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Fos and Jun Repress Transcription Activation by NF-IL6 through Association at the Basic Zipper Region

WEI HSU,[1] TOM K. KERPPOLA,[2] PHANG-LANG CHEN,[3] TOM CURRAN,[2] AND SELINA CHEN-KIANG[1]*

Brookdale Center for Molecular Biology, Mount Sinai School of Medicine, New York, New York 10029[1]; Department of Molecular Oncology and Virology, Roche Institute of Molecular Biology, Nutley, New Jersey 07110[2]; and Center of Molecular Medicine, Institute of Biotechnology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284[3]

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NF-IL6 and AP-1 family transcription factors are coordinately induced by interleukin-6 (IL-6) in a cell-type-specific manner, suggesting that they mediate IL-6 signals in the nucleus. We show that the basic leucine zipper (bZIP) region of NF-IL6 mediates a direct association with the bZIP regions of Fos and Jun in vitro. This interaction does not depend on the presence of their cognate recognition DNA elements or the posttranslational modification of either partner. NF-IL6 homodimers can bind to both NF-IL6 and AP-1 sites, whereas Fos and Jun cannot bind to most NF-IL6 sites. Cross-family association with Fos or with Jun alters the DNA binding specificity of NF-IL6 and reduced its binding to NF-IL6 sites. NF-IL6 isoforms that differ in the site of translation initiation have distinct transcriptional activities. Activation of a reporter gene linked to the NF-IL6 site by NF-IL6 is repressed by Fos and by Jun in transient transfection assays. Thus, association with AP-1 results in repression of transcription activation by NF-IL6. The repression is NF-IL6 site dependent and may have a role in determining the promoter and cell type specificity in IL-6 signaling.

Interleukin-6 (IL-6) is a pleiotropic cytokine that induces differentiation and regulates cell growth in a lineage- and cell-type-dependent manner, such as the differentiation of B cells and the induction of acute-phase responses in hepatocytes (5, 21, 37). Despite extensive descriptions of its biological activities, the mechanism that underlies the cell type specificity of IL-6 signaling is not understood. In most cases, signals mediated by IL-6 receptors on the cell surface lead to transcriptional regulation of genes that are specific to the differentiated phenotype. This implies that transcription factors are selectively and coordinately regulated in response to signals initiated by IL-6.

NF-IL6 is a human transcription factor of the C/EBP family whose mRNA synthesis is regulated by IL-6 and by other cytokines (1). NF-IL6 contains a bipartite DNA-binding domain consisting of a region rich in basic residues adjacent to five heptad repeats of leucine residues (basic leucine zipper [bZIP]) at its carboxyl terminus, analogous to LAP, its rodent counterpart also known as IL-6DBP, AGP/EBP, C/EBPβ, rNF-IL6, or CRP-2 (1, 3, 4, 7, 26, 35, 50). Through the bZIP region, NF-IL6 dimerizes with itself or other members of the C/EBP family and regulates target genes by binding as homo- or heterodimers to specific sequences in the promoters (22, 48). Most genes that are targets of the IL-6 signal transduction pathway contain NF-IL6-binding sequences in their promoters and enhancers (5), implying a potential role for NF-IL6 in mediating the signals.

The transactivating domain(s) of LAP lies outside the bZIP region (8). LAP functions as an activator or repressor, depending on the ratio of different forms of the protein that are translated from in-frame AUGs of the same mRNA species, at least with respect to a minimal albumin promoter linked to a LAP-responsive element (8). The abundance and ratio of the different forms of NF-IL6 vary according on the cell type and can be altered to favor the activator form by IL-6 and by other extracellular signals such as retinoic acid (13). Thus, IL-6 may mediate its signals by attenuating the ratio, and hence the interplay, of the activator and repressor forms of NF-IL6. In addition, transcriptional activation by NF-IL6 can be regulated by the C/EBPs with which it dimerizes, augmented by NF-IL6β in human cells (20) and antagonized by CHOP-10 in rodent cells (38). It can be enhanced by phosphorylation near and within the bZIP region (30, 49). Translocation of NF-IL6 from cytoplasm to the nucleus (26) and association of NF-IL6 with the c-*rel* domain of NFκB in vitro (23) imply that the functions of NF-IL6 can be further modulated by its subcellular compartmentalization and by interactions with proteins outside the C/EBP family.

AP-1 family transcription factors are also induced by IL-6, depending on lineage and cell type. In B cells, *junB* is activated by IL-6 (29). In myeloid leukemia cells, c-*jun*, *junB*, and *junD*, but not c-*fos*, are activated during macrophage differentiation (25), whereas in PC12 cells, c-*fos* is induced upon neuronal differentiation by IL-6 (40). Once activated, the expression of mRNAs encoding Jun, as well as NF-IL6 , is maintained at elevated levels in the presence of IL-6 (25, 32). The coordinate regulation of NF-IL6 and members of the Fos and Jun families raises the possibility that they can interact to mediate responses to IL-6 signals.

Both AP-1 and C/EBP family proteins form dimers via a leucine zipper interface by coiled-coil interactions and bind to DNA via a basic DNA contact surface (10, 22, 33), suggesting that NF-IL6 may associate with Fos and Jun. Fos and Jun form stable dimers with selective members of the ATF family according to their affinity for association (12). Fos and Jun can also interact with proteins that do not

* Corresponding author. Phone: (212) 241-6588. Fax: (212) 860-9279.

possess a bZIP structure, thereby participating in the regulation of diverse differentiation functions. Transcription activity of the glucocorticoid receptor is regulated by association with Fos and Jun positively and negatively, depending on the composition of Fos and Jun (9, 18, 27). Likewise, transcription activation by helix-loop-helix proteins MyoD and myogenin is antagonized upon association with Jun and Fos (2, 24). Recent studies on the early events of T-cell activation further demonstrate that Fos and Jun are components of a complex that is important for activation of transcription of the IL-2 promoter (5, 45).

We have undertaken the present study to elucidate the interactions between IL-6-regulated bZIP proteins. We demonstrate that NF-IL6 associates with Fos and with Jun through their bZIP regions in the absence of DNA. This interaction leads to an altered DNA binding specificity and modifies transcription activation by NF-IL6. The regulation of NF-IL6-responsive genes by IL-6 may therefore be subject to combinatorial regulation by NF-IL6 and AP-1 family proteins.

MATERIALS AND METHODS

Recombinant plasmids. pBS(SK)NF-IL6 was constructed by isolating and inserting the *Hin*dIII-*Bam*HI fragment of pCMV(NF-IL6) (1), which contains the coding sequences of human NF-IL6, into the *Hin*dIII and *Bam*HI restriction sites of pBluescript (SK). Glutathione *S*-transferase (GST)–NF-IL6-1 was constructed by inserting the *Hin*dIII-*Bam*HI fragment of pBS(SK)NF-IL6 into the same restriction sites of pGEX-2T. GST–NF-IL6-2 was constructed by inserting the *Nco*I-*Bam*HI fragment, after blunt ending with a Klenow fragment, into the *Sma*I site of pGEX-3X. To construct 3'-deletion mutants of GST–NF-IL6-2, the plasmid was linearized with *Eco*RI, blunt ended, and partially digested with *Rsa*I. The fragments were separated on a 0.8% gel, isolated, and ligated, and the resultant plasmids were verified by sequencing. GST–NF-IL6-3 was constructed by inserting the *Sma*I-*Bam*HI fragment of pBS(SK)NF-IL6 into the *Sma*I site of pGEX-3X. GST-Myc has been described elsewhere (11). pCMVNF-IL6-2 was constructed by inserting the *Nco*I-*Bam*HI fragment of pBS(SK)NF-IL6, after blunt ending, into the *Hin*dIII site of a pCMV vector (obtained from F. Rauscher). pCMVNF-IL6-3 was constructed by inserting the *Sma*I-*Bam*HI fragment of pBS(SK)NF-IL6 into a blunt-ended *Hin*dIII site and the *Bam*HI site of a pCMV vector. pCMVfos and pCMVjun are as previously described (41).

