NF-IL6, a Member of the C/EBP Family, Regulates E1A-Responsive Promoters in the Absence of E1A

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A cDNA encoding NF-IL6, an interleukin-6 (IL-6)-regulated human nuclear factor of the C/EBP family, is demonstrated to complement the transactivation function of E1A. The endogenous NF-IL6 level varies according to cell type and correlates positively with an IL-6-regulated cellular E1A-substituting activity that was described recently (J. M. Spergel and S. Chen-Kiang, *Proc. Natl. Acad. Sci. USA* 88:6472-6476, 1991). When expressed by transfection in cells which contain low levels of NF-IL6 and are incapable of complementing the function of E1A proteins, NF-IL6 also transactivates the E1A-responsive E2ae and E1B promoters, to the same magnitude as E1A. Activation by NF-IL6 is concentration dependent and sequence specific: mutational studies of the E2ae promoter suggest that the promoter-proximal NF-IL6 recognition site functions as a dominant negative regulatory site whereas the promoter-distal NF-IL6 recognition site is positively regulated at low NF-IL6 concentrations and negatively regulated when the NF-IL6 level is high. Consistent with these functions, NF-IL6 alone is sufficient to complement an E1A deletion mutant *d1312* in viral infection, when expressed at appropriate concentrations. These results identify NF-IL6 as a sequence-specific cellular nuclear factor which regulates E1A-responsive genes in the absence of E1A.

The polypeptides encoded by the transforming gene E1A of adenovirus transactivate all early genes of the virus, as well as a subset of cellular genes, and repress simian virus 40, immunoglobulin heavy chain, and insulin enhancer-linked promoters (for a review, see references 3, 17, and 58). Although structural analyses have yielded insights into domains important for the multiple functions of the E1A gene products (48), the molecular mechanism of E1A action has remained elusive. It has been suggested that E1A proteins function in synergy with cyclic AMP (cAMP) (16, 43, 49); through TFIID (34, 68) or ATF-2 (44); or by sequestering repressors in the cell (2). E1A also enhances RNA polymerase III transcription by activating transcription factor IIIC (28). The complexity of delineating the mechanism of E1A action is at least in part due to its ability to induce some promoters and not others, without apparent sequence specificity. Furthermore, it functions in a highly cell type-specific manner as a transactivator or repressor with respect to the same immunoglobulin heavy-chain enhancer in different cells (5, 25). Although many cellular proteins are associated with the E1A polypeptides, most notably the retinoblastoma gene products (67) and cyclins (21), the putative cellular repressor has not yet been identified.

A cellular activity (or activities) which functionally substitutes for E1A in transactivating an E1A-responsive adenovirus E2ae promoter has been shown to be present in mouse embryonal carcinoma cells (31, 39), in oocytes, and during early development in preimplantation embryos (15). Its identity, however, has remained obscure. A functionally related cellular activity was recently found in HepG2 cells, a human hepatoblastoma cell line. In addition to the E2ae promoter, the E1A-responsive E1B promoter was active in HepG2 cells in the absence of E1A, when assayed by either

transfection or viral infection. Furthermore, HepG2 cells supported productive infection of E1A deletion mutant virus. Unlike the activity in mouse embryonal carcinoma cells, which diminishes upon cellular differentiation, the E1A-substituting activity in HepG2 cells increases dramatically upon induction by the cytokine interleukin-6 (IL-6), resulting in full complementation of infection of E1A deletion viruses (64).

The enhancement of the E1A-substituting activity in HepG2 cells by IL-6 suggests that a nuclear factor(s) regulated by or transducing the IL-6 signal may mediate, or in itself be, the cellular activity which functionally substitutes for E1A. IL-6 is a multifunctional cytokine which promotes growth, inhibits growth, and induces differentiation in a highly cell-type-specific manner (for a review, see reference 27). Among its many differentiation functions, IL-6 promotes terminal differentiation of human B cells by coordinate activation of immunoglobulin heavy- and light-chain genes at the level of transcription (57). Although the IL-6 signal transduction pathway is largely unknown, NF-IL6, a human nuclear factor which is regulated by IL-6, has recently been cloned and found to belong to the C/EBP leucine zipper family (1). It recognizes a consensus sequence present in nearly all IL-6-inducible genes, 5'-T(T/G)NNG NAA(T/G)-3', and transactivates promoters linked to this sequence in transfection. Here we show that NF-IL6 alone is sufficient to complement an E1A deletion mutant in viral infection and to regulate E1A-responsive promoters in the absence of E1A in transfection. Furthermore, it functions as a positive or negative regulator in a concentration-dependent manner via NF-IL6 recognition sequences.

