potentiates basal and inducible IL-6 promoter activation.** HEK293T cells were transiently transfected with wt and various point-mutated IL-6 promoter variants, together with plasmids for CBP and p65. The quantity of plasmid DNA used per 24-well was 80 ng (IL-6 promoter construct), 80 ng (p65 expression vector), and 350 ng (CBP expression vector); the total amount of plasmid DNA was kept constant in all set-ups by using empty-vector DNA. Cells were treated 60 h after transfection as described in the legend to Fig. 5.



**FIG. 7. HAT activity potentiates NF-κB-driven IL-6 gene expression.** HEK293T cells were transiently transfected with p1168hu.IL6P-luc+ together with various expression plasmids (+). The quantity of DNA per 24-well was 48 ng (IL-6 promoter construct), 0.4 ng (p65 expression plasmid), and 28 ng (p300 or p300HAT(Δ1472-1522) expression plasmid); the total amount of expression vectors was kept constant in all set-ups by using empty-vector DNA. Cells were lysed 60 h after transfection; the luciferase expression levels in the lysates were normalized for protein concentration.

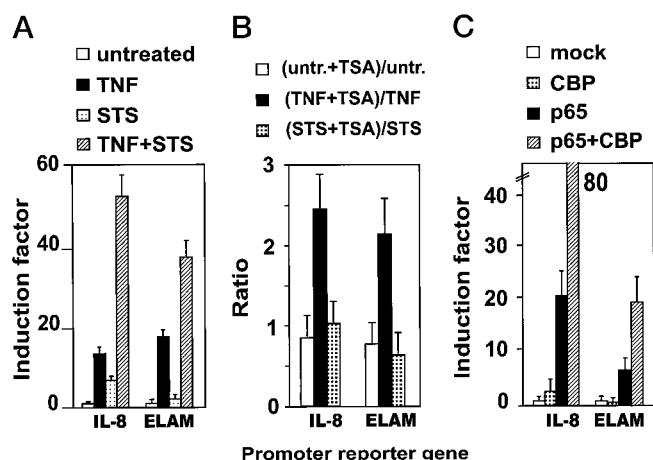
of the HAT domain; this is accompanied by a strong reduction of HAT activity (24, 31). In another approach, the impact of HAT/HDAC activity was tested with TSA. Immunofluorescence analysis demonstrates increased *in vivo* histone-4 acetylation after treatment with TSA (Fig. 8A, inset). As such, TSA effects were also evaluated in reporter gene analysis on TNF- and/or STS-driven IL-6 promoter induction. Since transiently transfected DNA often shows aberrant chromatin packaging, and since TSA effects have been shown to depend on native chromatin (32, 33), we used stable pools of the wt promoter, various point-mutated IL-6 promoter variants and other related recombinant constructs in those experiments (Fig. 8). The most striking observation, however, is that only the TNF-dependent promoter activation could be enhanced (2.5-fold) after treatment with TSA, while the basal or STS-dependent gene activity remained unaffected (Fig. 8A). The strong synergistic activity of TNF + STS could also be enhanced with TSA to the same



**FIG. 8. TSA potentiates NF-κB-dependent reporter gene expression.** Stable cell pools of wt (A and B), different point-mutated IL-6 promoter variants (B) and synthetic reporter gene constructs with multimerized responsive elements (B) were left untreated, or were treated with 2500 IU/ml TNF for 7 h, with 60 nM STS for 9 h, with TNF (7 h) + STS (9 h), whether or not in the presence of 100 nM TSA (7 h). Lysates were assayed for reporter gene expression and normalized for protein concentration. A, inset, immunofluorescent image of *in vivo* histone-4 hyperacetylation (serum-deprived L929sA cells were untreated or treated with 100 nM TSA for 2 h; HAT levels were revealed with anti-acetyl histone-4 antibody). B, TSA effects are expressed as relative increase caused by TSA, i.e. the ratio of induction factor of a treatment with or without TSA.

extent as TNF stimulation alone (i.e. 2.5-fold). These results suggest a close link between cofactor-dependent HAT activities and NF-κB-driven gene transcription. A comparative experiment with the point-mutated IL-6 promoter variants (Fig. 8B) showed that the TSA effect is limited to the NF-κB site. Indeed, none of the point mutations affected TSA-enhanced induction by TNF, whereas mutation of the NF-κB motif completely eliminated the up-regulation by TSA, as is the case for p50hu.IL6P-luc+. Since the IL-6-κB motif also constitutively binds the RBP-Jκ repressor complex, including the TSA-sensitive molecule HDAC-1 (5, 34, 35), we further investigated whether removal of this complex might influence the observed TSA effect. However, mutation of IL-6-κB to an I $\kappa$ B-responsive site that no longer binds RBP-Jκ (5) did not alter the observed TSA effects, which further points at an exclusive role of NF-κB. Similar conclusions can be drawn from synthetic reporter constructs containing multiple copies of AP-1-, CRE-, or NF-κB-responsive elements in front of a minimal promoter. Also in this case, a major TSA effect is observed only with the TNF-stimulated NF-κB reporter gene activity, while the STS-stimulated reporter gene activity remains almost fully unaffected (Fig. 8B). Finally, the constitutive 3-phosphoglycerate kinase promoter, which lacks NF-κB-responsive elements, is (as expected) completely unresponsive to TSA. These data clearly show an exclusive link between the increase in NF-κB-mediated IL-6 promoter activation and enhanced HAT activity.

