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NF-IL6 is a cellular homologue for adenovirus E1A proteins

Spergel, Jonathan Michael, Ph.D.

City University of New York, 1992

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300 N. Zeeb Rd.
Ann Arbor, MI 48106

NF-IL6 Is A Cellular Homologue For Adenovirus E1A Proteins

by

Jonathan M. Spergel

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, the City University of New York

1992

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

4/15/92
Date

Selina Chen-Kiang, Ph.D.
Chairman of Examining Committee

4/16/92
Date

Terry A. Krulwich, Ph.D.
Executive Officer

James J. Bieker, Ph.D.

Robert A. Lazzarini, Ph.D.

Lu-Hai Wang, Ph.D.

Hamisch Young, Ph.D.

Supervisory Committee

The City University of New York

Abstract

NF-IL6 Is A Cellular Homologue For Adenovirus E1A Proteins

by

Jonathan M. Spergel

Advisor: Selina Chen-Kiang, Ph.D.

The products of adenovirus E1A gene are transcription activators and repressors. Despite intense efforts, the molecular mechanism of E1A action remains to be elucidated. The complexity of delineating the mechanism of E1A action is compounded by the fact that it induces some promoters and not others, without apparent sequence specificity. A cellular activity (or activities) which functionally substitutes for E1A in transactivating an E1A-responsive adenovirus promoter has previously been shown to be present in embryonal carcinoma cells, in mouse oocytes, and during early development in preimplantation embryos. Its identity, however, has remained obscure. I have found a functionally related E1A-substituting cellular activity in HepG2 cells, of a human hepatoblastoma cell line. The E1A-responsive adenoviral promoters were 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. The E1A-substituting activity in HepG2 cells increases dramatically upon induction by the cytokine interleukin-6, unlike the activity present in embryonal carcinoma cells, which diminishes upon cellular differentiation.

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. Towards this end, I have found that NF-IL6, an IL-6 regulated transcription factor, can regulate E1A-responsive genes in the absence of E1A. It functions as a positive or negative regulator for the E1A-responsive *E2ae* promoter in a concentration-dependent manner via NF-IL6 recognition sequences. These results suggest that there is an overlap of signal transduction pathways elicited by the transforming gene E1A and by the cytokine IL-6. These results also represent the first demonstration of a defined cellular factor which substitutes for E1A.

FORMAT OF THESIS

This thesis was prepared in accordance with guidelines of the City University of New York. Chapter III contains results published as Spergel and Chen-Kiang, Proc. Natl. Acad. Sci. USA (1991) **88**:6472-6476. Chapter IV contains results published as Spergel et al., Journal of Virology (1992) **66:** 1021-1030. Each section contains an introduction and discussion, with a general introduction and general discussion at the beginning and end of the thesis. To reduce redundancy, Materials and Methods sections have been consolidated, as have the Bibliography. Result presented in Figure 20 was obtained from collaborative efforts with Wei Hsu, a fellow graduate student.

ACKNOWLEDGMENTS

I thank the editors from the Journal of Virology for providing written permission allowing me to include the article [(1992) **66:** 1021-1030] in my thesis.

I thank my mentor, Selina, for her advice on how to succeed and what results are worth pursuing, i.e. IL-6 regulation of *dl*312 replication. The lessons that I learn from my experiences in lab will help me succeed in what ever the future has in store for me, for this I'm eternally grateful.

I would also like to thank the members of the Chen-Kiang lab, past and present, Dan, Yaso, Ziying, Wei, Josina, Lyris, and Bill, for providing advice, support, and friendship throughout my graduate training especially when experiments did not work, which seem like the majority of the time.

I have to thank both the graduate and medical students, whom have lent a supportive ear when times were hard and help with the difficult decisions. I can not neglect to thank the many post-docs, who have help me with technical questions and have supplied me with necessary reagents when our supplies were low.

The deepest and warmest thanks must goes to my family without whom I would not have had the strength to finish: my parents, Martin and Rochelle, my brother and sister-in-law, David and Laura, and my sister, Lauren. A special thanks goes to Sydeny, whose friendship and companionship will be missed.

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Tables and Figures

I. INTRODUCTION

The functions of E1A gene products

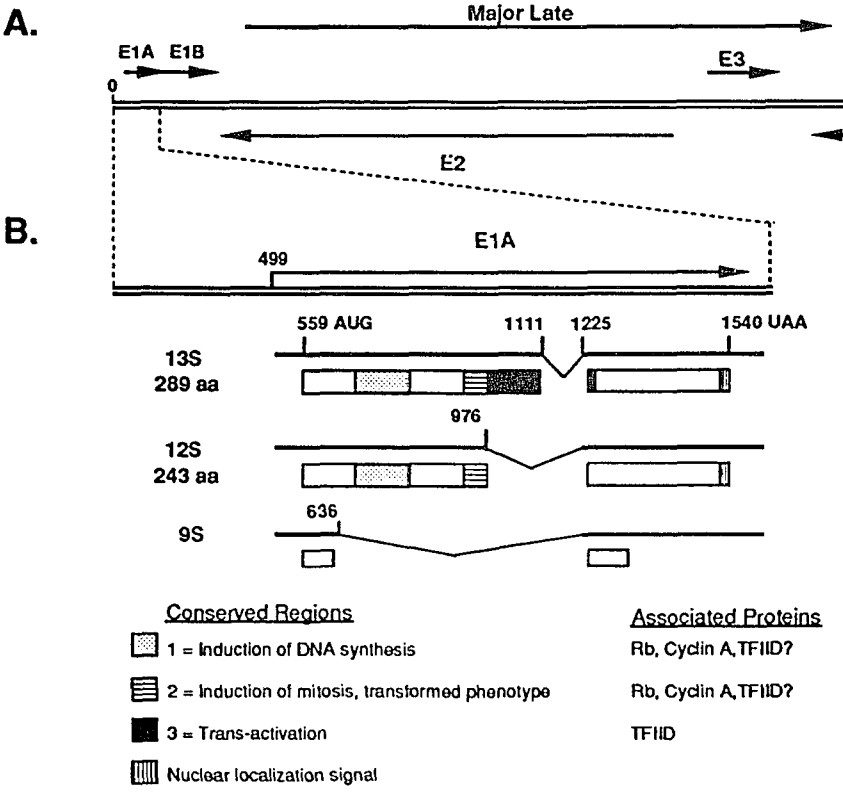
The products of the *E1A* oncogene are among the most extensively investigated proteins. E1A proteins have many functions (Table 1). The *E1A* gene is the first gene to be transcribed after adenovirus infection, enabling E1A polypeptides to activate the early viral transcription units (*E1A, E1B, E2, E3* and *E4*; for reviews see Berk, 1986; and Flint and Shenk, 1989). The *E1A* primary RNA transcripts are processed into three major mRNA species, which share the same 3' exon, but differ in the use of 5' splice donor sites. These mRNA species are called 13s (1.2 kilobases [kb]), 12s (1.0 kb) and 9s (0.6 kb) *E1A* mRNAs (Figure 1; Green, 1986). The two early E1A proteins, (289 and 243 amino acids in length), are translated from the same reading frame. The sole difference in the primary structure between the two proteins is the inclusion of a contiguous 46-amino acid domain in the 289 amino acid protein. Late in infection, a third *E1A* mRNA of unknown function, termed the 9S mRNA, is also synthesized.

Comparisons of the various adenovirus serotypes have suggested conservation in the primary structure of the E1A proteins. Genetic and mutational studies indicated that the activation of viral and cellular gene transcription by E1A can largely be attributed to the 46-amino acid conserved region 3 (Figure 1). Repression of certain enhancer-driven genes, the induction of cellular DNA synthesis, and the transformation phenotype have been attributed to conserved regions 1 and 2 (Figure 1; Moran and Mathews, 1987; and Green et al., 1988). Further investigation of the conserved region 3 by extensive mutational analysis has identified two amino

Table 1: Multiple functions of E1A Polypeptides

Transcriptional activation	Transcriptional repression
Adenovirus	SV40, Polyoma enhancers
c-myc	α-actin, myosin (myoblasts)
hsp 70	MHC promoter
Ig enhancer (L cells)	Ig Enhancer (plasmacytoma)
ß-tubulin	Insulin
Association with Cellular Proteins	
Retinoblastoma gene product, p105	
p107	
TFIID	
cyclin A	
cyclin-dependent kinase 2	
Association with Kinase Activities	
serine/threonine protein kinase	
histone H1 kinase	
Induction of cellular DNA synthesis	
Transformation of primary rat fibroblasts	
(with ras or E1B)	

Figure 1. Schematic representation of E1A gene products. A) The viral early transcription units of the adenovirus chromosome are designated by arrows, which also indicate the orientation of transcription. The adenovirus chromosome is conventionally divided into 100 units, with the left-hand terminus designated as 0. Each unit is approximately 360 bp depending on serotype (Flint, 1977). B) The *E1A* mRNAs and proteins. RNA splice sites and transcription start sites are expressed as nucleotides from the left-hand end of the viral genome (Flint, 1977). The major translation products of the *E1A* genes are also shown. The conserved regions are as defined by Moran and Mathews, 1987. The regions of E1A protein that bind to cellular proteins are based on the work of Whyte et al., 1989 for the retinoblastoma gene product, (Rb); Lee et al., 1991 for TFIID; and Mudryj et al., 1991 for cyclin A.



sequences that are necessary for trans-activation: the finger region (amino acids 147-177), and the carboxyl region (amino acids 183-188) (Webster and Ricciardi, 1991).

The adenovirus life cycle

E1A proteins activate early adenoviral gene promoters, thus allowing the viral infectious cycle to proceed (Figure 1). The *E1B* gene products have been shown to protect viral and cellular DNA from degradation, to induce preferential accumulation of viral mRNA in the cytoplasm, and to confer the oncogenic potential in cooperation with E1A (Babiss and Ginsberg, 1984; Babiss et al., 1985; Pilder et al., 1984; White et al., 1984; and Pilder et al., 1986). The *E2* gene, which encodes the viral DNA polymerase, single-stranded DNA binding protein (DBP), and the precursor to the terminal protein (Kruijer et al., 1981; Stillman et al., 1981; and Stillman et al., 1982), is essential for viral DNA replication. In addition to functioning during DNA replication, the E2 DBP can transcriptionally activate the *E1A, E1B, E2, E3,* and *major late* transcription promoters but represses *E4* gene transcription (Handa et al., 1983; and Chang and Shenk, 1990). The terminal protein, which is covalently linked to the 5' ends of the viral DNA, acts as a primer for viral DNA replication. It is also thought to mediate the attachment of viral chromosome to the nuclear matrix (Stillman et al., 1981; and Schaack et al., 1990).