MATERIALS AND METHODS

Cells and viruses. HepG2 cells, a human hepatoblastoma cell line (35), were cultured in Eagle's minimal essential

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medium. Jurkat cells, a human T-cell line, were cultured in RPMI 1640. All media were supplemented with 10% heat-inactivated fetal calf serum L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and nonessential amino acids (GIBCO). Infections and propagation of adenovirus type 2 (Ad2) and *d*/312 were performed as described previously (40). HepG2 cells were induced with 500 U of IL-6 per ml (57).

Plasmids and oligonucleotides. Plasmid 4×NF-IL6pA₁₀-CAT contains four copies of the NF-IL6 responsive element [5'-GATC(ACATGGCACAATCT)₄-3'] 5' to an enhancerless simian virus 40 promoter linked to the reporter CAT gene (32). The pE2ae-CAT plasmid contains 346 bp of the Ad5 E2ae promoter (from -284 to +62) cloned into pSVO-CAT. Linker-scanning (LS) mutants of E2 are as described previously (50). pAd5E1A contains bp 1 to 1764 of the adenovirus type 5 genome and expresses all three E1A proteins (65). The pE1B-CAT plasmid contains the promoter region of E1B from -365 to +1 (13). The positive control for transfection, pRSV-CAT, contains a 524-bp fragment (*Pvu*II site to *Bst*NI site) of the 3' long terminal repeat of Rous sarcoma virus (RSV) (22). The negative control, pJYM-CAT, contains the simian virus 40 TATA box but no enhancer (45). RSV-βgal contains the β-galactosidase gene driven by the RSV long terminal repeat enhancer-promoter (4). pCMV-NF-IL6(+) contains a cDNA encoding NF-IL6 directed by the cytomegalovirus promoter, and pCMV-NF-IL6(-) is its antisense derivative (1).

Synthetic DNA oligonucleotides were prepared by using an automated DNA synthesizer (Applied Biosystems, Inc.) and purified by electrophoresis in a 8 M urea-12% polyacrylamide gel. DNA was eluted in a buffer consisting of 0.1 M NaCl, 10 mM Tris-Cl (pH 7.4), and 1 mM EDTA; bound to a NENSORB column (New England Nuclear); and subsequently eluted in 50% methanol. The eluted DNA was dried under vacuum, dissolved in H₂O, and quantified by its A₂₆₀. Double-stranded oligonucleotides were 5' end labeled with T4 DNA polynucleotide kinase and [γ-³²P]ATP under standard conditions (61).

DNA probes used are as follows (the core recognition sequence of each DNA probe is underlined).

NF-IL6 (1)	5'-AGATTGTGCAATCT-3' 3'-TCTAACACCTTAGA-5'
Octamer (62)	5'-AGCAACACCACTGGGTAATTCGATTCTAAATAAGTTGAGGAT-3' 3'-TCGTTTGGGTGGACCCATTAAACGTAAGATTTCCTCAACTCCTA-5'
SP-1 (LTR) (8)	5'-GCGAGGCGCTGGCCTGGGCGGGAGTGGC-3' 3'-CTCCGACCGGACCGGCTGACCCCTACCGGCC-5'

Transfection procedures and assays. HepG2 cells at 24 h after plating (10⁶ cells per 100-mm dish) were transfected by the calcium phosphate method (23) with 20 µg of total DNA (including carrier salmon sperm DNA) per dish. The electroporation method (55) was used for transfection of Jurkat cells, by using the Gene-Pulser (Bio-Rad) at 1.5 kV and 25 µF, with 15 µg of total DNA for 3 × 10⁶ cells. The cell extracts were prepared 48 h after transfection. The cells were washed twice with phosphate-buffered saline, pelleted and resuspended in 100 µl of 0.25 M Tris-Cl (pH 7.8), and then subjected to freezing and thawing three times. Cellular debris was pelleted, and the supernatant was aliquoted and stored at -20°C.

The transfection efficiency is normalized by the β-galactosidase activity expressed in the cotransfected cells. Transfected-cell extracts (50 µl) were added to 1 ml of buffer consisting of 60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgCl₂, and 50 mM β-mecaptoethanol, to which 200 µl of 2.0-mg/ml ONPG (*o*-nitrophenyl-β-D-galactopyran-

oside; Sigma) had been added as the colorimetric substrate. The reaction was incubated at 37°C until a yellow color appeared, in approximately 10 h. The β-galactosidase activity was measured as the A₄₂₀ (26).