**CBP Engagement and HAT Activity May Be Generally Required for NF-κB-dependent Promoter Activation**—Since NF-κB appears to play a unique role in switching on IL-6 gene expression, by engagement of CBP and HAT-dependent activities, we investigated whether other NF-κB-containing promoters, such as the IL-8 and ELAM promoters, respond similarly to a treatment with TNF, STS, and/or TSA, or to overexpression of CBP. Stable cell pools of IL-8 and ELAM promoter reporter gene constructs were treated with TNF or STS alone or with their combination, and the lysates were assayed for corresponding reporter gene expression (Fig. 9A). Strongly enhanced luciferase expression levels were measured in response to TNF in both cases, which is in agreement with the presence



**FIG. 9. Engagement of CBP and sustained HAT activity reveal a general mechanism for NF- $\kappa$ B-dependent promoter activation.** L929sA cells stably transfected with NF- $\kappa$ B-containing promoter constructs p1481hu.IL8P-luc (*IL-8*) or with pELAMP-luc (*ELAM*) were treated either as described in the legend to Fig. 1 (A) or to Fig. 8 (B). HEK293T cells were transiently transfected with various reporter gene constructs as described in the legend to Fig. 5 (C).

of one or more NF- $\kappa$ B sites in the *IL-8* and *ELAM* promoters (20, 36). STS also stimulated both promoters, whereas TNF + STS synergistically increased the promoter activities, as also observed with the *IL-6* promoter. In addition, after stimulation of the pools with TNF or STS, whether or not in combination with TSA (Fig. 9B), effects similar to those obtained with the *IL-6* reporter gene were found, namely specific enhancement of NF- $\kappa$ B-mediated transcription *versus* non-NF- $\kappa$ B-driven gene expression. Transient transfection experiments carried out with the *IL-8* and *ELAM* promoters clearly showed that the strong up-regulation of promoter activity after coexpression of p65 and CBP is maintained by the different NF- $\kappa$ B-driven promoters (Fig. 9C).

#### DISCUSSION

Transcription factor-selective and signal-specific cofactor and/or HAT/HDAC recruitment have recently become a prime focus of investigation (8, 37–40). In the present work, we explored how the multiresponsive *IL-6* promoter may be differentially modulated by TNF or STS and what the underlying mechanism for promoter stimulation might be at the transcription factor and cofactor levels. We clearly demonstrate that TNF- and STS-dependent *IL-6* gene transcription can be distinctly discriminated. Whereas NF- $\kappa$ B alone is responsible and sufficient for responsiveness to TNF, neighboring binding factors such as AP-1, CREB, or C/EBP are crucial but redundant for STS-dependent gene activation. Investigation into the cofactor regulation in *IL-6* promoter stimulation revealed a strong synergism between p65 and CBP/p300, which is highly dependent on its HAT properties. Under conditions of sustained histone-4 acetylation following treatment with TSA, TNF-dependent (*i.e.* NF- $\kappa$ B-mediated) gene activation is distinctly increased, while basal or STS-stimulated *IL-6* promoter activity remains completely unchanged. The exclusive link between the effect of TSA and activated NF- $\kappa$ B was further strengthened by the fact that only the NF- $\kappa$ B mutation completely eliminates the TNF/TSA synergism.

So one may postulate that the *IL-6* promoter is dynamically regulated at the NF- $\kappa$ B site by an equilibrium of a coactivator complex including CBP/p300, interacting with NF- $\kappa$ B, and a corepressor complex containing HDAC-1, associated with RBP-J $\kappa$ . In the absence of activated NF- $\kappa$ B, the *IL-6*- $\kappa$ B site is

occupied by RBP-J $\kappa$  (5). Hence, the moderate CBP effects by AP-1, CRE, and C/EBP might result from a dominant RBP-J $\kappa$  repressor complex, shielding the RNA polymerase holoenzyme from CBP effects. Furthermore, the strong synergistic effect of p65 + CBP may be the consequence of dislocation of RBP-J $\kappa$  and its associated corepressor complex by NF- $\kappa$ B, followed by efficient engagement and/or activation of CBP/p300. The observed TSA effects might originate from inhibited HDAC activity associated with RBP-J $\kappa$ , in addition to sustained HAT activity engaged by p65. However, inhibition of HDAC activity in the basal state or after treatment with STS does not lead to enhanced gene activity, which suggests a minor contribution of the repressor complex. Replacement of the RBP-J $\kappa$ -binding site in the corresponding *IL-6* promoter mutant does not alter the effects of TSA on TNF induction. This observation further minimizes the role of the repressor complex in TSA phenomena linked to the NF- $\kappa$ B site. In addition, other NF- $\kappa$ B-dependent promoters, in which binding of RBP-J $\kappa$  does not occur or was at least not reported, respond in the same way to CBP and TSA as the *IL-6* promoter. Therefore, cofactor (CBP)- and HAT-dependent regulation converge exclusively at the factor NF- $\kappa$ B, and may represent two different aspects of the same activation mechanism. TSA-effects have now been specifically linked to transactivation by the factors RXR/RAR in an RA-responsive promoter (41), to Sp1 in the *WAF1/Cip1* promoter (42), to C/EBP and Stat5 in the  $\beta$ -casein promoter (43), as well as to NF-Y in the *MDR1* and *hsp70* gene promoters (44, 45). Our observations permit to extend the list of linkage of TSA effects to transactivation by NF- $\kappa$ B in *IL-6*- and other NF- $\kappa$ B-dependent gene promoters.