Transcription and translation in vitro. RNAs were transcribed in vitro from pSP65-fos, pGEM4-jun (36), and pBS-oct-1 (13) by SP6 or T7 RNA polymerase in 100 μl of a transcription buffer containing 200 mM Tris-HCl (pH 7.5), 50 mM NaCl, 30 mM $MgCl_2$, 10 mM spermidine, 10 mM dithiothreitol (DTT), 0.5 mM nucleoside triphosphate, 0.5 mM ^{7}GpppG (New England Biolabs), 100 U of the RNase inhibitor RNasin (Promega), and 2 μg of linearized DNA templates at 37°C for 60 min. Following digestion of the plasmid DNA with 5 μl of RNase-free DNase I (Promega) at 37°C for 15 min, RNA was precipitated with ethanol. Proteins were translated in vitro from 1 μg of RNA in 40 μl of nuclease-treated, RNA-dependent rabbit reticulocyte lysates (Promega) at 30°C for 60 min. Each reaction mixture was supplemented with 1 mM amino acid mixture without methionine and 20 μCi of [^{35}S]methionine for the synthesis of labeled proteins or with 1 mM of amino acid mixture with methionine for the synthesis of unlabeled proteins.

Expression of recombinant proteins. GST and GST fusion proteins were expressed in *Escherichia coli* BL21(DE3) cells by using the T7 expression system (44). To express soluble fusion proteins, overnight cultures of bacteria that were newly transformed with the recombinant plasmids were diluted with 10 volumes of medium, cultured for an additional 2 h (optical density at 600 nm of 0.4), and induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG; Sigma) at 37°C for 2 h. Cells from 3 ml of culture were harvested by centrifugation in an Eppendorf centrifuge (14,000 rpm for 5 min) and resuspended in 0.5 ml of phosphate-buffered saline (PBS) containing 1% Triton X-100 (PBS-Triton). The lysates were sonicated three times with 15-s pulses (Vibra Cell Sonicator at 40% duty cycle), and the debris was pelleted by centrifugation (14,000 rpm for 5 min). The supernatants (10 μl) were mixed with 50 μl of glutathione-Sepharose (Sigma), equilibrated and suspended in PBS-Triton according to the manufacturer's specification, and incubated at 4°C for 30 min. The Sepharose was collected by centrifugation (5,000 rpm for 2 min), washed three times with PBS-Triton, resuspended in 10 μl of sodium dodecyl sulfate (SDS) sample loading buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 100 mM DTT, 0.1% bromphenol blue, 10% glycerol), and assayed by SDS-polyacrylamide gel electrophoresis (PAGE). The GST fusion proteins were purified to greater than 90% homogeneity by this procedure, as determined by silver staining and Coomassie blue staining, and can be eluted with a buffer containing 5 mM Tris-HCl (pH 8.0) and 5 mM glutathione.

Association of proteins in vitro. Fos, Jun, and Oct-1 (0.3 fmol) translated in vitro and labeled with [^{35}S]methionine were incubated with 2 pmol of bacterially expressed GST or GST fusion proteins in 300 μl of buffer A (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 2 mM EDTA, 5 mM DTT, 2 mg of leupeptin per ml, 2 mg of antipain per ml, 0.5% Nonidet P-40) or in radioimmunoprecipitation assay buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 2 mg of leupeptin per ml, 2 mg of antipain per ml, 0.1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, and 1% sodium deoxycholate). The reaction mixtures were incubated at 37°C for 10 min and then at 4°C for 10 min. The protein complexes were precipitated with 50 μl of glutathione-Sepharose, washed three times with the same incubation buffer, and analyzed by SDS-PAGE (12% gel) and autoradiography.

Immunoprecipitation and immunoblotting. Nuclear extracts were prepared from HepG2 or Jurkat cells as described previously, and the protein concentration was determined by the Bradford assay (43). In each assay, 100-μg aliquots of nuclear extracts were incubated, with or without 30 ng of purified recombinant Fos or Jun (kindly provided by C. Abate) (6, 36), in 300 μl of buffer A at 37°C for 10 min. Protein complexes were recovered by the addition of anti-Fos or anti-Jun (1:333 dilution), each of which is a polyclonal rabbit antiserum directed against recombinant full-length Fos or Jun, respectively (36), and incubation at 4°C for 1 h. An anti-Oct-1 antibody (13) was used as a control. Protein A-Sepharose (75 μl of a 40-mg/ml suspension; Sigma) was added. After incubation at 4°C for 30 min, the immune complexes were precipitated by centrifugation (5,000 rpm for 3 min), washed three times with buffer A, and resuspended in 20 μl of SDS sample loading buffer. After boiling for 5 min, the supernatant was analyzed by SDS-PAGE (12% gel). The proteins were transferred onto nitrocellulose membranes by electrophoresis at 4°C, after which the membranes were incubated with an anti-NF-IL6 antiserum (anti-P4; 1:250 dilution) for 30 min at room temperature as described

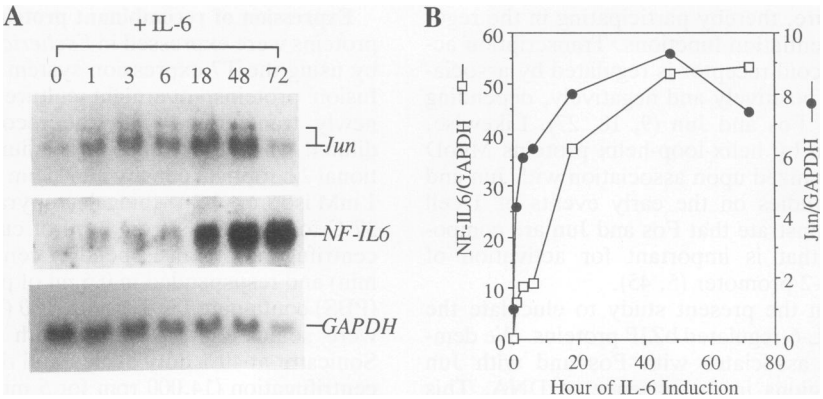

FIG. 1. NF-IL6 and c-Jun mRNAs are coordinately induced by IL-6. (A) Total RNA was isolated from 10^6 M1 cells that were cultured without (0) or with IL-6 for the times (in hours) indicated and analyzed by Northern blotting as described elsewhere (13). The blot was hybridized sequentially with ^{32}P-labeled probes specific to NF-IL6 [the *Sma*I-*Bam*HI fragment of pBS(SK)NF-IL6], c-Jun (36), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which serves as a control for sample loading (13). (B) The hybridization signals were quantified by densitometry scanning of the autoradiograph shown in panel A and expressed as the ratios of NF-IL6 to GAPDH and Jun to GAPDH as a function of time of IL-6 treatment.

previously (13). The bound antibody was then detected with an alkaline phosphatase-conjugated secondary antibody (Promega) and visualized by the chromogenic substrate reaction. As controls for immunoblotting, 20-μg aliquots of nuclear extracts were analyzed directly without immunoprecipitation.