Chloramphenicol acetyltransferase (CAT) activity was determined by the diffusion of [³H]acetyl coenzyme A into organic-miscible scintillation fluid. Equivalent amounts of cell extracts based on β-galactosidase activity were combined with chloramphenicol (final concentration, 1.25 mM; Sigma), and [³H]acetyl coenzyme A (final concentration, 1 µM; New England nuclear) in a buffer containing Tris-Cl (pH 7.4) in a final volume of 200 µl. Econofluor scintillation fluid (New England Nuclear) was overlaid onto the reaction mixture in 7-ml glass scintillation vials and incubated at 37°C. The organic-miscible [³H]acetyl coenzyme A-chloramphenicol was counted at 20, 40, 60, 120, and 180 min of incubation. The slope of the organic-miscible radioactivity as a function of time was calculated and used to represent the CAT activity (53, 61), the slope of the RSV CAT activity was used as a positive control, and the slope of the JYM CAT activity was used as a negative control:

$$\% \text{ RSV-CAT} = \frac{(\text{experimental CAT} - \text{JYM CAT})}{(\text{RSVCAT} - \text{JYM CAT})} \times 100$$

Electrophoretic mobility shift assay. Nuclear extracts were prepared at 4°C essentially as described by Mattila et al. (47). The protein concentration was determined by the Bradford assay (6) with a protein assay kit (Bethesda Research Laboratories). The mobility shift assays were based on the methods of Fried and Crothers (18) and Garner and Revzin (20). Nuclear extract (2 to 3 µg) was used for binding in a buffer containing 5 mM Tris-Cl, 10 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 50 mM NaCl, 1 mM dithiothreitol, 10% glycerol, and 1 µg of poly(dI-dC) (Boehringer Mannheim Biochemicals) to 1 ng of the NF-IL6 probe in the presence of 15 mM EDTA. Competition with oligonucleotides, at a 100-fold molar excess, and preincubation with antibodies were performed by incubation of extracts with oligonucleotides or antibodies at room temperature for 10 min. The probe was then added, and the mixture was incubated for 20 min. The bound DNA was separated from the free DNA by electrophoresis on a 6% nondenaturing polyacrylamide gel, unless otherwise indicated, in 0.25× TBE (18 mM Tris base, 18 mM borate) at 250 V at 4°C. The gels were transferred to dampened DEAE 81 (DE81; Whatman), dried, and exposed to X-OMAT film.

Other assays. For the RNase protection assay, poly (A)⁺ RNAs were isolated and selected as previously described (40) and the assay was carried out as described by Marton et al. (46). For the immunofluorescence assay, indirect immunofluorescence microscopy was performed essentially as previously described (40) with a monoclonal antibody directed toward the adenovirus E2 72-kDa protein (72K protein) as the first antibody and fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin as the second antibody.

Antibodies and recombinant proteins. Polyclonal antisera directed against peptide 3 of NF-IL6 (α-NF-IL6 [1]), against recombinant LAP (α-LAP; kindly provided by U. Schibler [14]), and against a 14-amino-acid unique peptide of C/EBP (α-14; kindly provided by S. McKnight [37]) and monoclonal antibodies directed against the adenovirus E4 ORF6/7 polypeptide (α-E4; kindly provided by T. Shenk [46]) and against the adenovirus E2 72K protein (kindly provided by R.

FIG. 1. The NF-IL6 binding activity varies with cell lines and is reduced after adenovirus infection. (A) Binding of proteins in nuclear extracts to a NF-IL6 probe (1). Nuclear extracts were prepared from HepG2, Jurkat (JK), and IL-6-induced HepG2 cells at the indicated days postinduction, as well as from Ad2-infected HepG2 and Jurkat cells (10 PFU per cell) at 24 and 48 h postinfection, respectively. The oligomer probes used for binding and competition are described in Materials and Methods. We used 3 µg of nuclear extracts or 0.01, 0.1, or 1 µg of purified rLAP for each binding reaction as indicated. (B) HepG2 or Jurkat nuclear extract (3 µg) was preincubated with 1 µl of antiserum as indicated, prior to the binding reaction. The conditions for electrophoretic mobility shift assay were as described in Materials and Methods. X represents the relative exposure times of the autoradiographs. (C) Schematic diagram of NF-IL6 and LAP protein structures. Abbreviation: aa, amino acids. D represents the basic region, and L represents the leucine zipper domain.

Carroll) were used as indicated. Purified recombinant LAP protein (14) was kindly provided by U. Schibler.

RESULTS

The NF-IL6-binding activity correlates with the endogenous E1A-substituting activity. The marked enhancement of a cellular activity in HepG2 cells by IL-6 (64) and the lack of this activity in other human cells, including lymphoid cells which require very low levels of E1A for productive infection (41), suggest that a cellular factor regulated by IL-6 may functionally substitute for E1A. This hypothesis predicts that if NF-IL6 represents an E1A-substituting activity, its level may be cell type specific. This was shown to be the case. Binding of proteins in nuclear extracts of HepG2 cells to a synthetic oligonucleotide containing a NF-IL6 recognition sequence, 5'-AGATTGTGCAACT-3', was 15 to 20-fold greater than binding of protein in nuclear extracts of Jurkat cells to the same DNA probe, as assayed by the electrophoretic mobility shift assay (Fig. 1A). Jurkat cells do not express the 80-kDa IL-6 receptor (69) or an E1A-substituting activity, but can support productive adenovirus infection in the presence of E1A (40).