The *E3* gene products have been proposed to protect the virus from host immune defenses. It is therefore not surprising that *E3* expression is dispensable for infection of cells in tissue culture systems (Thimmappayya et al., 1982). Expression of E3 gene products suppresses some cellular defense functions such as lysis of infected cells by tumor necrosis factor-α (Gooding et al., 1988), down-regulation of epidermal growth

factor receptor (Tollefson et al., 1991) and expression of MHC class I antigen on the surface of infected cells (Burgert and Kvist, 1985). The functions of *E4* gene products remain ill-defined, but they include complexing with the *E1B* gene products (Halbert et al., 1985; Bridge and Ketner, 1989; and Sandler and Ketner, 1989), leading to selective accumulation of viral mRNA in the cytoplasm. Also, the E4 ORF6/7 gene product complexes with a cellular transcription factor, E2F, which binds to the regulatory region of the *E2* gene (Hardy et al., 1989; and Huang and Hearing, 1989).

After synthesis of these early viral gene products, the virus enters the late phase of infection, characterized by replication of its genome and synthesis of the viral structural proteins. Most of the structural proteins are encoded in a single transcription unit which is greater than 26 kilobases (kb), the major late transcription unit (MLTU). This remarkably long transcript was characterized in its start and end points by UV crosslink mapping, nuclear run-on, and chemical analysis of mRNA cap structures, thus defining the first eukaryotic transcription unit (Darnell, 1982). Through an intricate combination of RNA splicing and alternate polyadenylation events, the primary transcript can be processed into one mRNA from one of five late gene families. These families are defined by common polyadenylation sites, each of which contains multiple distinct members arising from RNA splicing events (Chen-Kiang et al., 1982). Spliced to the 5' end of each MLTU mRNA is a three (and occasionally four [Chow et al., 1979]) exon leader, the "tripartite leader". The addition of these segments was the first demonstration of RNA splicing events (Chow et al., 1977; and Berget et al., 1977).

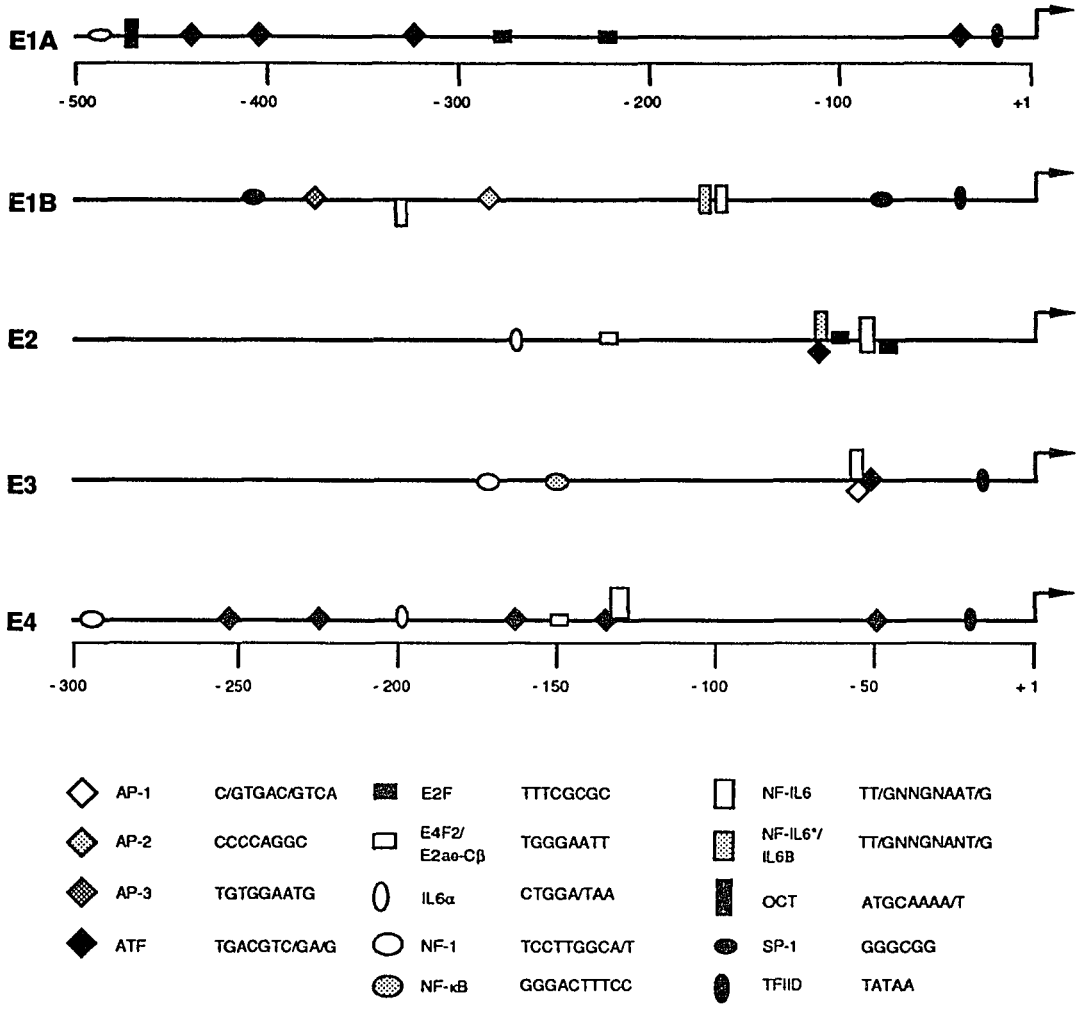
The proposed mechanisms of transcriptional regulation by E1A

In addition to transactivation of the viral promoters, the E1A proteins also interact with cellular promoters. They have been shown to activate the *heat shock 70 protein* and *ß-tubulin* promoters (Imperiale et al., 1984; and Gaynor et al., 1984), while they repress the *class I major histocompatibility complex* and the *insulin* promoters (Stein and Ziff, 1987). Ectopic expression of E1A activates the immunoglobulin heavy chain enhancer in fibroblasts (Borrelli et al., 1986), yet it represses the same enhancer in lymphoid cells (Hen et al., 1985). E1A protein also represses the simian virus (SV40), and polyoma enhancers (Velcich and Ziff, 1985; Imperiale et al., 1984; and Rochette-Egly et al., 1990).

The mechanism of E1A trans-activation is as yet unclear (for review, see Berk, 1986; and Flint and Shenk, 1989). *E1A* gene products stimulate transcription of both RNA polymerase II- and polymerase III-transcribed genes. However, E1A binds to DNA without sequence specificity (Ferguson et al., 1985; and Ko et al., 1986). Three basic models, which are not mutually exclusive, have been proposed for transcriptional activation and repression by E1A: (1) interaction with basal transcription factors; (2) interaction with cellular transcription factors; and (3) sequestration of transcriptional repressors.

In the first model, E1A polypeptides are suggested to interact with basal transcription factors by protein-protein interactions. The first indirect evidence for the first model came from the work of Berk and colleagues investigating a minimal adenoviral *E1B* promoter, which contains binding motifs for two transcriptional factors, SP-1 and TFIID (Wu et al., 1987, Pei and Berk, 1989; Figure 2). TFIID,

Figure 2. Schematic representation of adenoviral early promoters. The putative transcription factors' binding sites are based on either primary sequences or DNA-protein binding assays as reviewed in Jones et al., 1988; and Flint and Shenk, 1989.



which interacts with polymerase II, binds to a TATA motif present within 30 nucleotides 5' to the transcription start site (for a review, see Sawadogo and Sentenec, 1990). SP-1 is a ubiquitous transcription factor that binds to a GC rich sequence present 50 nucleotides 5' to the *E1B* transcription start site (Dynan and Tjian, 1983). The minimal E1B promoter is responsive to E1A in co-transfection experiments, and mutation of the TATA motif abolished this activity (Pei and Berk, 1989). In support of this model, certain TATA box sequences from the *heat-shock 70* promoter, the *c-fos* promoter and the HIV enhancer can mediate E1A activation (Kao and Nevins, 1983; Wu et al., 1987; Simon et al., 1988; Nabel et al., 1988; and Simon et al., 1990). For the *c-fos* promoter, substitution of the *c-fos* TATA element (TATAA) with the SV40 early promoter TATA sequence (TATTTAT) abolished E1A transactivation (Simon et al., 1990), giving circumstantial evidence that E1A interacts with certain TFIID motifs.

However, the most compelling evidence for this model is derived from recent findings that the conserved region 3 of the adenovirus E1A 289 amino acid protein directly contacts recombinant TFIID (Kao et al, 1990; Horikoshi et al., 1991; and Lee et al., 1991). Earlier evidence that E1A polypeptides might influence basal transcription units comes from work by Lillie and Green by the use of E1A-Gal4 hybrids. They showed that E1A stimulates transcription when it is brought into the vicinity of a promoter with a Gal4 binding motif (Lillie and Green, 1989).

The second model suggests that E1A interacts with or regulates transcription factors of which the ATF/CREB family is one example. The ATF/CREB factor is a family of transcription factors (Hai et al., 1989) that recognize a common sequence

element, TGACGTCA (Hai et al., 1988). This family includes the cyclic AMP regulatory protein (Montminy et al., 1986; Hardy and Shenk, 1988; Lin and Green, 1988; and Hurst and Jones, 1987). The ATF/CREB recognition elements appear in many adenoviral early promoters (see Figure 2). For example, the ATF/CREB binding sites in the adenovirus E4 promoter are required for E1A responsiveness in co-transfection experiments (Lee and Green, 1987; and Leza and Hearing, 1988). Also, an E4 DNA fragment containing two ATF/CREB sites can confer E1A inducibility onto a heterologous gene (Lee and Green, 1987). However, no differences have been noted in either the characteristics of binding or the amount of binding activity of ATF/CREB after adenovirus infection.

Since the ATF/CREB motifs are identical to cAMP elements, the viral promoter might also respond to cAMP. Transcription from the adenoviral early promoters, (*E1A*, *E2*, *E3* and *E4*) is stimulated by cAMP in adenovirus infected cells (Leza and Hearing, 1988; and Engel et al., 1988). E1A and cAMP activate *E1* and *E4* promoters synergistically at the same ATF/CREB elements in S49 and HeLa cells but in a non-synergistic fashion using different motifs in the *E4* promoter in HepG2 cells (Engel et al., 1988; Lin and Green, 1988; and Leza and Hearing, 1988). These results suggest a cell line specificity in the transactivation of the E4 promoter by cAMP and E1A. Since ATF/CREB is a family of proteins (Hai et al., 1989), each cell line may contain a different subset of proteins to interact with E1A and cAMP, generating the above results.