Electrophoretic mobility shift assay. Purified GST–NF-IL6-3 fusion protein and recombinant Fos and Jun, or in vitro-translated Fos and Jun, as indicated, were incubated in 10 μl of binding buffer containing 5 mM Tris-HCl (pH 7.5), 10 mM *N'*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 50 mM NaCl, 15 mM EDTA, 5 mM DTT, and 10% glycerol at 37°C for 15 min. To each reaction mixture, poly(dI-dC) (1 μg in 1 μl of H_2O) was added, and incubation was continued at room temperature for 5 min before the addition of ^{32}P-labeled oligonucleotide probe (70 pmol). After incubation at room temperature for 10 min, the protein-DNA complexes formed were separated from free probes by electrophoresis on a 6% native polyacrylamide gel in 0.25 × TBE (22 mM Tris base, 22 mM borate, 0.625 mM EDTA) at 250 V at 4°C for 3 h. Confirmation of the presence of Fos and Jun was performed by incubating anti-Fos and anti-Jun antisera (36) with in vitro-translated or recombinant proteins for 10 min at room temperature prior to the addition of oligonucleotide probes. The oligonucleotide probes used are as follows (the core recognition sequence of each DNA probe is underlined):

NF-IL6(IL-6), 5'-AGATTGTGCAATCT-3'
3'-TCTAACACGTTAGA-5'

NF-IL6(E2), 5'-TTAAATTTGAGAAAGGGCGCGAAACTAGTCCTT-3'
3'-AATTTAAACTCTTTCCCGCGCTTTGATCAGGAA-5'

ATF/NF-IL6*, 5'-GAGATGACGTAGTTTTCGCGCTT-3'
3'-CTCTACTGCATCAAAAGCGCGAA-5'

Cells and transfections. NT2/D1 cells, which are from a clonal human embryonal carcinoma cell line, were cultured in Dulbecco's modified essential medium (13). Jurkat, a human T-lymphoblastoid cell line, and HepG2 cells, which are from a human hepatoblastoma cell line, were cultured as described previously (43). M1 cells, which are from a mouse myeloid leukemia cell line (kindly provided by G. Yancopoulos), were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (HyClone), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and nonessential amino acids (GIBCO). In some M1 cell cultures, recombinant murine IL-6 (100 U/ml; R&D Systems) was added. Transfection of NT2/D1 cells and analysis of the chloramphenicol acetyltransferase (CAT) activity expressed by the reporter plasmid, 4xNF-IL6pA10-CAT (4xNF-IL6-CAT), in the transfected cell were performed as described previously (13, 43). Briefly, the CAT activity was determined by the diffusion of [^{3}H]acetyl coenzyme A into organic-miscible scintillation fluid. The slope of the organic-miscible radioactivity as a function of time was calculated and used to represent the CAT activity, with the slope of Rous sarcoma virus CAT as a positive control and that of JYM CAT (43) as a negative control. The transfection efficiency was normalized by the β-galactosidase activity expressed in the cotransfected cells.

RESULTS

NF-IL6 and Jun are coordinately induced by IL-6. Upon treatment with IL-6, M1 myeloid leukemia cells undergo macrophage differentiation and growth arrest within 72 h (25). Preceding phenotypic differentiation, NF-IL6 mRNA increases (32), as do mRNAs encoding members of the Jun family (25). To investigate the simultaneous temporal regulation of NF-IL6 and AP-1 by IL-6, we analyzed RNA isolated from M1 cells following IL-6 induction by Northern (RNA) blotting. The levels of mRNAs encoding NF-IL6 and Jun increased concomitantly, to 9-fold (Jun) and 55-fold (NF-IL6) above those of uninduced cells by 48 h (Fig. 1). Fos mRNA was not detected or induced by IL-6 (data not shown). Increases of NF-IL6 mRNAs appear to be biphasic. The initial increase occurs within 1 h after IL-6 treatment, which is followed by a second increase occurring between 6 and 18 h (Fig. 1). Although the significance of this reproducible pattern of regulation of NF-IL6 mRNA remains to be elucidated, NF-IL6 and Jun are activated immediately upon IL-6 induction. Unlike transient activation of immediately-early genes by growth factors, the elevated levels of NF-IL6 and Jun mRNA are maintained in the presence of IL-6.

NF-IL6 associates with Jun and with Fos. The coordinate

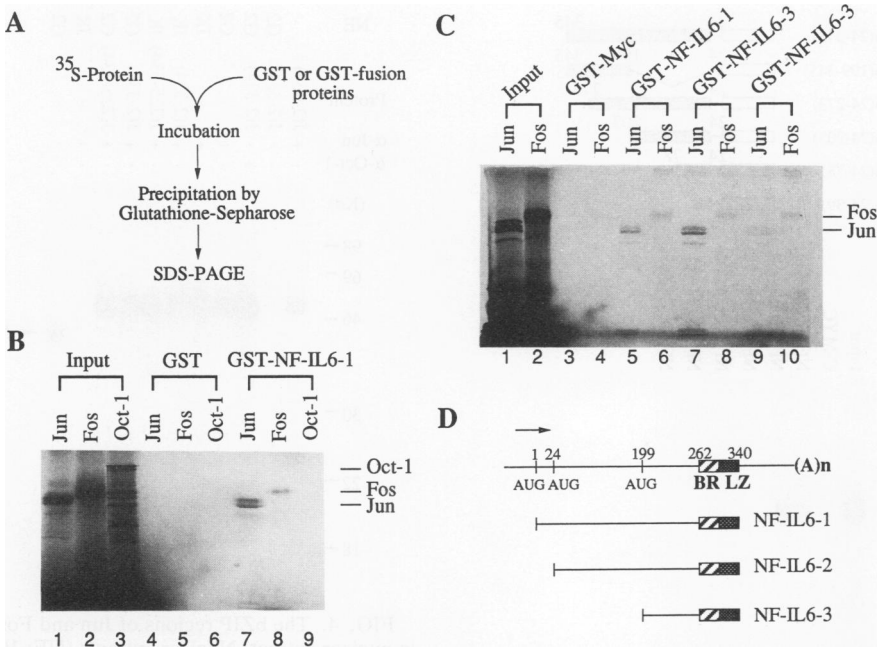

FIG. 2. NF-IL6 associates with Jun and with Fos in vitro. (A) Schematic representation of the protocol for the analysis of association of GST fusion proteins of NF-IL6 and in vitro-translated [^{35}S]methionine-labeled Jun and Fos. (B) Analysis of association of Jun, Fos, or Oct-1 with GST–NF-IL6-1 fusion protein (lanes 7 to 9) or with GST (lanes 4 to 6). The input Jun, Fos, and Oct-1 were analyzed directly by SDS-PAGE (lanes 1 to 3). (C) Analysis of association of Jun or Fos with GST-Myc (lanes 3 and 4), GST–NF-IL6-1 (lanes 5 and 6), or GST–NF-IL6-3 (lanes 7 and 8) in buffer A or with GST–NF-IL6-3 in radioimmunoprecipitation assay buffer (lanes 9 and 10). The input Jun and Fos were analysis directly by SDS-PAGE (lanes 1 and 2). (D) Schematic representation of the NF-IL6 mRNA and proteins. The three translation initiator AUGs, the corresponding amino acid residues, and the three NF-IL6 proteins initiated from the first (NF-IL6-1), second (NF-IL6-2), and third (NF-IL6-3) in-frame AUGs are as indicated. BR, basic region; LZ, leucine zipper region.

regulation of NF-IL6 and Jun suggests that they may interact when activated by IL-6. To test this possibility, we investigated the interactions between NF-IL6 and Fos and Jun in vitro, using (i) bacterially expressed GST fusion proteins of NF-IL6 and Myc and (ii) Jun, Fos, and Oct-1 proteins that were translated in vitro in the presence of [^{35}S]methionine (Fig. 2A). Analysis of protein complexes precipitated by gluthathione-Sepharose show that NF-IL6 associates with both Jun and Fos but not with the homeobox-containing Oct-1 (Fig. 2B). The association is specific to NF-IL6 because none of the three proteins precipitated with GST or with a GST-Myc fusion protein which contains a basic helix-loop-helix zipper structure (Fig. 2B and C).