In agreement with regulation of both NF-IL6 (1) and an E1A-substituting activity (64) in HepG2 cells by IL-6, the

formation of DNA-protein complexes was increased two to threefold in extracts prepared after IL-6 induction. The complexes formed were specific, since they were not inhibited by DNA of unrelated sequences, and they comigrated with those formed with recombinant LAP (rLAP), a reference protein, on the same NF-IL6 probe. LAP (14), also known as IL-6 DBP and AGP/EBP (10, 54), is a rodent transcription factor of the C/EBP family that is inducible by IL-6 (54). Highly homologous in its amino acid sequence and almost identical in its DNA-binding and leucine zipper domains to NF-IL6, it is most probably the rat homolog of human NF-IL6 (Fig. 1C).

Consistent with these results, α-NF-IL6, an antiserum which recognizes NF-IL6, inhibited binding. So did α-LAP, an antiserum directed against recombinant LAP; however, α-E4, a control antibody directed against the 19-kDa polypeptide product of the adenovirus E4 gene, and α14, a peptide antiserum specific for the rat C/EBP, did not. In fact, α14 reproducibly enhanced protein-NF-IL6 complex formation (Fig. 1B). Although the significance of this enhancement requires further investigation, C/EBP appeared not to be a component of protein-NF-IL6 complexes at equilibrium. Taken together, these data suggest a positive correlation of the NF-IL6-binding activity with the ability of the cells to activate E1A-responsive promoters in the absence of E1A.

FIG. 2. NF-IL6 and E1A transactivate E1A-responsive E2ae and E1B promoters in transfection. (A) Schematic diagram of the plasmids used in transfection. (B and C) Transfections were performed with 1 μ g of pE2ae-CAT or pE1B-CAT DNA per 10^6 cells and various amounts of pCMV-NFIL6(+) DNA as indicated. In panel B the calcium phosphate precipitation method (23) was used to transfect HepG2 cells, and in panel C the electroporation method was used for Jurkat cells (55). (D) pAd5E1A DNA was cotransfected, at various amounts as indicated, with 1 μ g of pE2ae-CAT or pE1B-CAT per 10^6 cells into Jurkat cells as in panel C. The cells were harvested at 48 h after transfection and assayed for CAT activity as described in Materials and Methods.

NF-IL6 transactivates E1A-responsive promoters in a concentration-dependent manner. There is no sequence homology between E1A and NF-IL6. Although E1A activates genes without apparent sequence specificity, NF-IL6 and LAP bind to specific sequences in the promoters and activate target cellular genes (1, 14). The NF-IL6 recognition sequence is present in the promoters of nearly all E1A-responsive viral and cellular genes. The mechanism of transactivation of E1A-responsive genes by NF-IL6 is therefore unlikely to be the same as that of E1A activation. Since NF-IL6 binding activity was dramatically reduced in both HepG2 and Jurkat cells after virus infection, when the E1A-responsive promoters were active (Fig. 1A), it is also unlikely that NF-IL6 serves as an obligatory intermediate for the transactivation function of E1A.

To investigate whether NF-IL6 activates E1A-responsive genes at the level of transcription initiation, pCMV-NFIL6(+), an expression vector of NF-IL6, was cotransfected with pE1B-CAT or pE2ae-CAT (E1B or E2ae promoters, respectively, linked to the CAT reporter gene) into HepG2 and Jurkat cells (Fig. 2). It is clear that when expressed at lower concentrations [1 to 5 μ g of pCMV-NFIL6(+)] transfected into 10^6 cells, NF-IL6 transactivated E2ae and E1B promoters in both cell lines (Fig. 2B and C). The increases of E2ae and E1B promoter activities by NF-IL6, 15- to 20-fold in Jurkat cells and 2- to 4-fold in HepG2 cells, appeared to be inversely correlated with the basal promoter activities in these cell lines. Furthermore, in Jurkat cells the levels of transactivation of these promoters by NF-IL6 approached those seen with E1A proteins, expressed by cotransfecting pAd5E1A (Fig. 2C and D). However, whereas transactivation by E1A did not diminish significantly at higher E1A protein concentrations, NF-IL6 at concentrations above a

threshold had a markedly reduced ability to transactivate both promoters (Fig. 2C and D).