Liu and Green have proposed a possible mechanism for activation of ATF/CREB transcription factors by E1A proteins. In co-transfection, the ATF-2

fusion protein (ATF-2/Gal4) was shown to activate transcription when the E1A protein is expressed. However, E1A does not activate transcription when ATF-2 was substituted by ATF-1 in this assay (Liu and Green, 1990). Together with the first model, these results suggest that E1A may form a molecular bridge between ATF-2 and TFIID to stimulate transcription.

Another proposed mechanism for activation by E1A via the ATF/CREB element involves transcriptional factors in the related family, AP-1. As in the case of transcription factors in the CREB family, Fos and Jun protein dimerize (for review see Abate and Curran, 1990). The AP-1 recognition element, TGACTCA, differs by only one nucleotide from the ATF/CREB recognition sequence, TGACGTCA (Hai et al., 1989). However, AP-1 can bind to the ATF/CREB site, albeit with lower affinity than the ATF proteins. For the *E4* promoter, cyclic AMP (cAMP) and E1A appear to act synergistically to activate the transcription factor AP-1 at the mRNA level by stimulating *c-fos* and *jun-B* mRNA (Müller et al., 1989). Additionally, both the 12S and the 13S proteins of adenovirus E1A can induce *c-fos* mRNA and AP-1 DNA binding activity (Engel et al., 1991). However, activation of AP-1 by E1A is not universal. van der Eb and colleagues found that members of the *jun* family were activated or repressed by the E1A proteins depending on the cell type and the serotype of adenovirus (van Dam et al., 1990).

The third variation of this model involves modification of existing proteins by E1A. The strongest evidence for this model comes from the work on the RNA polymerase III transcription of adenovirus VA RNA. Roeder and colleagues have correlated an E1A-induced shift in electromobility shift assay (EMSA) of the

polymerase III transcription factor, TFIIIC, with an increase in the transcription of the *VAI* gene in a cell-free system (Hoeffler et al., 1988). The alteration in DNA-protein mobility is apparently due to phosphorylation of TFIIIC, suggesting that this process may play a role in E1A activation. Other transcription factors have been suggested to exhibit E1A-inducible phosphorylation such as E2F (Yee et al., 1989) or ATF-3/E4F (Raychaudhuri et al., 1989); however, E1A-inducible phosphorylation of E2F was not reproduced in another laboratory (Marton et al., 1990).

The third model suggests that E1A interacts with cellular repressors. One set of results implicates that Rb may function as a repressor for E1A-responsive promoters. The inhibitory effect is postulated to be mediated through its interaction with the E2F transcription factor (Bagchi et al., 1991; and Chellappan et al., 1991) or a related factor termed DRTF1 (Bandara et al., 1991). E2F is a partially purified cellular factor with two recognition sites in the *E2* promoter as defined by DNase I assay (Kovesdi et al., 1986a, 1986b). Additionally, E2F-dependent transcription is stimulated by E1A in co-transfection assays (Yee et al., 1988; Pei and Berk, 1989; and Bagchi et al., 1990). The important feature of this model is the finding that E2F complexes with the retinoblastoma gene product, p105, and these complexes can be disrupted by E1A in gel mobility shift assays (Bagchi et al., 1991; Chellappan et al., 1991; and Chittenden et al., 1991). Since E1A polypeptides interact with Rb (Whyte et al., 1988), E1A may activate the E2F transcription factor from the inactive E2F-Rb complex by binding to Rb and dissociating the complex (Bagchi et al., 1991; and Chellappan et al., 1991). Furthermore, in transient transfection, expression of a wild-type Rb resulted in a five-fold reduction of E2-CAT expression in a cell line deficient

for wild-type Rb (Hiebert et al., 1992). However, since E2F has not been cloned and antibodies against E2F are not available, this model lacks support from direct experimental evidence derived from assaying E2F. Additionally, recent work has shown that purified E2F does not bind to Rb, as an associated factor is necessary for Rb to complex with E2F (Hiebert et al., 1992).

More direct evidence that E1A interacts with a cellular repressor comes from the study of the YY-1 gene. YY-1 is a GL1 Krüppel-like zinc-finger protein, that binds to the adeno-associated virus *P5* promoter (Shi et al., 1991). It is identical to the δ factor which binds to the mouse ribosomal promoter (Hariharan et al., 1991) and to NF-E1, which binds to the immunoglobulin κ 3' enhancer and the μE1 site (Park and Atchison, 1991). YY-1 protein can activate transcription *in vitro* from the adeno-associated virus *P5* element (Seto et al., 1991). YY-1 protein can also repress transcription of the NF-E1 site in κE3' enhancer and immunoglobulin enhancer (Park and Atchison, 1991). When YY-1 is expressed as a hybrid protein, YY-1-Gal4, it represses transcription from a promoter containing Gal4 DNA-binding sites. However, the 243 aa E1A gene product can relieve this repression, while the 289 aa E1A gene product both relieves the repression and activates the promoter (Shi et al., 1991). This is the first cloned transcription factor that E1A interacts with and relieves its repression.

E1A-Substituting Activities

The search for cellular factors that substitute for E1A has mainly involved the detection of complementation of E1A function by *E1A* deletion viruses or activation of E1A-responsive viral promoters in transfection. *dl*312, which contains a 73%

deletion of the *E1A* coding region, from bp 448 to 1349, which one of the first *E1A*-deletion viruses generated (Jones and Shenk, 1979). HeLa cells were the first cell line found to be permissive for *dl*312 viral replication at high multiplicity of infection (moi) (Shenk et al., 1979). This activity in HeLa cells could be due to constitutive expression of the integrated copies of the human papilloma virus (HPV) type 18 genome (Scheider-Gadicke and Schwarz, 1986). The HPV *E7* gene products have been shown to transactivate the *E2* early promoter of adenovirus (Phelps et al., 1988), implying that the HPV genome could influence adenoviral replication and function in HeLa cells. E7 protein has another E1A-substituting activity: binding to the retinoblastoma gene product (Dyson et al., 1989) and interacting with E2F via the Rb gene product (Phelps et al., 1991).

Additional evidence for the existence of a cellular E1A-substituting activity comes from studies examining the *E2* promoter in F9 cells. The *E2* promoter is transcriptionally active in undifferentiated F9 cells, a mouse teratocarcinoma cell line. However, differentiation of F9 cells by dibutyryl cyclic AMP or retinoic acid causes F9 cells to lose this ability to activate the *E2* promoter (Imperiale et al., 1984; and La Thangue and Rigby, 1987). Furthermore, the *E2* promoter is transcriptionally active in pre-implantation mouse embryos and oocytes, but again, activity diminishes upon differentiation (Dooley et al., 1990). These results suggest that an E1A-substituting activity exists in undifferentiated cells but not in the differentiated cells. DRTF1 (differentiation regulated transcription factor 1) is a biochemical fraction of F9 nuclear extracts that binds to the E2F motif (Shivji and La Thangue, 1991). The binding of DRTF1 to the *E2* promoter resulted in two protein-promoter complexes,

DRTF1a and DRTF1b (Shivji and La Thangue, 1991). The ratio of the DRTF1 complexes in the cell lines varied with the ability of the cell line to stimulate E2-CAT transcription (Reichel et al., 1987; Partridge and La Thangue, 1991). For example, DRTF1b is abundant in F9 embryonal carcinoma cell line, embryonal stem cells, blastocystes, and embryos, and is down-regulated during differentiation; the reverse is true for DRTF1a (Partridge and La Thangue, 1991). As mentioned previously, E2F/DRTF1 complexes with cyclin A and Rb proteins; however, only the DRTF1a protein complexes with Rb, while DRTF1b protein does not (Bandara et al., 1991). This circumstantial evidence suggests that the protein(s) that could account for the difference between DRTF1a and DRTF1b may be an E1A-substituting factor. However, similar to E2F, DRTF1 is not cloned; therefore, no direct evidence linking DRTF1 with Rb and with E2 transcription activity has been provided.

Despite all this circumstantial evidence, no cloned factors have been shown to substitute for E1A. In this thesis, I will demonstrate that an E1A-deletion virus replicates in HepG2 cells and that this activity is inducible by interleukin-6 (IL-6; see Chapter 3). I will also describe the first cloned transcription factor that can substitute for the E1A transactivating activity to stimulate E1A-inducible viral promoters (Chapter 4).

The multiple functions of interleukin-6

IL-6 is a pleiotropic cytokine that is synthesized in a variety of cells, exerting growth-inducing, growth-inhibitory, and differentiation-inducing effects, depending on the nature of the target cells (for review see Hirano and Kishimoto, 1989; and Van Snick, 1990; see Table 2). According to its biological functions, IL-6 was initially

known as B cell stimulatory factor-2 (BSF-2, Hirano et al., 1986), interferon-$ß_2$ (IFN-$ß_2$, Zilberstein et al., 1986), 26-kDa protein (Haegeman et al., 1986), and hepatocyte stimulating factor (HSF, Andus et al., 1987; and Gauldie et al., 1987) before, molecular cloning showed that all the molecules were one and the same. Also, IL-6 has been implicated as a causative factor in a variety of diseases such as autoimmune diseases, and neoplasia (see Table 3 for review).

However, little is known of the IL-6 signal transduction pathway. IL-6 binds to its receptor, an 80kDa protein belonging to the immunoglobulin superfamily and the cytokine receptor family (Yamasaki et al., 1988), which then associates with a 130-kDa transducer which transmits the signal into the cytoplasm (Taga et al., 1989; and Hibi et al., 1990). In mouse B lymphoma cells, IL-6 appears to induce a rapid and transient phosphorylation of a cellular 160kDa cellular protein and activate transcription of TIS11 and *jun-B* genes (Nakajima and Wall, 1991). IL-6 also induces NF-IL6, a member of the CCAAT/enhancer binding protein (C/EBP) family in hepatocytes and many other cell types (Akira et al., 1990).