Like most genes of the C/EBP gene family, the NF-IL6 gene does not contain introns, but it can encode three proteins translated in the same reading frame from different AUGs on the same mRNA (13). The abundance and the ratio of the three proteins, called NF-IL6-1, NF-IL6-2, and NF-IL6-3 (Fig. 2D), depend on the cell type and can be regulated by IL-6 (13). Studies of LAP (8) suggest that NF-IL6-3, which lacks the putative transactivating domain present in NF-IL6-1 and NF-IL6-2, may dimerize with the other isoforms and function as a repressor. It is therefore of interest to determine whether Fos and Jun associate with the three NF-IL6 proteins selectively. Fos and Jun associated with NF-IL6-3 as stably as or more strongly than with NF-IL6-1 when the two NF-IL6 proteins were equimolar (Fig. 2C). The interaction took place even under stringent conditions in the presence of 1% deoxycholate and 1% Triton X-100 (Fig. 2C, lanes 9 and 10) and was dependent on the concentrations of Fos and Jun present in the reaction (data not shown). Taken together, these results show that the carboxyl region shared by the three naturally occurring NF-IL6 proteins mediates cross-family association with Fos and with Jun.

The bZIP regions of NF-IL6 and AP-1 are necessary and sufficient for their association. The domain(s) of NF-IL6 that mediates association with Fos and with Jun was analyzed further by serial deletions of the carboxyl terminus of NF-IL6-2, using a GST fusion construct encoding residues 24 to 345 of NF-IL6 [GST–NF-IL6(24-345); Fig. 3A]. Deletion of the bZIP region in GST–NF-IL6(24-273) completely abolished its ability to associate with Jun (Fig. 3B, lane 5). Progressive deletions of the carboxyl-terminal portion of NF-IL6-2 confirm that the bZIP region is required for interaction with Jun (Fig. 3B, lanes 6 to 8).

The interactions of NF-IL6 and AP-1 were investigated independently by immunoprecipitation with anti-Jun or anti-Fos antibodies followed by immunoblotting with anti-P4, which specifically recognizes the amino terminus of NF-IL6-1 and NF-IL6-2 and does not cross-react with other C/EBPs (Fig. 4). HepG2 cells express a high level of NF-IL6 proteins that are detected by immunoblotting, whereas Jurkat cells do not (Fig. 4, lanes 10 and 11). NF-IL6 was not detected by immunoblotting following immunoprecipitation of HepG2 nuclear extract with anti-Jun, suggesting either that NF-IL6 does not associate with Jun in vivo or that the abundance of NF-IL6–Jun complexes formed in vivo does not permit detection by this method (lane 4). Preincubation of the HepG2 nuclear extract with either a nearly full length Jun (lane 8) or a truncated version of Jun containing the bZIP region (lane 9) resulted in the formation of Jun–NF-IL6 complexes that could be precipitated by anti-Jun but not by anti-Oct-1 antibodies (lane 3). This interaction is attributed to NF-IL6 in the nuclear extracts, since preincubation of Jun

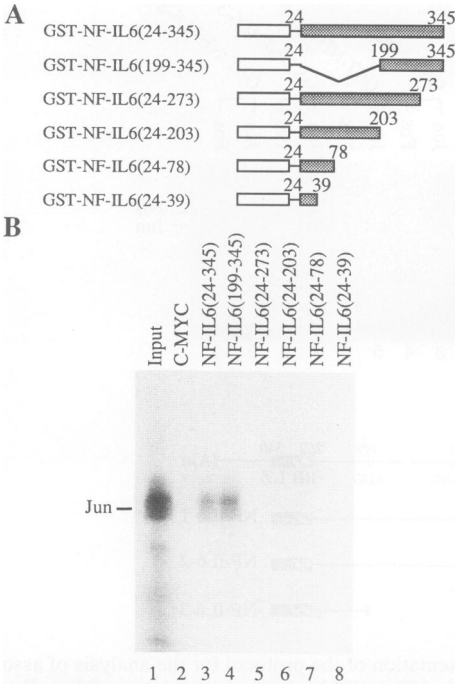

FIG. 3. The bZIP region of NF-IL6 mediates association with Jun. (A) Schematic representation of the GST–NF-IL6-2 fusion protein [(GST–NF-IL6(24-345)] and the deletion mutants. The open box represents GST, and the shaded box represents NF-IL6, indicated by the amino acid residues of its coding region. (B) Analysis of the association of [^{35}S]methionine-labeled Jun with GST–NF-IL6 proteins diagrammed in Fig. 2A.

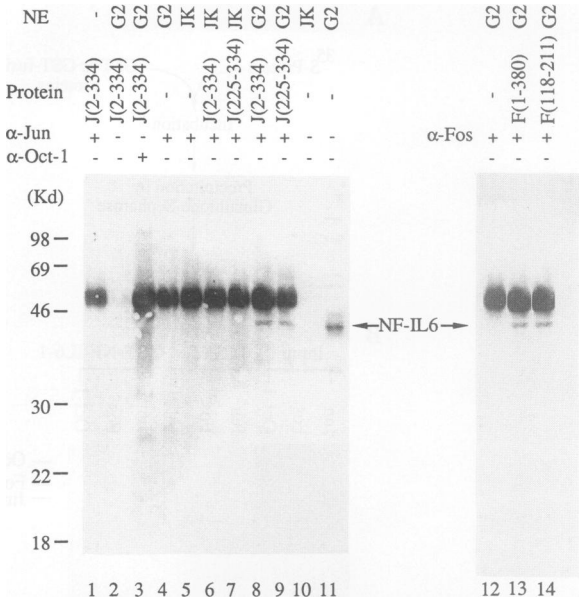

FIG. 4. The bZIP regions of Jun and Fos associate with NF-IL6 in nuclear extract. Nuclear extracts (NE; 100 μg) from HepG2 (G2) or Jurkat (JK) cells were incubated with (+) or without (−) purified recombinant Jun or Fos. J(2-334) is a nearly full length Jun, and J(225-334) contains the bZIP region of Jun. F(1-380) is a full-length Fos, and F(118-211) contains the bZIP region of Fos (6). The protein complexes were recovered by the addition of an anti-Jun (α-Jun), anti-Fos (α-Fos), or anti-Oct-1 (α-Oct-1) antibody as a control, as indicated by +, and analyzed by immunoblotting with an anti-NF-IL6 antiserum (anti-P4) as described in Materials and Methods. Lanes 10 and 11 represent direct immunoblotting of 20 μg of nuclear extracts without immunoprecipitation. The arrow indicates the migration of NF-IL6-1. The signal above the arrow is due to the interaction of the alkaline phosphatase-conjugated second antibody with the first antibody used in immunoprecipitation.

proteins with Jurkat nuclear extracts did not give rise to any Jun–NF-IL6 complexes (lanes 5 to 7). Similarly, the bZIP region of Fos is sufficient for association with NF-IL6 in the HepG2 nuclear extracts (lanes 12 to 14). Thus, by two criteria, the bZIP regions of NF-IL6 and AP-1 family proteins mediate their interactions.