Although transcriptional activation by nucleosomal relaxation due to local hyperacetylation of histones by CBP/p300 is now generally accepted (32, 46, 47), it cannot be ruled out that other nuclear factors, corecruited to the *IL-6* promoter, are also responsible for (part of) the acetylation process and/or chromatin remodeling activities (48–52). It may also be noted that nonhistone proteins, like transcription factors, have also been reported to serve as substrates for HAT activity (17). In view of the enhanced NF- $\kappa$ B-driven gene transcription in response to sustained HAT activity, one may expect acetylation of p50 and p65 by CBP/p300; such acetylation has, however, not been demonstrated (53). Alternatively, cross-talk of acetylation with phosphorylation (54, 55), methylation (56) and caspase regulation (57) has been reported. But neither the p38 mitogen-activated protein kinase inhibitor SB203580 nor the methylation inhibitor azacytidine, nor the caspase inhibitor benzyloxycarbonyl-Asp(O-Me)-Glu(O-Me)-Val-Asp(O-Me)-fluoromethylketone (zDEVD-fmk) were able to revert the TSA effects observed at the *IL-6* promoter level in L929sA cells.<sup>2</sup>

Our experimental data support a model for synergistic transcription in which the cointegrator CBP/p300 may be recruited to the multiresponsive *IL-6* promoter by multiple protein-protein interactions with the DNA-bound factors AP-1, CREB, C/EBP, and NF- $\kappa$ B, sequentially arranged along the promoter sequence (Fig. 10). In this context, the ultimate switch for gene induction is achieved by TNF-induced NF- $\kappa$ B, which engages the available CBP/p300 for maximal transcriptional stimulation. Whether this engagement relies on conformational changes of CBP by interaction with NF- $\kappa$ B (58, 59) or whether it is the result of concomitant posttranslational modification of either NF- $\kappa$ B or CBP, for example by TNF induction, is not clear (17, 30, 60–63). We have indeed reported that activation of extracellular signal-regulated kinase and p38 mitogen-activated protein kinases is a necessary step for *IL-6* gene expres-

<sup>2</sup> W. Vanden Berghe, unpublished results.

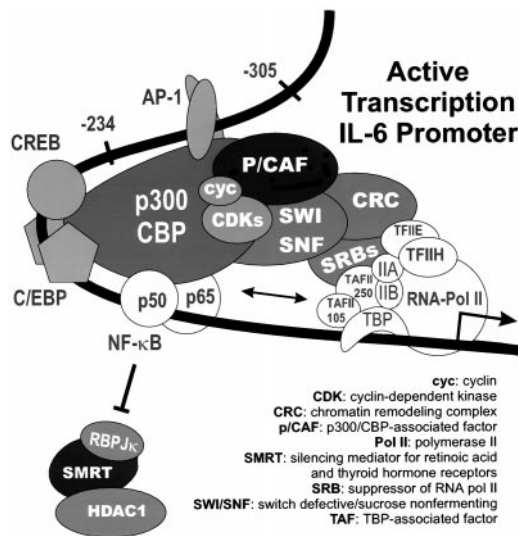


FIG. 10. Hypothetical model of the IL-6 promoter-specific transcriptional complex.

sion in response to TNF (3); we have, however, not found p65 itself to be a substrate for TNF-induced mitogen-activated protein kinase phosphorylation.<sup>3</sup> To fully understand IL-6 gene regulation, it will be needed to determine how CBP can simultaneously integrate functions of various transcriptional activators present in the IL-6 promoter. A similar model with multiple transcription factor-CBP interactions and a strictly stereospecific arrangement has been suggested for virus-induced stimulation of the interferon- $\beta$  promoter enhanceosome (9). Alternatively, a coactivator sequestration model has also been proposed in which multiple transcription factors are competing for limiting amounts of CBP and become opponents to mediate CBP effects (64). So far, our experimental data favor the first model since all factors are required for optimal promoter activation in synergy with CBP. The dominant role of p65 in the engagement of CBP within the IL-6 promoter context is representative of an enhanceosome-like structure and function different than those proposed for the interferon- $\beta$  gene promoter.

In conclusion, our results suggest an essential role for NF- $\kappa$ B in engaging CBP and HAT-responsive transcription from the IL-6 promoter and other NF- $\kappa$ B-driven promoters *in vivo*. For the first time, we identify (histone) acetylation as a new player in specific modulation of NF- $\kappa$ B-mediated gene induction on chromatin-embedded promoters.

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