C/EBP Transcription Factor Family

C/EBP (CCAAT/enhancer binding protein), originally identified in rats (Landschulz et al., 1988a), is primarily expressed in liver, fat and intestinal tissues (Birkenmeier et al., 1989). C/EBP, now termed C/EBP-α, activates many different cellular promoters, including albumin, α_1 anti-trypsin, and transthyretin promoters, as well as viral promoters including the murine sarcoma virus long terminal repeat, and the herpesvirus thymidine kinase promoter (reviewed in Johnson and McKnight, 1989). Other members of the C/EBP family have been cloned recently

Table 2: Interleukin 6 Activities

Growth and Differentiation

IN LYMPHOCYTES:

Induces B-cell differentiation

Promotes myeloma/plasmacytoma/hybridoma cell growth

Induces IL-2 and IL-2 receptor expression in T cells

Enhances the proliferation and differentiation of T cells

IN MYELOID CELLS:

Inhibits cell growth of certain myeloid leukemia cell lines

Differentiates myeloid leukemia cell lines into macrophages

IN HEMATOPOIETIC CELLS:

Stimulates the maturation of megakaryocytes

Enhances IL-3-induced multipotential colony cell formation

IN OTHER CELL LINES:

Induces acute phase proteins in liver cells

Promotes mesangial cell and keratinocytes growth

Differentiates PC12 cells into neuronal cells

SYSTEMIC REACTIONS:

Increases body temperature

Increases circulating level of glucocorticoids

Alters tissue metabolism

After Hirano et al., 1990; Sehgal, 1990.

Table 3: IL-6 and Disease

INFECTIONS:
Elevated levels in bacteremia
Intraamniotic infections
MALIGNANCIES:
Plasmacytoma
Myeloma
Lymphoma
Leukemia
Renal Cell Carcinoma
Ovarian Cell Carcinoma
Kaposi's Sarcoma
OTHER DISEASES:
Alcoholic liver cirrhosis
Cardiac myxoma
Rheumatoid arthritis
Castleman's disease
AIDS
Psoriasis
Mesangial Proliferative Glomerulonephritis

After Hirano et al., 1990; Sehgal, 1990.

(Figure 3).

Analysis of the C/EBP protein has identified some important structural features. Landschulz and colleagues were first to notice that C/EBP and several other transcription factors have a heptad repeat of four to five leucines termed the leucine zipper (Landschulz et al., 1988). Comparison of the proteins in the C/EBP family shows a strong similarity, 55% to 88% homology, in the basic and leucine zipper (bZIP) with unique amino termini, based on conserved substitution of amino acids (Figure 3). The conserved substitution groups were defined as Leu, Ile, Val and Met; Lys, Arg and His; Pro, Gly, Ser, Thr and Ala; Asp, Asn, Glu, and Gln; Phe, Trp, and Tyr; and Cys.

LAP appears to be the rodent homolog of NF-IL6, as it is highly homologous in its amino terminus and nearly identical in the DNA binding domain (Akira et al., 1990; Figure 3). The amino acid homology is 96% between NF-IL6 and LAP when the 6 segments of amino acid insertion in the NF-IL6 protein are not considered. Both NF-IL6 and LAP appear to be involved in the IL-6 signal transduction pathway (Akira et al., 1990).

Proteins in the C/EBP family can form homo- and heterodimers using the leucine zipper for dimerization in a coiled-coiled parallel fashion (O'Shea et al., 1989; Gentz et al., 1989; Descombes et al., 1990; Roman et al., 1990; and Cao et al., 1991). The heterodimerization of the C/EBP family is similar to heterodimerization of the fos, jun and ATF/CREB transcription factor families, generating a large array of transcription factors to mediate control of promoters.

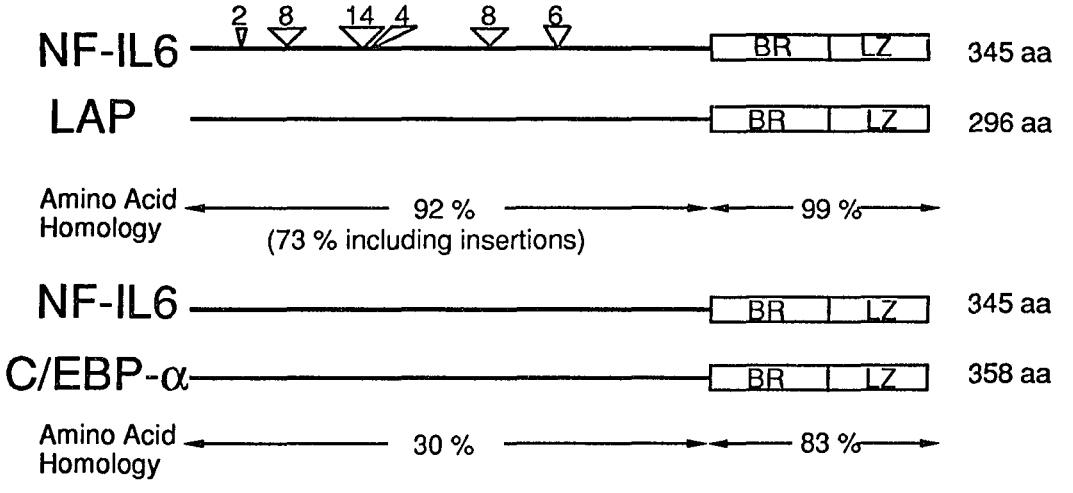
The NF-IL6 mRNA and its rodent equivalents are detected in most tissues and

Figure 3. C/EBP transcription family. (A) Comparison of the transcription factors based on the cDNA sequences. The bZIP (basic and leucine zipper region) homologies are in comparison to C/EBP-α with conserved substitution of amino acids as described in the text. (B) Schematic representation of NF-IL6, LAP and C/EBP protein structures. Abbreviations: aa, amino acids.

A. C/EBP Transcription Factor Family

Transcription Factor	mRNA Distrubition	Size	bZIP Homology (to C/EBP-α)	Reference
C/EBP-α	Liver, adipose	358 aa	100%	Landschulz et al., 1988
NF-IL6 (LAP, IL-6DBP, C/EBP-β, CRP2, AGP/EBP)	Liver, lung, adipose (Rodent NF-IL6)	296 aa	83%	Akira et al., 1990 (Descombes et al., 1990; Poli et al, 1990; Cao et al., 1991; Williams et al., 1991; Chang et al., 1990)
Ig/EBP-1, C/EBP-γ	Lung, adipose, kidney, spleen	268 aa	79%	Roman et al., 1990; Cao et al. , 1991;
C/EBP-δ, CRP3, NF-IL6β	Lung, adipose, liver	268 aa	74%	Cao et al., 1991; Williams et al., 1991; Kinoshita et al., 1992
CRP1	no data	249 aa	88%	Williams et al., 1991
CHOP	Testis, muscle, liver, heart	168 aa	55%	Ron and Habener, 1992

B.



is stimulated by either lipopolysaccharide (LPS), IL-1, or IL-6 (Akira et al., 1990; and Poli et al., 1990). However, the LAP protein is only abundant in liver and adipose tissue, suggesting post-transcriptional regulation of the gene. Schibler and colleagues recently showed that translational regulation of *LAP* mRNA can result in the production of 3 different proteins from the same mRNA species. *LAP* mRNA encodes both a transcriptional activator, LAP, and a transcriptional repressor, LIP, presumably by a leaky ribosome scanning mechanism. Alteration of the ratio of LAP to LIP proteins was made by a series of mutagenesis experiments by substituting ATGs with TTGs, or by altering sequences surrounding the ATG from imperfect to perfect Kozak consensus motifs (Kozak, 1989). LIP can repress transcription from a construct containing the D element from the albumin promoter, while LAP activates transcription from the same promoter-construct in CAT assays (Descombes et al., 1991). The D element of the albumin promoter interacts with liver-specific factors and can direct a high level of liver-specific transcription expression (Maire et al., 1989).

NF-IL6 protein interacts with many of the acute phase promoters and NF-IL6 binding activity is enhanced by induction with IL-6 (Akira et al., 1990; and Chapter 4) suggesting a role for NF-IL6 in the regulation of the acute phase genes (Isshiki et al., 1991). Further evidence for the role of members of the C/EBP family in regulating the acute phase response is that *NF-IL6* mRNA dramatically increases after IL-6 stimulation in an acute phase reaction; in contrast, the *C/EBP-α* mRNA level decreases in mouse liver after IL-6 stimulation (Isshiki et al., 1991). The C/EBP family also plays a role in 3T3/L1 adipocyte differentiation. C/EBP-ß (NF-

IL6) and C/EBP-δ reached a maximal level during the first two days of differentiation and declined sharply before the onset of C/EBP-α accumulation in the late phase of adipocyte differentiation (Cao et al., 1991); while, the reversal of adipocyte phenotype by TNF-α causes a decrease of C/EBP-α and concomitant increase of LAP (Ron et al., 1992).

In this thesis, I demonstrate another activity for NF-IL6. NF-IL6 can complement an E1A deletion mutant in viral infection and regulate E1A-responsive promoters in the absence of E1A. Furthermore, it functions as a positive or negative regulator in a concentration-dependent manner via NF-IL6 recognition sequences (Chapter 4). These results suggest there is an overlap of signal transduction pathways elicited by the transforming gene E1A and by the cytokine IL-6.

II. MATERIALS AND METHODS

Cells and viruses:

HepG2 cells, a human hepatoblastoma cell line (Aden et al., 1979; and Knowles et al., 1980), were cultured in Eagle's minimal essential medium supplemented with 10% (vol/vol) fetal calf serum (HyClone). HeLa cells were maintained as suspension cultures for viral infection in Joklik modified minimal essential medium supplemented with 5% fetal calf serum, or as monolayer cultures for transfection. Monolayer cultures, including HeLa cells, 293 cells, human embryonic kidney cells transformed with the left hand 15% of adenovirus genome (Graham et al., 1977), HEL cells, a human embryonic lung fibroblastic cell line, and HA22T/VGH (HA22T) cells, a human liver cell line (Chang et al., 1983) were maintained in Dulbecco's modified minimal essential medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum. Jurkat cells, a human T cell line, were cultured in RPMI-1640 supplemented with 10% (vol/vol) heat-inactivated fetal calf serum. Fetal calf serum was heat-inactivated at 56°C for thirty minutes. All media were supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and non-essential amino acids (GIBCO). Human fetal liver explants were prepared by Joachim Bauer as described in Sells et al., 1985. Cells were used for experiments on day 3 after explant, when major dedifferentiation had not yet occurred.