NF-IL6 binds to AP-1 sites, but Fos and Jun do not bind to NF-IL6 sites. The bZIP regions mediate the binding of NF-IL6 to consensus DNA sequences of 5'-T(T/G)NNG NAA(T/G)-3' (1) and the binding of AP-1 proteins to 5'-TGACTCA-3' (12). Fos and Jun also bind to ATF-binding sites with reduced affinity (12). The interactions of NF-IL6 with NF-IL6 and AP-1 recognition sequences were analyzed by mobility shift assays. Recombinant NF-IL-3 bound to NF-IL6 sites present at the adenovirus E2 promoter [NF-IL6(E2) site; 5'-TTGAGAAAG-3'] and the IL-6 promoter [NF-IL6(IL-6) site; 5'-TTGTGCAAT-3'] with comparable efficiency (Fig. 5, lanes 1 to 5 and 11 to 14). It also bound, albeit less efficiently, to an ATF/AP-1-binding site present at the E2 promoter (5'-ATGACGTAGT-3'; called the ATF/NF-IL6* site) (lanes 6 to 10). These results showed that NF-IL6 can bind to an ATF/AP-1-binding site. Binding of truncated recombinant Jun and Fos proteins that contain the bZIP regions to the ATF/NF-IL6* site was predicted and confirmed by the inhibition of DNA-protein complex formation by antisera directed against Fos and Jun (Fig. 6A, lanes 1 to 7). However, Fos and Jun did not bind to either the NF-IL6(E2) site (lanes 8 to 11) or the NF-IL6(IL-6) site (14). Identical results were obtained with full-length Fos and Jun proteins translated in vitro (Fig. 6B). The inability of Fos and Jun to bind to NF-IL6 recognition sequences is therefore an intrinsic property of this protein-DNA interaction.

Association of NF-IL6 with AP-1 alters the DNA binding specificity of NF-IL6. Cross-family dimerization of Fos and Jun with ATF family proteins generates a novel DNA binding specificity that is distinct from that of either parental molecule (12). This suggests that the DNA binding specificity of NF-IL6 may be altered upon association with Jun and with Fos. Binding of NF-IL6-3 to either NF-IL6 site, to which AP-1 cannot bind, was inhibited in the presence of recombinant truncated Fos and Jun in a concentration-dependent manner (Fig. 7, lanes 1 to 6 and 15 to 20). Consistent with dimerization with NF-IL6 via the leucine zipper region, Fos and Jun mutants containing deletions in the basic region were as effective as the wild-type proteins in reducing the NF-IL6 DNA-binding activity (lanes 7 to 11 and 21 to 25). Association of NF-IL6 with Fos or Jun via the leucine zipper region therefore leads altered DNA binding specificity of NF-IL6.

Fos and Jun repress transcription activation by NF-IL6. NF-IL6-1 and NF-IL6-2 likely function as transcription activators and NF-IL6-3 as a repressor analogous to LAP (Fig. 2D). Reduction of binding of NF-IL6 to an NF-IL6 site may lead to reduction or enhancement of transcription mediated by this site, depending on which of the functionally distinct NF-IL6 isoforms is sequestered by Fos and by Jun. The functional properties of NF-IL6 proteins and the influence of Fos and Jun were investigated by transient transfections in human embryonal carcinoma NT2/D1 cells. NF-

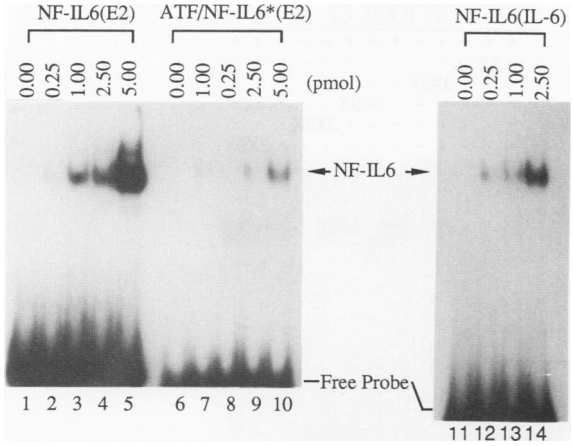

FIG. 5. NF-IL6 binds to an ATF/NF-IL6* site with reduced affinity, as determined by electrophoretic mobility shift assay of binding of NF-IL6-3, in increasing concentrations as indicated, to an oligonucleotide probe (70 pmol) which contains the NF-IL6-binding site at the E2 promoter [NFIL-6(E2)] or the IL-6 promoter [NF-IL6(IL-6)] or the ATF/NF-IL6* site at the E2 promoter [ATF/NF-IL6*(E2)]. The probes have comparable specific activity (1.3×10^5 cpm/nmol).

IL6-3, Fos, and Jun are not detected and NF-IL6-2 is expressed at an extremely low level in these cells (13, 14). We found that NF-IL6-2 activates transcription of a reporter CAT gene linked to four tandemly repeated copies of NF-IL6(IL-6) (4xNF-IL6-CAT) (Fig. 8A). Analogous to the repressor form of LAP, NF-IL6-3 alone did not activate 4xNF-IL6-CAT and repressed transcription activation by NF-IL6-2 (Fig. 8A). Consistent with their inability to bind to this site (Fig. 7B), Fos and Jun did not activate NF-IL6(IL-6) site-directed transcription (Fig. 8A). Fos and Jun, however, repressed transcription activation by NF-IL6-2 in a concentration-dependent manner (Fig. 8). These results show that NF-IL6 functions as an activator or repressor, depending on the ratio of the isoforms in human cells. Correlated with diminution of the NF-IL6 DNA-binding activity by association with Fos and with Jun, NF-IL6 site-dependent transcription activation by the activator forms of NF-IL6 is antagonized by Fos and by Jun.

DISCUSSION

A member of the C/EBP family associates with Fos and Jun. This study shows that NF-IL6 directly associates with Fos and with Jun in vitro in the absence of DNA. Mutational analysis of NF-IL6 demonstrates that the bZIP region of NF-IL6 is essential and that regions outside the bZIP region are not necessary for this interaction. A truncated Jun or Fos, containing only the bZIP region, associates with NF-IL6 in nuclear extracts from human cells. Since amino acid substitution in the leucine zipper regions of Fos and Jun abolishes their association with NF-IL6 (data not shown), NF-IL6 and AP-1 family proteins likely associate via the leucine zipper region by coiled-coil interactions.