Thus, although the magnitude of transactivation by NF-IL6 is cell type dependent, NF-IL6 can transactivate E1A-responsive promoters in transfection, contingent upon its concentration and to levels comparable to those for transactivation by E1A proteins.

NF-IL6 functions as a negative or positive regulator in a site-specific and concentration-dependent manner. Nuclear factors of the C/EBP family are known to bind to specific DNA sequences in dimerized form with various affinities (14, 38, 59), and LAP can dimerize with C/EBP (14). Since dimerization is concentration dependent, it raises the possibility that formation of specific NF-IL6-promoter complexes and the promoter activity depends upon endogenous levels of NF-IL6 and the DNA sequences to which NF-IL6 binds. Two NF-IL6 recognition elements are present in the E2ae promoter, at -46 and -69 (designated as NF-IL6 and NF-IL6* sites respectively [Fig. 3A]). The NF-IL6* site overlaps with an ATF recognition element to which members of the AP-1 family can also bind, albeit with reduced relative affinity (51, 56). One of them, *junB*, has been shown to be regulated by IL-6 signaling (52).

The functional significance of NF-IL6 and NF-IL6* sites in the E2ae promoter was explored by cotransfecting linker-scanning (LS) mutants of the E2ae promoter with the NF-IL6 expression vector, pCMV-NFIL6(+), or its antisense derivative, pCMV-NFIL6(-), into Jurkat and HepG2 cells (Fig. 3). In Jurkat cells, in the absence of additional NF-IL6, transcription from the E2ae promoters of LS mutants -40/-50 and -49/-59, in which the NF-IL6 site (-46 to -54) was mutated, was 10- to 20-fold greater than that from the wild-type E2ae promoter (Fig. 3B). The magnitude of de-

FIG. 3. NF-IL6 functions as a negative or positive regulator in a site-specific manner. (A) Schematic diagram of representative LS mutants of the E2ae promoter used in panels B and C. The NF-IL6 and NF-IL6* sites are represented by rectangles, and the two E2F sites are represented by ovals. The E2F sites are binding sites for the E2F factors (24, 36). (B and C) Jurkat cells (panel B) and HepG2 cells (panel C) were cotransfected with pE2ae-CAT (WT) or its LS mutants (50) at 1 μ g of DNA per 10^6 cells and the NF-IL6 expression vector pCMV-NF-IL6(+) or its antisense derivative, pCMV-NF-IL6(-), at 10 μ g of DNA per 10^6 cells as indicated.

pression by alteration of the NF-IL6 site, as displayed by the LS -49/-59 mutant, ranged from 10- to 25-fold in seven transfection experiments and was not influenced by the presence of the cotransfected pCMV-NF-IL6(-) (Fig. 3B and 4A). This result is reminiscent of an earlier observation in HeLa cells, in which LS -49/-59 exhibited a fivefold-greater promoter activity than the wild-type promoter (50). Derepression was not exhibited by LS mutant -63/-72 or -74/-85, in which the NF-IL6* site was mutated (Fig. 3B). These results suggest that the two NF-IL6 recognition sites differ in their functions: the NF-IL6 site functions primarily as a dominant negative regulatory site, while the NF-IL6* site is necessary for transactivation.

It was also apparent that derepression was insignificant in HepG2 cells, which express a high level of endogenous NF-IL6-binding activity (Fig. 1) and form additional E2ae promoter complexes in reference to Jurkat cells in vitro (28a). When expressed at a high level by transfection with pCMV-NF-IL6(+) at 10 μ g or greater per 10^6 cells, NF-IL6 abrogates the phenotype of the LS mutants in Jurkat cells (Fig. 3B and 4A). These results suggest that NF-IL6 at high levels may play a role as a repressor. They also suggest that

the promoter-distal NF-IL6* site, which is intact in LS mutants -49/-59 and -40/-50, may be the major site for mediating the activation or repression function of NF-IL6, contingent upon its concentration. Repression was E2 promoter specific, since at a high concentration (15 μ g/ 10^6 cells) NF-IL6 significantly transactivates 4 $\times$ NF-IL6pA₁₀-CAT, a reference plasmid containing four copies of the NF-IL6 recognition element of the IL-6 promoter linked to the reported gene (Fig. 4A). It was not due to sequestering of transcription factors by the strong cytomegalovirus promoter used for expressing NF-IL6, since transfecting pCMV-NF-IL6(-) at a concentration of 10 μ g/ 10^6 cells did not interfere with the promoter activity of either LS mutant -49/-59 or -40/-50 (Fig. 3B). Moreover, repression was NF-IL6 specific, since E1A proteins, which did not activate the control promoter in 4 $\times$ NF-IL6pA₁₀-CAT at all concentrations, maintained their ability to transactivate LS -49/-59 and wild-type E2ae promoters, even when expressed at high levels by cotransfecting 15 μ g of pAd5E1A per 10^6 cells (Fig. 4B).