Adenovirus type 2 (Ad-2), wild-type virus, was propagated as described and plaqued on A549 cells (Lavery et al., 1987). *dl*312, an adenovirus type 5 E1A mutant virus containing a deletion from base pair 448 to 1349 of the left-hand of the

adenovirus genome (Jones and Shenk, 1979) was plaqued and propagated on 293 cells. *dl*343, an adenovirus type 5 E1A mutant, which contains a 2 base pair deletion (661-662) in the E1A codon region, resulting in a frame-shift in translation and no synthesis of functional E1A polypeptides was also plaqued and propagated on 293 cells (Hearing and Shenk, 1985). Viral infections were performed essentially as described previously (Lavery et al., 1987), at a moi of 10 plaque forming units (pfu) per cell, except where noted.

Cytokine induction:

The cells were induced with appropriate cytokines at concentrations as indicated, at 24 hours prior to infection and continuing throughout viral infection. Interleukin 6 (IL-6, provided by T. Kishimoto and T. Hirano) was used at 100 units per ml on HepG2 cells. Tumor necrosis factor-α (TNF-α), a gift from A. Cerami, was used at 200 units per ml, and dibutyryl cyclic AMP (Bt_2cAMP), obtained from Sigma, was used at a concentration of 1 mM. For nuclear extract preparation and transfections, IL-6 was used at 500 units/ml.

Southern and Northern blot analyses:

Total DNA from 2 x 10^4 cells was isolated at times post-infection (p.i.) by proteinase K digestion, cleaved with *Hind*III, and separated on a 1% agarose gel. After blotting, hybridization with a [^{32}P]labeled DNA probe was performed in the presence of 50% formamide at 42°C overnight as previously described (Lavery et al., 1987). Adenovirus DNA was detected using a [^{32}P]-random primed probe of plasmid p3WT18 (Ruether et al., 1986), which contains the left-hand 5.4 kilobases of Ad-5 and detects E1A and E1B sequences.

Total RNAs were isolated using the guanidinium thiocyanate method (Chirgwin et al., 1979), and polyadenylated RNAs were selected by poly(U)-agarose chromatography (Chen-Kiang and Lavery, 1989A). Steady state RNAs were fractionated on 1.4% agarose-formaldehyde gels in MOPS buffer (20 mM morpholinopropane sulfonic acid [Sigma Chemical Company], 5 mM sodium acetate, 1 mM EDTA, pH 7.0) and analyzed by Northern blotting. Hybridization was performed in the presence of 50% formamide at 42° C overnight as described (Thomas et al., 1980), and blots washed four times with 2X SSC, 0.2% SDS for ten minutes at 42°C, and two times with 0.1X SSC, 0.2% SDS for 30 minutes at 55°C (1X SSC = 150 mM NaCl, 15 mM sodium citrate). Probes were [^{32}P]-labeled by a random priming reaction (Feinberg and Vogelstein, 1984).

The specific probes for Northern analysis were c131, an E1A cDNA clone (Perricaudet et al., 1979), c244, an E1B cDNA clone (Perricaudet et al., 1980), the Ad-2 *Hind*III D fragment for the L2 family (41.8-50.1 map units [m.u.] on the Ad-2 genome), and the Ad-2 *Bal*I K fragment for E2 (63.2-66.3 m.u.). (The Ad-2 genome is 36 kb and conventionally divided into 100 units. The 0 at the left end is the 5' end of the rightward transcribed strand [Flint, 1977]). For quantification of mRNA loading, a rat cDNA for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a probe (Fort et al., 1985).

Polymerase Chain Reaction:

1.5 μg of cellular DNA, corresponding to 10^5 cells from 293, HepG2, or HeLa or the molar equivalent of 10, 1, 0.1 and 0.01 copy per cell of the E1A gene from p3WT18, plus 1.5 μg of DNA isolated from HEL cells was added to a 50 μl reaction

mix consisting of 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.5 μM primers, 200 μM dNTPs, and 3 U Taq polymerase (New England Biolabs). The primer sequences were GTGAGGAGTTTGTGTTAGAT (974- 993) for primer 1 for rightward reading and TTATGGCCTGGGGCGTTTACAG (1521 - 1542) for primer 2 for leftward reading as shown in Figure 7. The reaction was cycled for 20 or 80 times under the conditions of denaturing at 94°C for 1 minute, annealing at 47°C for 2 minutes, and extending at 72°C for 3 minutes in each cycle. The end products were analyzed by electrophoresis on a 1% agarose gel and stained with ethidium bromide.

Transfection procedures and assays

For IL-6 induction, HepG2 cells were transfected essentially as described by Won and Baumann, 1990. Briefly, HepG2 cells at 24 hours after plating (6 X 10^5 cells per 100 mm dish) were transfected with 20 μg of total DNA, including carrier and 3 μg of RSV-ßgal, by the calcium phosphate method (Graham and van der Eb, 1973). After dispersion by trypsinization on the following day, equal numbers of cells were plated onto two collagen (Boehringer Mannheim)-coated 6 well dishes. One set of cells served as a control; and the other was induced with IL-6 (500 units/ml) in serum-free medium at four hours after plating. Transfection of monolayer cells without cytokine induction was performed by the calcium phosphate method with desired amounts of experimental plasmid DNA in a total amount of DNA (including carrier DNA and 3 μg of RSV-ßgal) empirically determined to be optimal for 6 x 10^5 cells for each cell line: 10, 20, 30, 30 and 30 μg of total DNA for 293, HepG2, HeLa, HA22T and HEL cells respectively (Graham and van der Eb, 1973). The

electroporation method (Potter et al., 1984) was used for transfection of Jurkat cells, using the Gene-Pulser (Bio-Rad) at 1.5 kV and 25 μF, with 15 μg of total DNA for 3 x 10^6 cells.

The cell extracts were prepared 48 hours after transfection. The cells were washed twice with phosphate-buffered saline (PBS), 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 was normalized by the ß-galactosidase activity expressed in the co-transfected cells. Transfected cell extracts (50 μl) were added to 1 ml of buffer containing 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$, and 50 mM ß-mercaptoethanol, to which 200 μl of 2.0-mg/ml O-NPG (*o*-nitrophenyl ß-D-galactopyranoside; Sigma) had been added as the colorimetric substrate. The reaction was incubated at 37°C until a yellow color appeared, in approximately 10 hours. The ß-galactosidase activity was measured as the A_{420} (Herbomel et al., 1987).

Chloramphenicol acetyltransferase (CAT) activity was determined by the diffusion of [^{3}H]acetyl coenzyme A into organic-miscible scintillation fluid. Equivalent amounts of cell extracts based on ß-galactosidase activity were combined with chloramphenicol (final concentration 1.25mM; Sigma), and ^{3}H-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 [^{3}H]acetyl-chloramphenicol was counted at

20, 40, 60, 120, and 180 minutes of incubation. The slope of the organic-miscible radioactivity as a function of time was calculated and used to represent the CAT activity (Newmann et al., 1987, Sambrook et al., 1989), the slope of the pRSV-CAT was used as a positive control and the slope of the pJYM-CAT was used as a negative control. After normalizing transfection efficiency with the ß-galactosidase activity, the CAT activity is expressed as the percentage of that of the positive control, pRSV-CAT. For example,

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

Plasmids used in transfection experiments

Plasmids (Figure 4) containing the chloramphenicol acetyltransferase (CAT) reporter gene were as follows: plasmid 4NFIL6pA_{10}-CAT contains four copies of the NF-IL6 responsive element [5'-GATC(ACATTGCACAATCT)$_4$-3'] 5' to an enhancerless simian virus 40 (SV40) promoter (Isshiki et al., 1991); the pE2ae-CAT plasmid contains the Ad5 E2ae promoter (-284 to +62) cloned into pSVO-CAT; the E2ae linker-scanner mutants contain the linker GGGCGTAGGCCC cloned into pE2ae-CAT (Murthy et al., 1985, Figure 5); the pE1B-CAT plasmid contains the Ad5 E1B promoter (-365 to +1 of the E1B promoter, Dery et al., 1987); 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 (LTR) of Rous sarcoma virus (RSV) (Gorman et al., 1982); and the negative control, pJYM-CAT, contains a TATA box from

Figure 4: Schematic maps of the plasmids used in transfection. The curve line represents the host plasmid, e.g. pBR322. The arrow in pCMV-NF-IL6 plasmids represents the direction of cDNA in the plasmid. For references, see text.

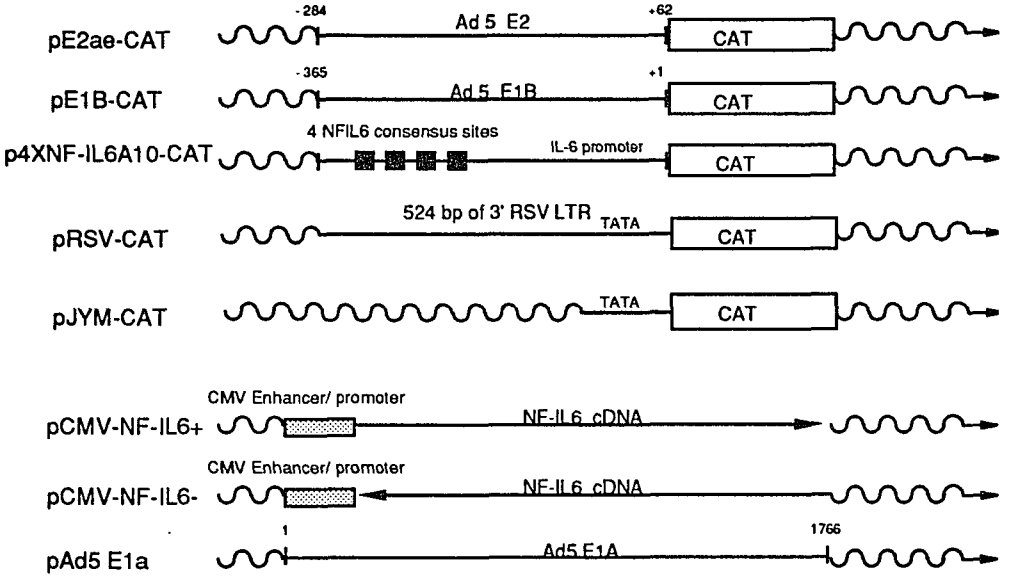
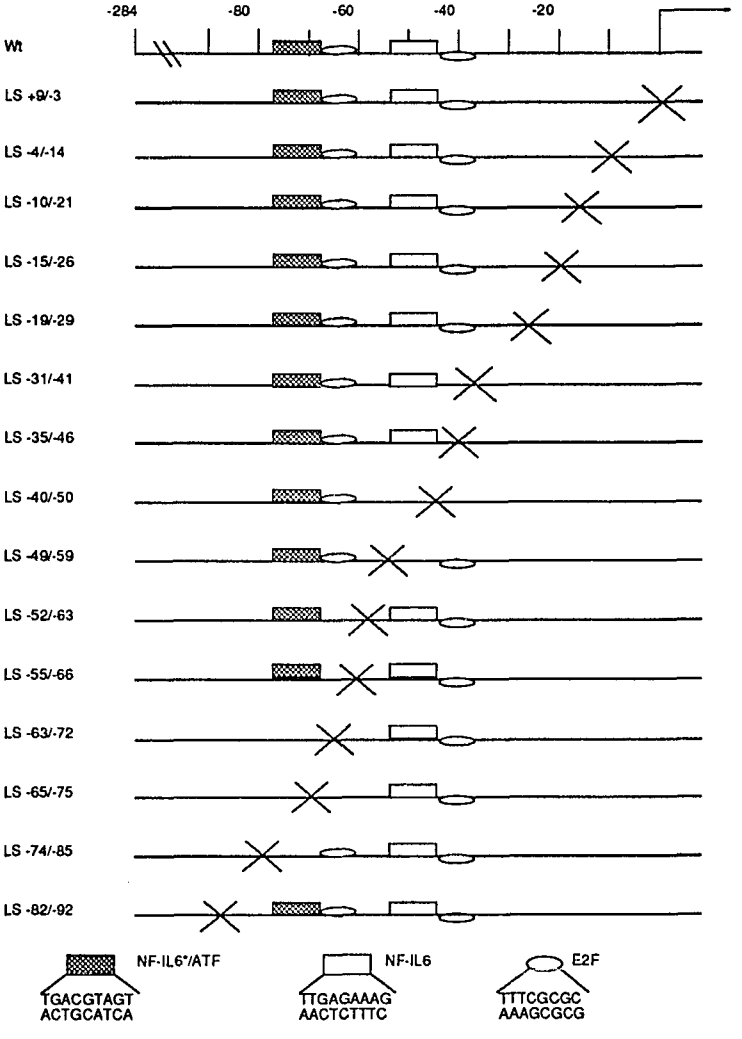