The bZIP region is highly conserved among members of the C/EBP family and the AP-1 family (5), raising the question of the specificity for cross-family association. Formation of homo- and heterodimers of Fos and Jun (36) is largely dictated by the thermostability of the dimers (34). Fos-Jun dimers are most stable and Fos-Fos dimers are extremely unstable at physiological pH as a result of the presence of acidic residues (34). NF-IL6 associates with Jun more stably than with Fos under certain conditions (Fig. 2). Whether this is due to the primary structure of the bZIP regions of the association partners or modifications of Fos and Jun in the reticulocyte lysate used to synthesize the

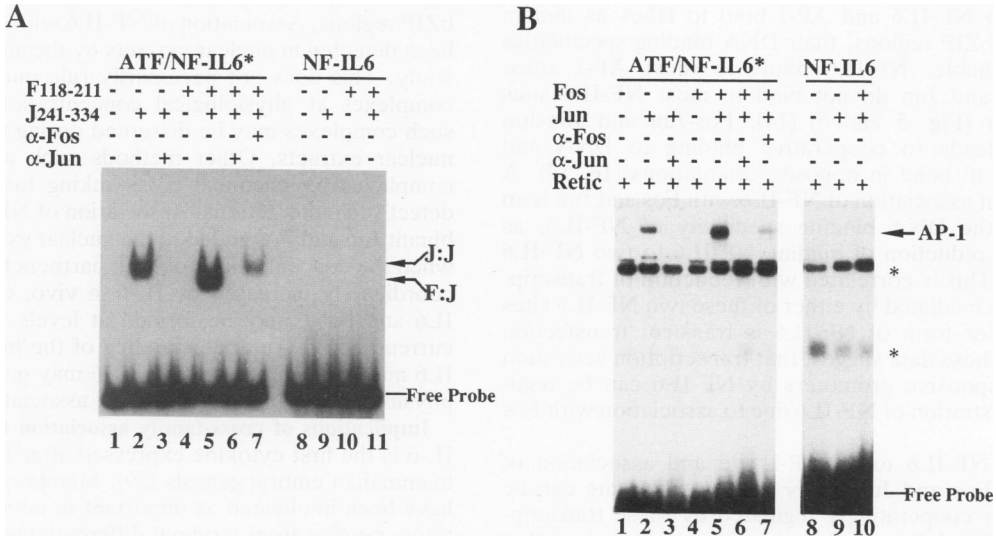

FIG. 6. Jun and Fos bind to the ATF/NF-IL6* site but not to the NF-IL6 site. (A) Electrophoretic mobility shift assay of binding of purified recombinant Fos ($F_{118-211}$) and Jun ($J_{241-334}$) proteins (2.5 pmol) to the ATF/NF-IL6* site and the site at the E2 promoter. The truncated Fos and Jun proteins contain amino acids residues indicated, which include the bZIP regions. The oligonucleotide probes and the antisera directed against Jun (α-Jun) and Fos (α-Fos) are as described in Materials and Methods. The positions of migration of DNA-protein complexes containing Jun-Jun homodimers and Fos-Jun heterodimers are marked J:J and F:J, respectively. (B) Electrophoretic mobility shift assay of unlabeled full-length Fos and Jun proteins translated in vitro (0.3 fmol) to the DNA probes used for panel A. The positions of migration of DNA-protein complexes containing Jun-Jun homodimers and Fos-Jun heterodimers are indistinguishable and marked AP-1. The nonspecific complexes formed as a result of proteins present in the reticulocyte lysate (Retic) used for translation are marked with asterisks.

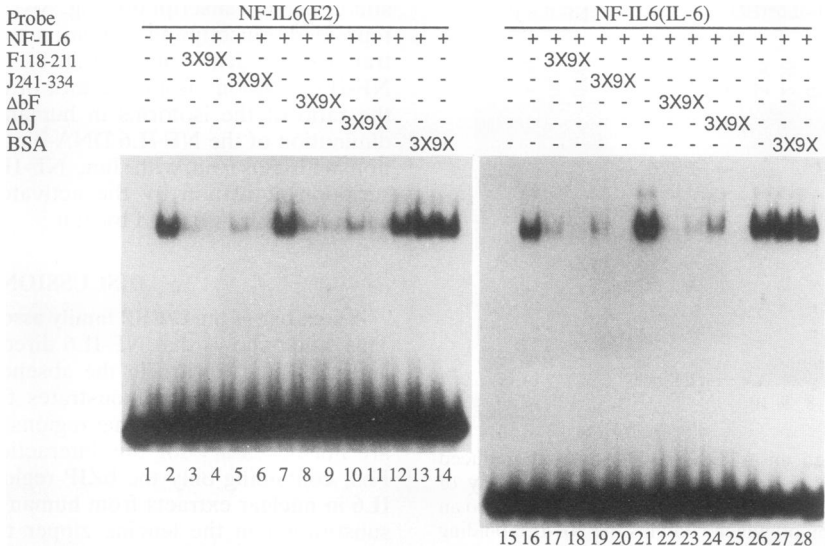

FIG. 7. The NF-IL6 DNA-binding activity is reduced by Jun and by Fos. Binding of purified recombinant NF-IL6-3 (2.5 pmol) to the NF-IL6(E2) and NF-IL6(IL-6) oligonucleotide probes (70 pmol) was performed in the presence (+) or absence (−) of purified recombinant Fos or Jun as indicated. $F_{118\text{-}211}$ and $J_{241\text{-}334}$ are as described in the legend to Fig. 6A. ΔbF is a derivative of F118-211 which lacks residues 139 to 144 in the basic region. ΔbJ is a derivative of $J_{255\text{-}344}$ which lacks residues 260 to 266 in the basic region. Fos and Jun proteins were added in three times (3×) or nine times (9×) the molar concentration of NF-IL6, as was the control, bovine serum albumin (BSA).

proteins remains to be elucidated. In addition, NF-IL6-3 appears to associate with Jun more stably than do the two larger forms of NF-IL6 (Fig. 2 and 3). This result implies that the amino terminus of NF-IL6, which is lacking in NF-IL6-3 and less conserved between C/EBP members, may influence the association of NF-IL6 with Jun. Elucidation of the stoichiometry of association and the sequences that influence this interaction may help to explain the specificity of cross-family association.

The functional consequences of NF-IL6 and AP-1 association. Although NF-IL6 and AP-1 bind to DNA as dimers through their bZIP regions, their DNA binding specificities are distinguishable. NF-IL6 binds to most AP-1 sites, whereas Fos and Jun do not bind to most NF-IL6 sites studied so far (Fig. 5 and 6) (14). Fos-Jun and Jun-Jun dimerization leads to cooperative binding to DNA and induces DNA to bend in opposite orientations (16, 17). A consequence of association of NF-IL6 with Fos and Jun is an alteration of the DNA binding specificity of NF-IL6, as shown by the reduction of binding NF-IL6 to two NF-IL6 sites (Fig. 7). This is correlated with reduction of transcription activation mediated by either of these two NF-IL6 sites by the activator form of NF-IL6 is transient transfection (Fig. 8) (14). These data suggest that transcription activation of NF-IL6-responsive promoters by NF-IL6 can be regulated by sequestration of NF-IL6 due to association with Fos and Jun.

Binding of NF-IL6 to an AP-1 site and association of NF-IL6 with Fos and Jun imply that an AP-1 site can be redundantly or cooperatively regulated by these transcription factors. Regulation of this type is reminiscent of that observed at composite DNA elements by AP-1 and glucocorticoid receptor (8, 18). Since NF-IL6 comprises activator and repressor forms, it may regulate AP-1 site-mediated transcription depending on the ratio of the functionally distinct forms. Preliminary results suggest that this is the case. In transient transfections, NF-IL6-3, but not NF-IL6-2, antagonizes transcription activation by Fos and Jun via the AP-1-binding sites present at the promoters of collagenase, the adenovirus E2 (14), and the tumor necrosis factor α-inducible *TSG-6* genes (19). NF-IL6 therefore can contribute to the regulation of AP-1-responsive promoters.

Recently, Vallejo et al. showed that NF-IL6 can dimerize with C/ATF, a member of the ATF family, and direct its binding to a subclass of asymmetric cyclic AMP response elements in vitro (47). Together, our results suggest that the functional diversity of C/EBP, AP-1, and ATF can be expanded greatly by cross-family association through their bZIP regions. Association of NF-IL6 with Jun or Fos has not been detected in nuclear extracts by the analysis used in this study. This does not necessarily rule out the formation of complexes at physiological concentrations in vivo, since such complexes may be disrupted during the preparation of nuclear extracts. Other methods such as stabilization of complexes by chemical cross-linking may be required to detect such interactions. Association of NF-IL6 with recombinant Jun and Fos added to the nuclear extracts suggest that when the concentrations of both partners for association are coordinately increased by IL-6 in vivo, complexes of NF-IL6 and AP-1 may be formed at levels detectable by the current assay. An understanding of the interactions of NF-IL6 and AP-1 in response to IL-6 may give insight into the physiological significance of their association in vitro.