The NF-IL6* site certainly also serves as a positive regulatory site in response to NF-IL6: LS mutant -49/-59

FIG. 4. NF-IL6, but not E1A, functions as an activator or repressor contingent upon its concentration. Jurkat cells were cotransfected with pE2ae-CAT (WT), its LS mutant -49/-59 (-49) or -63/-72 (-63), or 4×NF-IL6pA₁₀-CAT (4NF), which contains four copies of the NF-IL6 responsive element and serves as a control, at 1 μg of plasmid per 10⁶ cells, and pCMV-NF-IL6(+) (A) or pAd5E1A (B) at 1, 5, or 10 μg/10⁶ cells as indicated. Transfection and assay of CAT activity were as described in Materials and Methods.

was transactivated by NF-IL6 when it was expressed at a lower concentration [1 to 5 μg of pCMV-NF-IL6(+) per 10⁶ Jurkat cells] (Fig. 4A). Corroborating the transactivating function of the NF-IL6* site, LS mutant -63/-72, in which the NF-IL6* site was mutated, failed to be transactivated by NF-IL6 at all concentrations (Fig. 4A). Transactivation of the wild-type E2ae promoter by NF-IL6, in which both NF-IL6 and NF-IL6* sites are present, demonstrated that the promoter activity is the sum of the dominant negative regulatory function of the NF-IL6 site and the NF-IL6 concentration-dependent positive and negative regulatory functions of the NF-IL6* site (Fig. 4A). Together, these results provided compelling evidence that NF-IL6 transactivates and represses an E1A-responsive promoter in a site-specific and concentration-dependent manner.

NF-IL6 reconstitutes the IL-6-regulated E1A-substituting cellular activity in viral infection. To investigate whether NF-IL6 can mediate the IL-6 signals and complement E1A functions in viral infection, we transfected pCMV-NF-IL6(+) into human Jurkat T cells prior to their infection with an E1A deletion mutant virus, *dl312*. *dl312* carries a 902-bp deletion within the E1A gene, rendering it incapable of E1A expression (33).

Indirect immunofluorescent staining of the 72K single-stranded DNA-binding protein, encoded by the viral transcription unit E2ae which is dependent on E1A for activation and is essential for viral replication, showed that expression of NF-IL6 led to the synthesis of the 72K protein in the absence of E1A (Fig. 5). Complementation of the E1A transactivation function by NF-IL6 appeared to be efficient and specific. Synthesis of the 72K protein either in the presence of E1A in wild-type infection or in cells transfected with NF-IL6 prior to infection with *dl312* was comparable, at 2 days after viral infection prior to the onset of viral replication, in approximately 15% of cells (Fig. 5). In cells infected with *dl312* alone or transfected with the antisense derivative of NF-IL6 pCMV-NF-IL6(-) prior to infection with *dl312*, the 72K protein was undetectable.

Similarly, NF-IL6 activates the E1B transcription unit,

FIG. 5. NF-IL6 activates the E2ae gene in infection by an E1A deletion mutant virus. Jurkat cells were infected with Ad2 (Wild Type) or *dl312* at 20 PFU per cell, with or without transfection with pCMV-NF-IL6(+) (NF-IL6+/*dl312*) or pCMV-NF-IL6(-) (NF-IL6-/*dl312*) at 5 μg/10⁶ cells, 8 h prior to infection. Infected and uninfected (Control) cells were subjected to indirect immunofluorescence staining at 2 days postinfection as described in Materials and Methods. Abbreviations: IF, immunofluorescent; Phase, phase-contrast microscopy.

FIG. 6. NF-IL6 activates the synthesis of E1B mRNA in infection by an E1A deletion mutant virus. Jurkat cells were infected with Ad2 or *d/312* with or without transfection as described in the legend to Fig. 5. In the left panel, RNA was isolated at 48 or 72 h after infection with Ad2 or *d/312*, respectively. The poly(A)⁺ RNA was selected from 5×10^6 cells and subjected to RNase protection analysis as described in Materials and Methods. Lanes represent results obtained from uninfected (lane 1), AD2-infected (lane 2), *d/312*-infected (lane 3), pCMV-NF-IL6(+)-transfected and *d/312*-infected (lane 4), and pCMV-NF-IL6(-)-transfected and *d/312*-infected (lane 5) cells and DNA size markers (lane M). In the right panel is a schematic diagram of the probe (46) used in the assay and the predicted protected E1A and E1B-specific RNAs.

which is also dependent on E1A for activation in viral infection. RNase protection assays demonstrated that expression of NF-IL6 in the absence of E1A resulted in the accumulation of E1B mRNAs to the wild-type level, albeit delayed by 1 day after viral infection (Fig. 6). The protected RNAs corresponding to the positive controls, the common 3' exon, and the 5' exons of alternatively spliced E1A 13S and 12S mRNAs were also evident in wild-type virus-infected cells, but not in NF-IL6-transfected *d/312*-infected cells. Likewise, control cells infected with *d/312*, with or without transfection of the antisense construct of NF-IL6, showed no synthesis of E1A and E1B mRNA (Fig. 6).