Figure 5. pE2ae-CAT Linker-Scanner Mutants. These mutants which contain replacement of *E2* promoter with a linker GGGCGTAGGCCC cloned into pE2ae-CAT were obtained from Murthy et al., (1985). The NF-IL6 (TTTGAGAAAG) and NF-IL6* sites (TGACGTAGTT) are in rectangles whereas the two E2F sites are in ellipses. The E2F sites (CGCGCTTT) are binding sites for the E2F factors (Kovesdi et al., 1986) which are thought to be necessary for the function of the E2ae promoter. The ATF site overlaps with the NF-IL6* site (Murthy et al., 1985; SivaRaman et al., 1986). The X represents the area where the sequences are replaced by the linker.



simian virus 40 (SV40) but no enhancer (Lusky et al., 1983). pAd5E1A contains bp 1 to 1764 of the adenovirus type 5 genome including the *E1A* promoter and expresses all three E1A proteins (Tibbetts et al., 1986). pCMV-NF-IL6(+) contains a cDNA encoding NF-IL6 directed by the CMV promoter and pCMV-NF-IL6(-) is its antisense derivative (Akira et al., 1990). RSV-ßgal contains the ß-galactosidase gene driven by the RSV LTR enhancer-promoter (Bonnert et al., 1987).

Nuclear Extract Preparation:

Nuclear extracts were prepared at 4°C essentially as described by Mattila et al., 1990. 3 x 10^7 cells were collected at 250xg and washed twice with 1 X PBS. The cells were washed with 1 ml of Buffer A: 10 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.8; Sigma), 15 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT, Sigma), 0.1 mM EDTA, 1% non-fat milk, 1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma), 1 µg/ml antipain (Sigma) and 0.3 µg/ml leupeptin (acetyl-leucine-leucine-arginine-al; Sigma). The cells were pelleted at 750xg, and resuspended in one ml of buffer A containing 0.2% nonidet-P40 (NP-40; Sigma) to lyse the cells. The nuclei were then isolated by pelleting through a 10% sucrose cushion in buffer A. The pelleted nuclei were resuspended in 100 µl of Buffer B and the final volume was brought to 162 µl with Buffer B: 50 mM HEPES (pH 7.8), 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF and 10 % glycerol. 13 µl of 4M ammonium sulfate was added to the resuspended nuclei to a final concentration of 0.3 M and the mixture was rocked for thirty minutes at 4°C. The mixture was centrifuged for fifteen minutes at 75,000 rpm using a TLA-100.3 rotor in a Beckman TLA-100 ultracentrifuge. 125 µl of the supernatant was transferred to

an eppendorf tube, mixed with 75 μl of 4 M ammonium sulfate (final concentration 1.5 M), and then centrifuged at 50,000 rpm for ten minutes at 4°C in a TLA-100.3 rotor in a TLA-100 ultracentrifuge. The pellet was resuspended in 50 - 100 μl of Buffer B and stored at - 80 °C. The protein concentration was determined by the Bradford assay (Bradford, 1976) using a protein assay kit (Bethesda Research Laboratories).

Electrophoretic mobility shift assay:

The mobility shift assays were performed based on the methods of Fried and Crothers (1981) and Garner and Revzin (1981). Nuclear extract (2 to 3 μg) was used for binding of NF-IL6 complexes in a buffer containing 5 mM Tris-Cl, 10 mM HEPES (pH 7.9), 50 mM NaCl, 1 mM dithiothreitol (DTT), 10 % glycerol, 1 μg of poly(dIdC) (Boehringer Mannheim Biochemicals), to 1 ng of the NF-IL6 probe or the E2 probe, in the presence of 15 or 1 mM EDTA, respectively. For E2F complex binding, nuclear extracts (8 μg) were incubated with end-labeled E1 or E2 probe (1 ng) in 16 μl of binding buffer containing 20 mM HEPES (pH 7.9), 40 mM NaCl, 50 mM KCl, 0.5 mM $MgCl_2$, 1 mM EDTA, 4% Ficoll, and 2 μg of sheared salmon sperm DNA, at room temperature for thirty minutes. For SP-1 complex binding, nuclear extracts (5 μg) were incubated with the SP-1 probe in a buffer containing 20 mM Tris, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 4% Ficoll, 3 μg poly(dIdC), and 1 ng probe at 4°C.

The dissociation rate of protein-DNA complexes were performed according to Hardy and Shenk (1989). The DNA-protein complexes were formed on the specific labeled probe under the reaction conditions as above for NF-IL6 or E2F

complexes without any competitors. The complex was incubated for one hour prior to addition of 300-fold molar excess of unlabeled identical probe. Equal portions of the reaction mixture were analyzed by EMSA as a function of time after addition of the unlabeled competitor DNA (Hardy and Shenk, 1989).

Competition with oligonucleotides, at a 100-fold molar excess unless otherwise indicated, and preincubation with antibodies were performed by incubation of extracts with oligonucleotides or antibodies at room temperature for 10 minutes. The probe was then added, and the mixture was incubated for twenty minutes. The bound DNA was separated from the free DNA by electrophoresis on a 6% nondenaturing polyacrylamide gel for NF-IL6 and SP-1 and 4% nondenaturing polyacrylamide gel for E2F complexes in 0.25 X 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 Cronex x-ray film (DuPont).

Oligonucleotides for Electrophoretic Mobility-Shift Assay

Synthetic DNA oligonucleotides were prepared using an automated DNA synthesizer (Applied Biosystems, Inc.), and purified by electrophoresis in an 8M urea-12% polyacrylamide gel. The DNA was eluted in a buffer containing 0.1 M NaCl, 10 mM Tris-Cl (pH 7.4), 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 H_2O, and quantified by A_{260}. Double-stranded oligonucleotides were 5'-end-labeled by T4 DNA polynucleotide kinase with [γ-^{32}P]ATP under standard conditions (Sambrook et al., 1989).

DNA probes used are as follows: (The E2F and other core recognition sequence of each DNA probe is shaded, while the NF-IL6 site is underlined).

E2 (*Nar*I-*Bssh*II) (-96 to -14)

5'-CGCCGGGTGTGGCCTCTGGAGATGACGTAGTTTC-
3'-GGCCCACACCGGAGACCTCTACTGCATCAAAAG-
-GCGCTTAAATTTGAGAAAGGGCGCGAAACTAGTCCTT-
-CGCGAATTTAAACTCTTTCCCGCGCTTTGATCAGGAA-
-TAGAGTCACGCG-3'
-TTCTCAGTGCGCGC-5'

E2M2 (-72 to -40) (NF-IL6 mutant)

5'-AGTTTTCGCGCTTAAATCTAAAGACGGGCGCGA-3'
3'-TCAAAAGCGCGAATTTAGATTTCTGCCCGCGCT-5'

E2M3 (-82 to -60) (NF-IL6* and E2F)

5'-GAGATGACGTAGTTTTCGCGCTT-3'
3'-CTCTACTGCATCAAAAGCGCGAA-5'

E2M4 (-59 to -29) (NF-IL6 and E2F)

5'-AAATTTGAGAAAGGGCGCGAAACTAGTCCTT-3'
3'-TTTAAACTCTTTCCCGCGCTTTGATCAGGAA-5'

E1 (-229 to -198)-(E2F) Hardy et al., (1989).

5'-CCATTTTCGCGGGAAACTGAATAAG-3'
3'-GGTAAAAGCGCCCTTTGACTTATTC-5'

NF-IL6 (IL-6 promoter) Akira et al., (1990).

5'-AGATTGTGCAATCT-3'
3'-TCTAACACGTTAGA-5'

CREB/ATF (Fibronectin) Dean et al. (1987).

5'-ACAGTCCCCCGTGACGTCACCCGGGAGCCC-3'
3'-TGTCAGGGGGCACTGCAGTGGGCCCTCGGG-5'

Octamer (Mouse Ig heavy chain enhancer) Schreiber et al. (1988)

5'-AGCAAACACCACCTGGGTAATTTGCAT-
3'-TCGTTTGTGGTGGACCCATTAAACGTA-
-TTCTAAAATAAGTTGAGGAT-3'
-AAGATTTTATTCAACTCCTA-5'

SP-1 (HIV LTR) Buh et al., (1989).

5'-GGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGC-3'
3'-CTCCGCACCGGACCCGCCCTGACCCCTCACCGCC-5'

AP-1 (Collagenase) Angel et al., (1987).