Implications of cross-family association for IL-6 signaling. IL-6 is the first cytokine expressed after fertilization during mammalian embryogenesis (39). Members of C/EBP family have been implicated as important in many biological functions, ranging from terminal differentiation of adipocytes in rodents and B cells in humans (31, 37, 46) to cell migration during oogenesis in *Drosophila melanogaster* (28). IL-6 also induces a cellular activity that functionally substitutes for the adenovirus E1A protein in transcriptional activation, and NF-IL6 reconstitutes this activity (42, 43), suggesting a functional overlap between viral and IL-6 pathways. Although it is believed that NF-IL6 has a role in mediating IL-6

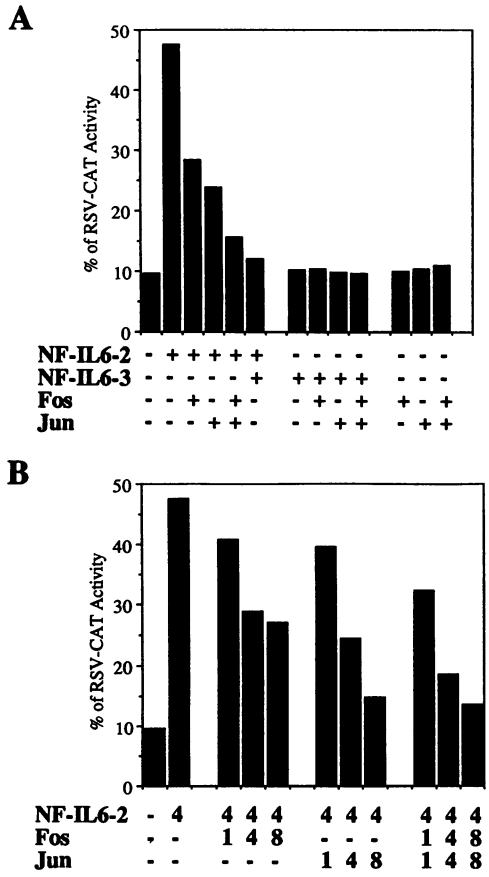

FIG. 8. Fos and Jun repress transcription activation by the activator form of NF-IL6. (A) The reporter plasmid, 4xNF-IL6-CAT (43), was cotransfected with pCMVNF-IL6-2 or pCMVNF-IL6-3, with or without pCMVfos and pCMVjun, into NT2/D1 cells as described previously (13). In each transfection, 10^6 cells and 20 μg of total DNA, including 4 μg of 4xNF-IL6-CAT, 4 μg of each of the expression plasmids as indicated (+), and carrier pBluescript DNA. (B) The reporter plasmid 4xNF-IL6-CAT (4 μg) was cotransfected with pCMVNF-IL6-2 (4 μg) and pCMVfos or pCMVjun, or both, in amounts as indicated. In each transfection, a total of 30 μg of DNA and 10^6 cells were used. The cells were harvested at 48 h after transfection and assayed for CAT activity as described in Materials and Methods. The results are representative of three experiments. RSV, Rous sarcoma virus.

signals, the mechanism underlying the promoter and cell type specificity of IL-6 signaling is not understood.

The interactions of NF-IL6 and AP-1 family proteins may contribute to differential regulation of promoters following IL-6 stimulation. NF-IL6 is regulated by IL-6 at multiple levels: the mRNA synthesis (Fig. 1) (25, 32), the abundance and the ratio of the isoforms of NF-IL6 (13), and the translocation into the nucleus (26). The regulation of NF-IL6-responsive promoters during IL-6 signaling undoubtedly will be modulated by the concentrations and the ratios of NF-IL6 isoforms in the nucleus, and possibly by AP-1 family proteins through protein-protein interactions. Likewise, AP-1-dependent transcription may be subject to regulation by NF-IL6 as a result of the association of NF-IL6 with AP-1 family proteins and the resultant changes in the DNA binding specificity. These interactions, and hence the functional consequences, are likely to evolve as a function of time of IL-6 induction when the ratios of the activator form to the repressor form of NF-IL6 are altered and Fos and Jun are regulated, thereby providing a basis for promoter specificity in IL-6 signaling. As both NF-IL6 and AP-1 are regulated by many extracellular stimuli, the interactions between NF-IL6 and AP-1 may be important in many signal transduction pathways.

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REFERENCES

1. **Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto.** 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO J. **9**:1897–1906.
2. **Bengal, E., L. Ransone, R. Schaarfmann, V. J. Dwarki, S. J. Tapscott, H. Weintraub, and I. M. Verma.** 1992. Functional antagonism between c-Jun and MyoD proteins: a direct physical association. Cell **68**:507–519.
3. **Cao, Z., R. M. Umek, and S. L. McKnight.** 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev. **5**:1538–1551.
4. **Chang, C. J., T. T. Chen, H.-Y. Lei, D. S. Chen, and S.-C. Lee.** 1991. Molecular cloning of a transcription factor, AGP/EBP, that belongs to members of the C/EBP family. Mol. Cell. Biol. **10**:6642–6653.
5. **Chen-Kiang, S., W. Hsu, Y. Natkunam, and X. Shang.** 1993. Nuclear signaling by interleukin-6. Curr. Opin. Immunol. **5**:124–128.
6. **Cohen, D. R., and T. Curran.** 1990. Analysis of dimerization and DNA binding function in Fos and Jun by domain-swapping: involvement of residues outside the leucine zipper/basic region. Oncogene **5**:929–939.
7. **Descombes, P., M. Chojkier, S. Lichtsteiner, E. Falvey, and U. Schibler.** 1990. LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. Genes Dev. **4**:1541–1551.
8. **Descombes, P., and U. Schibler.** 1991. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. Cell **67**:569–579.
9. **Diamond, M. I., J. N. Miner, S. K. Yoshinaga, and K. Yamamoto.** 1990. Transcription factor interactions: selectors of positive or negative regulation from a signal DNA element. Science **249**:1266–1272.
10. **Gentz, R., F. J. Rauscher III, C. Abate, and T. Curran.** 1989. Parallel association of Fos and Jun leucine zippers juxtaposes DNA binding domains. Science **243**:1695–1699.
11. **Goodrich, D. W., and W.-H. Lee.** 1992. Abrogation by c-*myc* of G1 phase arrest induced by *RB* protein but not by p53. Nature (London) **360**:177–179.
12. **Hai, T., and T. Curran.** 1991. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. Proc. Natl. Acad. Sci. USA **88**:3720–3724.
13. **Hsu, W., and S. Chen-Kiang.** 1993. Convergent regulation of NF-IL6 and Oct-1 synthesis by interleukin-6 and retinoic acid signaling in embryonal carcinoma cells. Mol. Cell. Biol. **13**: 2515–2523.
14. **Hsu, W., and S. Chen-Kiang.** Unpublished data.
15. **Jain, J., P. G. McCaffrey, V. E. Valge-Archer, and A. Rao.** 1992. Nuclear factor of activated T cells contains Fos and Jun. Nature (London) **356**:801–804.
16. **Kerppola, T. K., and T. Curran.** 1991. DNA bending by Fos and Jun: the flexible hinge model. Science **254**:1210–1214.
17. **Kerppola, T. K., and T. Curran.** 1991. Fos-Jun heterodimers and Jun homodimers bend DNA in opposite orientations: impli-