Previously, we have shown that IL-6 signaling enhanced an E1A-substituting activity in HepG2 cells which led to a greater than 30-fold increase in the steady-state E1B and E2ae mRNA levels in viral infection in the absence of E1A (64). The results presented here provide direct evidence that NF-IL6 alone is sufficient to overcome the requirement for E1A by E1A-dependent viral promoters during infection.

DISCUSSION

NF-IL6 regulates E1A-responsive genes in the absence of E1A. The first conclusion we derive from this work is that NF-IL6, a cellular transcription factor mediating the signals transduced by the IL-6 receptor, can override the E1A dependence of two promoters. The physiological significance of NF-IL6 with respect to the virus is displayed by its

complementation of E1A functions in infection by E1A deletion mutant virus. This suggests a functional overlap of a cytokine pathway and the transactivation function initiated by the viral transforming gene E1A. Thus some of the E1A-dependent or E1A-inducible genes, collectively called E1A-responsive genes and including adenovirus E2ae and E1B, can also be viewed as genes regulated by a physiologically significant cellular signal transduction pathway. Indeed, the identification of NF-IL6 as a nuclear component responsible for the endogenous E1A-substituting activity in HepG2 cells began with the observation that this cellular activity was significantly enhanced by IL-6 induction (64).

Although other members of the C/EBP family, LAP and C/EBP, are enriched in terminally differentiated hepatocytes and adipocytes (11, 14), the NF-IL6 activity is not restricted to specific cell types. However, its level, as measured in an *in vitro* binding assay (Fig. 1) and by Western immunoblotting (29), is cell type dependent and positively correlates with the ability of the cells to support E1A-responsive promoters in the absence of E1A. These results imply that the cellular E1A-complementing activities found in embryonal carcinoma cells and during early mouse development (15, 31, 39) need not be different from NF-IL6. This possibility is amenable to verification. If the cellular E1A-substituting activity in these cells is proven to be NF-IL6 or an NF-IL6-related nuclear factor in the C/EBP family, the role of NF-IL6 during early development would be an intriguing question to address.

NF-IL6 functions as a positive and negative regulator via the NF-IL6 recognition sites. Mutational analyses of the E2ae promoter have yielded some initial understandings of the mechanism of NF-IL6 action. NF-IL6 functions as an activator and repressor in a concentration-dependent and site-specific manner. It appears to bind to the two NF-IL6 sites with different relative affinities and forms specific protein-E2ae promoter complexes (28a). In a cell type-dependent manner, NF-IL6 appears to complex with members of the AP-1 family (29), which are known to bind to DNA in dimerized form (12).

Complementation of E1A functions by NF-IL6 in viral infection has lent support to the physiological relevance of mutational analyses and raises some interesting questions. What is the biochemical nature of NF-IL6 when it functions as an activator and as a repressor? Genes in the C/EBP family do not contain introns and are incapable of generating diversity by alternative splicing but are subject to translation control, as shown by Descombes et al. (14). Friedman and McKnight (19) suggested that regions outside the DNA-binding and leucine zipper domains are important for the activating function of C/EBP. We have observed by Western blotting that the molar ratio of NF-IL6 proteins present in HepG2 cells, corresponding to its three translation start sites, was altered after IL-6 induction and different from that of Jurkat cells after transfection with the NF-IL6 expression vector (29). It is tempting to speculate that although all dimers of NF-IL6 can bind to DNA, they may be functionally distinct depending on the primary structure of their monomeric subunits.

The functional diversity of NF-IL6 can be further expanded by dimerization with other members of the C/EBP family, as LAP can dimerize with C/EBP (14), and/or by its association with transcription factors outside the C/EBP family. Although association of members in the C/EBP family with those in the AP-1 family has not been previously shown, AP-1 transcription factors are attractive candidates for modifying the function of NF-IL6 from a biological point

of view. Like NF-IL6, at least one member of the AP-1 family, *junB*, has been shown to be regulated by IL-6 signaling (52).