5'-GGATGTTATAAAGCATGAGTCACTCAGGGGCGCA-3'
3'-CCTACAATATTTCGTACTCAGAGAGTCCCCGCGT-5'

Antibodies and Recombinant Proteins

Polyclonal antisera directed against peptide 3 of NF-IL6, an 18-amino acid basic region, NH_3-CSKAKKTVDKHSDGYKIR-COOH, of NF-IL6 (α-NF-IL6; Akira et al., 1990); against carboxyl 177 amino acids of recombinant LAP (α-LAP, kindly provided by U. Schibler; Descombes et al., 1990); against a 14-Kd peptide, NH_3-AGPHPDLRTGGGGGGGA-COOH of C/EBP (α-14, kindly provided by S. McKnight; Landschulz et al., 1988); against recombinant c-fos (α-fos 2.2), against the amino terminal 131 amino-acid of c-fos (α-alu) and against recombinant c-jun (α-jun 2.2, all kindly provided by T. Curran; Abate et al., 1991; and Cohen and Curran, 1990) and monoclonal antibodies directed against the synthetic peptide of adenovirus E4 ORF6/7, NH_3-SRDLPPFETETGGY-COOH (α-E4, kindly provided by T. Shenk; Marton et al., 1990), against adenovirus DBP (kindly provided by R. Carrol) were used as indicated. Purified recombinant LAP protein (Descombes et al., 1990) was kindly provided by U. Schibler.

Ribonuclease protection assay

The RNase protection assay was carried out as described by Marton et al., (1990). Poly A (+) RNAs selected from 5 x 10^6 cells were hybridized with the specific RNA probe, digested with RNases A and T1, and separated on denaturing polyacrylamide gel. The E1A/E1B-specific probe contains the first 1833 bp of the Ad5 left end transcribed from a T7 RNA promoter. The RNAs were prepared from Jurkat cells which were uninfected, infected with Ad2 or *dl*312 at 20 pfu per cell, or transfected with pCMV-NFIL6(+) [NF-IL6+/*dl*312] or pCMV-NFIL6(-) [NF-IL6-/*dl*312] at 5 μg/10^6 cells, 8 hours prior to infection with *dl*312.

Indirect immunofluorescence microscopy

Infected or control cells were pelleted by centrifugation, washed three times with ice-cold PBS/BSA/Azide (phosphate buffered saline (pH 7.4), supplemented with 1 mM $MgCl_2$, 1% BSA [Bovine Serum Albumin, fraction V, Boehringer Mannheim] and 0.2% azide), and resuspended in PBS/BSA/Azide. Samples of cell suspensions, 1 x 10^5 cells in 100 μl, were then spun onto ethanol-cleaned glass slides at 200 rpm for 2 min in a Cytospin II centrifuge (Shandon-Southern). The attached cells were fixed in 95% methanol, 5% glacial acetic acid at -20°C for 20 minutes, and quickly rinsed in distilled water. The fixed cells were pre-incubated with PBS/BSA/Azide for 15 minutes, then incubated with 100 μl of diluted (1:500 dilution in PBS/BSA/Azide, pH 7.4) mouse monoclonal antibody against the adenovirus E2 DNA binding protein (kindly provided by R. Carroll) for 45 min at 37°C in a humidified chamber. After incubation, the slides were washed three times for 15 minutes each with PBS/BSA/azide. The slides were than incubated with 100 μl of diluted (1:1000 dilution in PBS [pH 8]) fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin G (IgG; kindly provided by R. Carrol) for 45 min at 37°C in a humidified chamber. The slides were washed two times in PBS/BSA/azide and once with 1X PBS (pH 7.8), for 20 min each time, and sealed in 2.5% w/v DABCO (1,4-diazabicyclo[2.2.2]octane, Sigma) in glycerol under cover slips. Photography under phase-contrast and UV microscopy was carried out with a Zeiss UV microscope.

III. Interleukin-6 enhances a cellular activity that functionally substitutes for E1A protein in transactivation.

Portion of this section was published in Proc. Natl. Acad. Sci. USA. 1991, **88**: 6472-6476.

ABSTRACT

An interleukin 6 (IL-6)-regulated cellular activity in HepG2 cells is found to functionally substitute for the transcriptional transactivator product of the adenovirus transforming gene *E1A* in transactivating E1A-dependent and E1A-responsive viral early genes. Mutant viruses deficient in *E1A* expression replicate in HepG2 cells. Induction with IL-6 leads to significant enhancement of synthesis of viral early E1B and E2ae mRNAs by >30-fold and increases viral replication to the wild-type levels. The E1A-substituting activity activates E1A-responsive promoters in transient transfection and this transcriptional activity is regulated by IL-6 induction. Formation of distinct protein-promoter complexes by binding of proteins in nuclear extracts prepared from HepG2 cells to the E1A-dependent *E2ae* promoter further supports the possibility that this activity may be a nuclear component in the IL-6 signal transduction pathway.

INTRODUCTION

Many viral transforming genes encode transcription regulators, among them the *E1A* gene product (E1A) of adenovirus (Ad) which regulates viral genes and a subset of cellular genes (for reviews, see Berk, 1986; and Flint and Shenk, 1989). Despite significant efforts, however, the mechanism of E1A action remains to be

elucidated. This is due at least in part to the lack of sequence specificity in genes regulated by the E1A proteins and to the strong cell-type dependence of E1A action. Furthermore, although the efficiency of transactivation by E1A appeared to be correlated with the levels of E1A mRNAs (Brunet and Berk, 1988), it also has been observed that replication of mutant viruses synthesizing very little E1A proteins was unabated in HeLa cells (Hitt and Graham, 1990). We have found that transactivation of E1A-dependent viral genes is as efficient in some cultured human lymphoid cells, when the E1A proteins are expressed at a level 1/50th of that of HeLa cells (Lavery and Chen-Kiang, 1990). Nevertheless, E1A is indispensable for the expression of Ad early genes in lymphoid cells (Lavery and Chen-Kiang, 1990) and in nearly all cell types so far studied.

It has been noted, however, that HeLa cells support a low level of replication of *dl*312, an *E1A*-deletion mutant virus, when the multiplicity of infection (moi) is high (Shenk et al., 1979). Whereas this activity in HeLa cells may be attributed to complementation by human papilloma virus type 18 integrated in HeLa cells and expressing its *E7* gene, which can substitute for *E1A* in transfection (Schneider-Gädicke and Schwarz, 1986; and Phelps et al., 1988), a cellular activity (or activities) that functionally substitutes for E1A protein has been described in mouse embryonal carcinoma F9 cells (Imperiale et al., 1984; and La Thangue and Rigby, 1987). The E1A-dependent *E2ae* promoter is active in F9 cells in transfection and correlates with the presence of E2F, a cellular factor that binds to the *E2* promoter (Reichel et al., 1987), and the formation of distinct *E2* promoter-protein complexes (Hardy et al., 1989; and Jansen-Durr et al., 1989). However, the activity in F9 cells diminishes

upon cellular differentiation (Reichel et al., 1987; and Jansen-Durr et al., 1989). A similar activity has been described in mouse oocytes and in preimplantation embryos (Dooley et al., 1990). The nature of these activities and whether they are one and the same, has remained obscure.

Here, I report a cellular activity in human hepatoblastoma HepG2 cells, which, in the absence of E1A, transactivates E1A-dependent viral genes in viral infection. This activity can be significantly increased by induction of HepG2 cells with the cytokine interleukin 6 (IL-6), resulting in full complementation in the replication of *E1A*-deletion mutant virus *dl*312. Regarding the two E1A-dependent promoters investigated, promoter elements of the *E2ae* and *E1B* genes, IL-6 regulates them in transfection, thereby confirming that the cellular activity functions at the transcriptional level. These results suggest that a component in the IL-6 transduction pathway may be the cellular activity that regulates the E1A-dependent promoters in the absence of E1A.

RESULTS

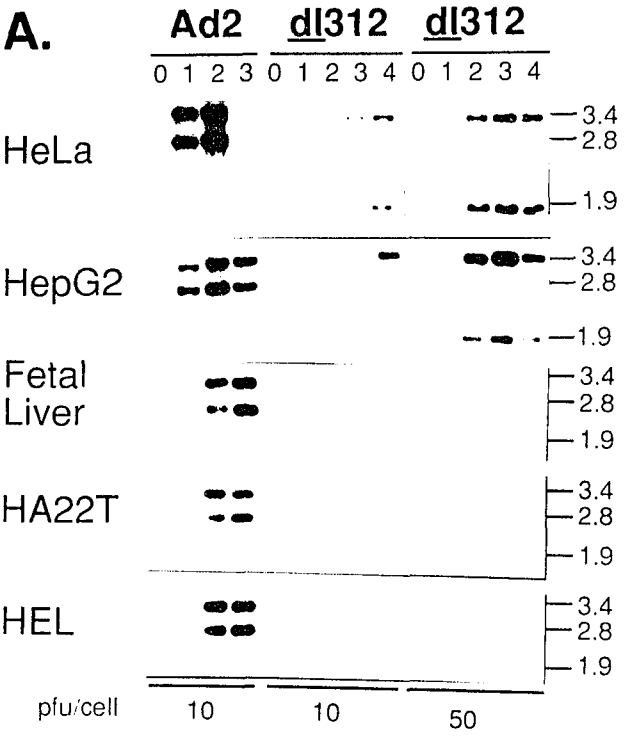
A Cellular Activity Functionally Substituting for E1A Is Present in HepG2 Cells. The E1A proteins are required for transactivation of all early genes of adenovirus. Thus *E1A* deletion mutants cannot express the E1A-dependent viral early genes and do not replicate in infected human cells with the exception of HeLa cells (see Introduction). However, the ability of E1A proteins to efficiently transactivate E1A-dependent viral early genes at very low protein concentrations in lymphoid cells (Lavery and Chen-Kiang, 1990) raises the possibility that in some

differentiated human cells other than HeLa cells, cellular activities may be sufficient to complement the transactivation function of E1A. Using replication of an *E1A*-deletion mutant virus *dl*312 as an assay, it is apparent that HepG2 cells possess an activity that functionally substitutes for E1A and permits replication of *dl*312 (Fig. 6A). As quantified by the intensity of distinct viral genomic fragments after digestion with restriction enzyme *Hind*III in Southern blot analysis, maximal replication of *dl*312, infecting at 10 pfu per cell, reached 5-10% of that of wild type virus, albeit after a 2-day delay. When the moi was increased from 10 to 50 pfu per cell, replication of *dl*312 was proportionally increased and approached that of the wild-type virus at 10 pfu per cell (See Table 4). This activity was confirmed by another E1A-mutant virus *dl*343. *dl*343 replicates in HepG2 cells in a similar fashion (data not shown). Equivalent levels of input virus were detected in all cell lines for both *dl*312 and *dl*343 (data not shown).