cations for transcription factor cooperativity. Cell **66**:317–326.
18. **Kerppola, T. K., D. Luk, and T. Curran.** 1993. Fos is a preferential target of glucocorticoid receptor inhibition of AP-1 activity in vitro. Mol. Cell. Biol. **13**:3782–3791.
19. **Klampfer, L., J. Vilcek, and S. Chen-Kiang.** Unpublished data.
20. **Kinoshita, S., S. Akira, and T. Kishimoto.** 1992. A member of the C/EBP family, NF-IL6β, forms a heterodimer and transcriptionally synergizes with NF-IL6. Proc. Natl. Acad. Sci. USA **89**:1473–1476.
21. **Kishimoto, T., S. Akira, and T. Taga.** 1992. Interleukin-6 and its receptor: a paradigm for cytokines. Science **258**:593–597.
22. **Landschulz, W. H., P. F. Johnson, and S. L. McKnight.** 1989. The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. Science **243**:1681–1688.
23. **LeClair, K. P., M. A. Blanar, and P. A. Sharp.** 1992. The p50 subunit of NF-kB associates with the NF-IL6 transcription factor. Proc. Natl. Acad. Sci. USA **89**:8145–8149.
24. **Li, L., J.-C. Chambard, M. Karin, and E. N. Olson.** 1992. Fos and Jun repress transcriptional activation by myogenin and MyoD: the amino terminus of Jun can mediate repression. Genes Dev. **6**:676–689.
25. **Lord, K. A., A. Abdollahi, B. Hoffman-Liebermann, and D. A. Lieberman.** 1993. Proto-oncogene of the Fos/Jun family of transcription factors are positive regulators of myeloid differentiation. Mol. Cell. Biol. **13**:841–851.
26. **Metz, R., and E. Ziff.** 1991. cAMP stimulates the C/EBP-related transcription factor rNF-IL6 to translocate to the nucleus and induce c-fos transcription. Genes Dev. **5**:1754–1766.
27. **Minor, J. F., and K. Yamamoto.** 1992. The basic region of AP-1 specifies glucocorticoid receptor activity at a composite response element. Genes Dev. **6**:2491–2501.
28. **Montell, D. J., P. Rorth, and A. C. Spradling.** 1993. *slow border cells*, a locus required for a developmentally regulated cell migration during oogenesis, encodes drosophila C/EBP. Cell **71**:51–62.
29. **Nakajima, K., and R. Wall.** 1991. Interleukin-6 signals activating *junB* and *TIS11* gene transcription in a B-cell hybridoma. Mol. Cell. Biol. **11**:1409–1418.
30. **Nakajima, T., S. Kinoshita, T. Sasagawa, K. Sasaki, M. Naruto, T. Kishimoto, and S. Akira.** 1993. Phosphorylation at threonine 235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcriptional activation of NF-IL6. Proc. Natl. Acad. Sci. USA **90**:2207–2211.
31. **Natkunam, Y., X. Zhang, and S. Chen-Kiang.** Unpublished data.
32. **Natsuka, S., S. Akira, Y. Nishio, S. Hashimoto, T. Sugita, H. Isshiki, and T. Kishimoto.** 1992. Macrophage differentiation-specific expression of NF-IL6, a transcription factor for interleukin-6. Blood **79**:460–466.
33. **O'Shea, E. K., R. Rutkowski, and P. S. Kim.** 1989. Evidence that the leucine zipper is coiled coil. Science **243**:538–542.
34. **O'Shea, E. K., R. Rutkowski, and P. S. Kim.** 1992. Mechanism of specificity in the Fos-Jun oncoprotein heterodimer. Cell **68**:699–708.
35. **Poli, P., F. P. Mancini and R. Cortese.** 1990. IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction defines a new family of leucine zipper protein related to C/EBP. Cell **63**:643–653.
36. **Rauscher, F. J., III, P. J. Voulalas, B. R. Franza, Jr., and T. Curran.** 1989. Fos and Jun bind cooperatively to the AP-1 site: reconstitution in vitro. Genes Dev. **2**:1687–1699.
37. **Raynal, M. C., Z. Lui, T. Hirano, L. Mayer, T. Kishimoto, and S. Chen-Kiang.** 1989. Interleukin-6 induces secretion of IgG1 by coordinated transcriptional activation and differential mRNA accumulation. Proc. Natl. Acad. Sci. USA **86**:8024–8028.
38. **Ron, D., and J. F. Habener.** 1992. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. Genes Dev. **6**:439–453.
39. **Rothstein, J. L., D. Johnson, J. A. DeLola, J. Skowronski, D. Solter, and B. Knowles.** 1992. Gene expression during preimplanation mouse development. Genes Dev. **6**:1190–1201.
40. **Satoh, T., S. Nakamura, T. Taga, T. Mastuda, T. Hirano, T. Kishimoto, and Y. Kaziro.** 1988. Induction of neuronal differentiation in PC12 cells by B-cell stimulation factor 2/interleukin-6. Mol. Cell. Biol. **8**:3546–3549.
41. **Sonnenberg, J. L., F. J. Rauscher III, J. Morgan, and T. Curran.** 1989. Regulation of proenkephalin by Fos and Jun. Science **246**:1622–1625.
42. **Spergel, J. M., and S. Chen-Kiang.** 1991. Interleukin-6 enhances a cellular activity that functionally substitutes for E1A protein in transactivation. Proc. Natl. Acad. Sci. USA **88**:6472–6476.
43. **Spergel, J. M., W. Hsu, S. Akira, B. Thimmappaya, T. Kishimoto, and S. Chen-Kiang.** 1992. NF-IL6, a member of the C/EBP family, regulates E1A-responsive promoters in the absence of E1A. J. Virol. **66**:1021–1030.
44. **Studier, F. W., A. H. Rosenberg, and J. J. Dunn.** 1990. Use of T7 RNA polymerase to direct the expression of cloned genes. Methods Enzymol. **185**:60–89.
45. **Ullman, K. S., J. P. Northrop, A. Admon, and G. R. Carbtree.** 1993. Jun family members are controlled by a calcium-regulated, cyclosporin A-sensitive signaling pathway in activated T lymphocytes. Genes Dev. **7**:188–196.
46. **Umek, R. M., A. D. Friedman, and S. L. McKnight.** 1991. CCAAT-enhancer binding protein: a component of a differentiation switch. Science **251**:288–292.
47. **Vallejo, M., D. Ron, C. P. Miller, and J. F. Habener.** 1993. C/ATF, a member of the activating transcription factor family of DNA-binding proteins, dimerizes with CAAT/enhancer-binding proteins and directs their binding to cAMP response elements. Proc. Natl. Acad. Sci. USA **90**:4679–4683.
48. **Vinson, C. R., P. B. Sigler, and S. L. McKnight.** 1989. Scissors-grip model for DNA recognition by a family of leucine zipper proteins. Science **246**:911–916.
49. **Wegner, M., Z. Cao, and M. Rosenfeld.** 1992. Calcium-regulated phosphorylation within the leucine zipper of C/EBP. Science **256**:370–373.
50. **Williams, S. C., C. A. Cantwell, and P. F. Johnson.** 1991. A family of C/EBP related proteins capable of forming covalently linked leucine zipper dimers in vitro. Genes Dev. **5**:1553–1568.