Why is an E1A-responsive promoter not active in most cells? The NF-IL6 binding activity is detectable by electrophoretic mobility shift assays in most cells at different levels. The presence of a dominant repressor site in the E2ae promoter offers a plausible explanation by which NF-IL6 primarily functions as a repressor unless appropriately modified. Inspection of sequences in the promoters of E1A-responsive adenovirus early genes reveals the presence of multiple NF-IL6-binding sites in almost all of them; one of these binding sites might serve as a repressor site. Since the E1B gene is regulated by NF-IL6 in a similar manner to the E2ae promoter, the regulation of E1A-responsive genes by NF-IL6 via specific sites may be more easily pursued by using this simpler promoter.

Functional overlap of the IL-6 signal transduction pathway and E1A transactivation. The total NF-IL6-binding activity was significantly reduced after adenovirus infection, suggesting that NF-IL6 is unlikely to be an obligatory intermediate for E1A function (Fig. 1A). The reduction of NF-IL6 activity is most probably due to modification or regulation of NF-IL6 by E1A, since it occurs early after infection, before virus induced host protein synthesis shutoff takes place (28a) (Fig. 1A). How, then, is an NF-IL6-regulated promoter activated when the NF-IL6 level is reduced to undetectable levels? First, reduction of NF-IL6 activity may occur after activation of the E1A-responsive genes. This possibility requires the supposition that once activation begins, NF-IL6 is not needed for maintenance of expression of E1A-responsive gene throughout the viral infectious cycle. Second, nuclear factors of the C/EBP family might be intricately regulated, resulting in a reciprocal increase of other members of the C/EBP family, which functionally replace the NF-IL6 activity when the level of NF-IL6 declines. An example of reciprocal expression of nuclear proteins in the C/EBP family has recently been found during adipose conversion of 3T3-L1 cells (9) and in mouse liver after injection of the mouse with IL-6 (32).

Third, the NF-IL6* site may be functionally redundant, allowing NF-IL6 to function cooperatively and competitively with members of the AP-1 or ATF transcription factor families. This view is supported by our results that in the absence of the dominant negative regulatory NF-IL6 site, LS mutants -49/-59 and -40/-50 were active in the presence of a level of NF-IL6 otherwise inadequate for transactivation (Fig. 3B and 4A). Together with the possibility that NF-IL6 cooperates with members of the AP-1 family and possibly also of the ATF family, in forming specific E2 promoter complexes (65), it is reasonable to speculate that in the absence of NF-IL6 after viral infection, some members of the AP-1 or ATF family may gain entry to the NF-IL6* site and activate transcription of the promoter. In this manner, E1A may function to modify and reduce NF-IL6 on the one hand and to increase the activities of activators such as AP-1 or ATF on the other hand, thereby sequestering a cellular pathway. In the presence of E1A, the presence of a dominant negative regulatory site, such as the NF-IL6 site at the E2ae promoter, provides an additional constraint in regulation but need not be obligatory.

This possibility is consistent with the results of Müller et al. (49), who showed that cAMP functions synergistically with E1A in transactivating E1A-responsive promoters during viral infection, possibly through *c-fos* and *junB*, whose syntheses are regulated by E1A (49, 66). It is also in

agreement with the observations that nuclear factors of the ATF/CREB family can participate in or mediate the E1A transactivation function (7, 24, 30, 42-44, 60, 63). Bagchi et al. (2) recently reported that E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor in vitro, suggesting that E1A may function by sequestering cellular repressors. Can NF-IL6 at high concentrations act as the repressor proposed by Bagchi et al. (2)? Alternatively, is there more than one repressor? These are important questions which remain to be investigated.

The biological significance of regulation of E1A-responsive genes by the IL-6 signal transduction pathway. What is the biological significance of regulating E1A-responsive genes by the IL-6 signal transduction pathway? The NF-IL6 recognition sequence is present in many viral enhancers and promoters, among them the transforming gene E1B, which, in the viral chromosome, is regulated by NF-IL6 in transfection and by IL-6 induction (Fig. 2 and 4) (64). This raises the question of whether the cooperation of E1A with E1B for transformation can be substituted by NF-IL6 or the IL-6 signaling pathway. Although the other members of the C/EBP family have been extensively studied in the terminal differentiation of hepatocytes and adipocytes, they have not previously been shown to function as repressors. The NF-IL6 recognition sequence is also present in the promoters and enhancers of cellular genes, including the immunoglobulin enhancer, which is activated or repressed by E1A depending on the cell type (5, 25). Since immunoglobulin genes are regulated at the level of transcription during IL-6-induced terminal differentiation of B cells (57), it is likely that NF-IL6 is pivotal in this regulatory pathway and that E1A may mimic the NF-IL6 function. As a cellular signaling pathway begins with the receptor for external stimuli, an understanding of the IL-6 signaling pathway and the extent of its overlap with E1A functions will undoubtedly give insights into the biological significance of regulation of E1A-responsive genes by NF-IL6.

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