Since the activity in HepG2 cells appeared to resemble that in HeLa cells, which express integrated human papilloma virus 18, the possibility that HepG2 cells may also contain viral sequences known to complement E1A function was addressed. To exclude the possibility that the replication of *E1A* deletion mutants in HepG2 cells was due to the presence of endogenous *E1A* sequences, polymerase chain reaction (PCR) was used to detect *E1A* sequences (Figure 7). Using primers for the *E1A* coding region of Ad2/5, base pair 974- 993 for primer 1 for rightward reading and base pair 1521 - 1542 for primer 2, DNA from HepG2 cells, HeLa cells and 293 cells was assayed for the presence of adenovirus. The polymerase chain reactions were in the linear range within twenty cycles of chain elongation. In 293 cells, which

Figure 6: Replication of the E1A deletion mutant *dl*312 in HepG2 cells. (A) Total DNA was isolated from HepG2, HeLa, HEL, HA22T cells, and human fetal liver explants infected with Ad type 2 [10 plaque forming units(pfu) per cell] or *dl*312 (10 or 50 pfu per cell) at indicated days after infection, except for HeLa cells in which Ad2 infection was completed at 2 days after infection. The DNA was digested with restriction enzyme *Hind*III, separated on a 1% agarose gel, and analyzed by Southern blotting (Lavery and Chen-Kiang, 1990) with a ^{32}P-labeled probe as shown in (B). Each lane contained 1 μg of DNA (approximately 6 x 10^4 cells). (B) The *Hind*III restriction fragments of Ad2 and Ad5 *dl*312 encompass the E1A and E1B transcription units. *dl*312 (Jones and Shenk, 1979) contains a 901 bp deletion in the *E1A* coding region which renders the virus incapable of expressing E1A. *dl*343 (Hearing and Shenk, 1985) contains 2 bp deletion at 661-662 in the E1A coding region generating no functional E1A mRNA or protein. Ad-2 and Ad-5 are phenotypically indistinguishable and nearly identical in this region of the genome. The DNA probe is the insert of p3WT18, which contains the left-hand 5.8 kilobases (kb) of the Ad5 genome (Lavery and Chen-Kiang, 1990).

Figure 6


A.
Ad2
dl312
dl312
0 1 2 3 0 1 2 3 4 0 1 2 3 4
HeLa
HepG2
Fetal Liver
HA22T
HEL
3.4
2.8
1.9
pfu/cell
10
10
50

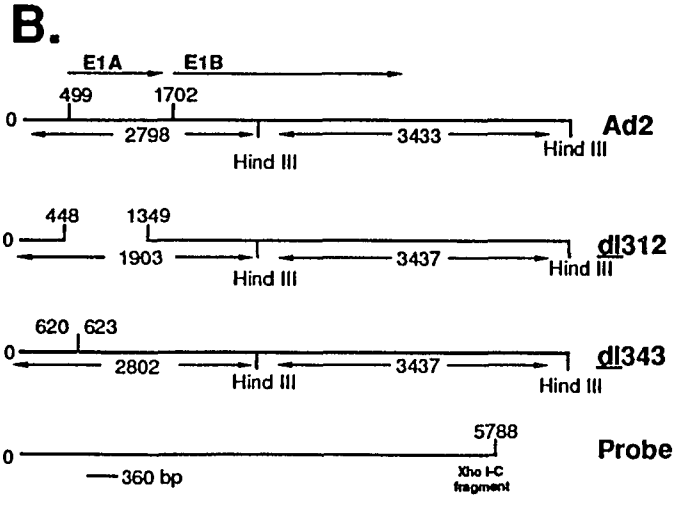
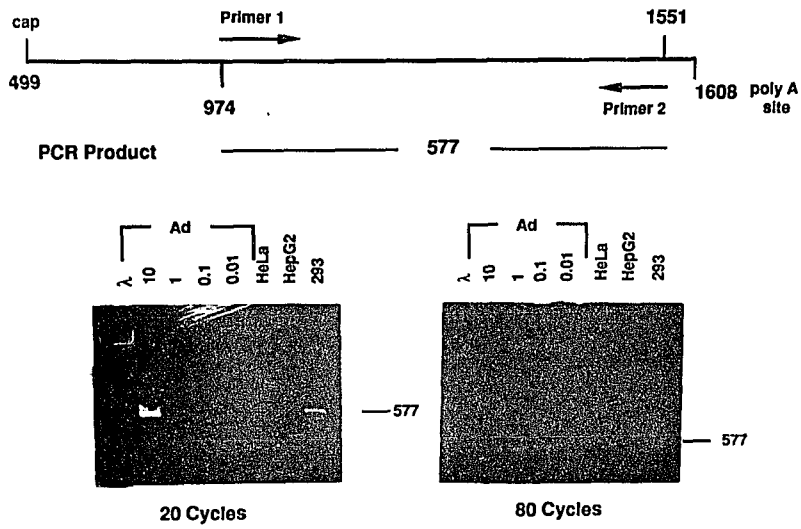

B.
E1A
E1B
499
1702
0
2798
3433
Hind III
Hind III
Ad2
448
1349
1903
3437
dl312
620
623
2802
dl343
5788
Probe
360 bp
Xho I-C fragment

Table 4: Induction of Viral DNA Synthesis by Cytokines

	Ad2	*dl*312
Uninduced	100	5.0
IL-6	550	55.0
Bt_2cAMP	490	10.0
TNF-α	145	6.5
TNF-α/IL-1	130	33.4

Relative replication of adenoviral DNA based on densitometry scanning of hybridization signals. The intensity of HepG2 infected with Ad2 at 72 hpi was arbitrarily defined as 100.

Figure 7: PCR analysis of endogenous *E1A* sequences. Total DNA from 10^5 293 cells, HeLa cells and HepG2 cells as indicated or, circular plasmid p3WT18 DNA corresponding to 10, 1, 0.1, or 0.01 copies of adenovirus DNA per cell as indicated was mixed with DNA from 10^5 HEL cells and analyzed by the polymerase chain reaction as described in Materials and Methods. The 577 bp expected reaction product is indicated. The end-products were analyzed on 1 % agarose gel with *Hind*III digested λ DNA as size markers and stained with ethidium bromide. The intensities of the ethidium bromide bands in the 20 cycle and 80 cycle gels were not comparable because the gels were run and stained separately.



contain integrated E1A and E1B sequences and served as the positive control, approximately 6 copies of E1A gene per cell were detected, consistent with results originally described by Graham et al. (1977). No E1A sequences were detected in HepG2 cells at 20 or 80 cycles of chain elongation, when the sensitivity of detection reached 0.1 copy per cell. Analyses with Southern blotting using probes for HPV and hepatitis B virus (HbV) which detected human papilloma virus sequence in HeLa cells and Caski cells harboring HPV type 16 and 18 or HBV, failed to detect any human papilloma virus in HepG2 cells (data not shown). This result is consistent with a report that HBV sequence were not detected in HepG2 (Aden et al., 1979).

The E1A-substituting activity was not present, however, in either liver explants, which retain differentiated liver functions or HA22T/VGH, another human liver cell line (Chang et al., 1983), because they did not support replication of *dl*312 (Fig. 6A). Replication of wild-type virus in these cells was identical to that in a human embryonic lung fibroblastic cell line, HEL, which served as a control. Taken together, these data suggest the existence of a cellular activity that functionally substitutes for E1A, is a property of HepG2 cells, and is not a liver-specific function.

E1A-Substituting Activity Is Regulated by IL-6. Among its pleiotropic effects, IL-6 induces cellular differentiation in a highly cell-type specific manner (for a review, see Hirano and Kishimoto, 1990). As in the case of induction of human B-cell differentiation by coordinated regulation of immunoglobulin genes at the level of transcription (Raynal et al., 1989), IL-6 induces acute phase genes in HepG2 cells transcriptionally (Gauldie et al., 1987), apparently in a promoter sequence-specific manner (Poli and Cortese, 1989; Majello et al. 1990; and Won and Bauman, 1990).

Relative to uninduced HepG2 cells, IL-6 clearly accelerated the onset of replication of *dl*312 virus by 1 day and increased the maximal replication 11-fold to levels approaching that of the uninduced wild-type virus (Fig. 8). Furthermore, enhancement of replication by IL-6 was not synergistic with E1A, since replication of wild-type virus was increased only 5.5-fold by IL-6 induction. Other cytokines were much less effective in enhancing replication of *dl*312. Bt_2cAMP and TNF-α increased *dl*312 replication by <3-fold (Fig. 8). These results showed that the requirement for E1A in viral replication can be compensated by an IL-6 regulated activity in HepG2 cells.

IL-6 Regulates the Synthesis of mRNAs Encoded by E1A-Dependent Viral Genes. Replication of *E1A* deletion mutants suggested, but did not prove, that the E1A-substituting activity in HepG2 cells functions by activating E1A-dependent viral genes transcriptionally. Analyses of mRNAs transcribed from E1A-dependent *E2ae* and *E1B* genes in the course of viral infection showed that the E1A-substituting activity in HepG2 cells functioned at the level of mRNA synthesis (Fig. 9). The mRNAs encoded by the *E1B* gene, which cooperates with E1A for transformation (Ruley, 1983) and is dependent on E1A for its transcription, were detectable in HepG2 cells 72 hr after infection with *dl*312, as compared with 24 hr after infection with Ad2 (data obtained from a 20-fold longer exposure of Fig. 9A, not shown).

Induction of *dl*312 infected HepG2 cells with IL-6 resulted in a >30-fold increase of E1B mRNA levels, such that 24 hr after infection the levels equaled that of wild-type Ad2 infection without IL-6 induction (Fig. 9B, compare lane 24+ of *dl*312 with lane 24- of Ad-2). Increases of E2ae mRNA levels by IL-6 induction in

Figure 8: Induction by IL-6 enhances replication of Ad2 and dl312. DNA isolated from HepG2 cells at the indicated hours after infection with Ad2 (10 pfu per cell) or *dl*312 (20 pfu per cell) was analyzed as described in Figure 5. Induction with IL-6 (100 units/ml), Bt_2cAMP (1 mM), or TNF-α (200 units/ml) were as described. "X" represents the relative length of exposure time of the autoradiographs.

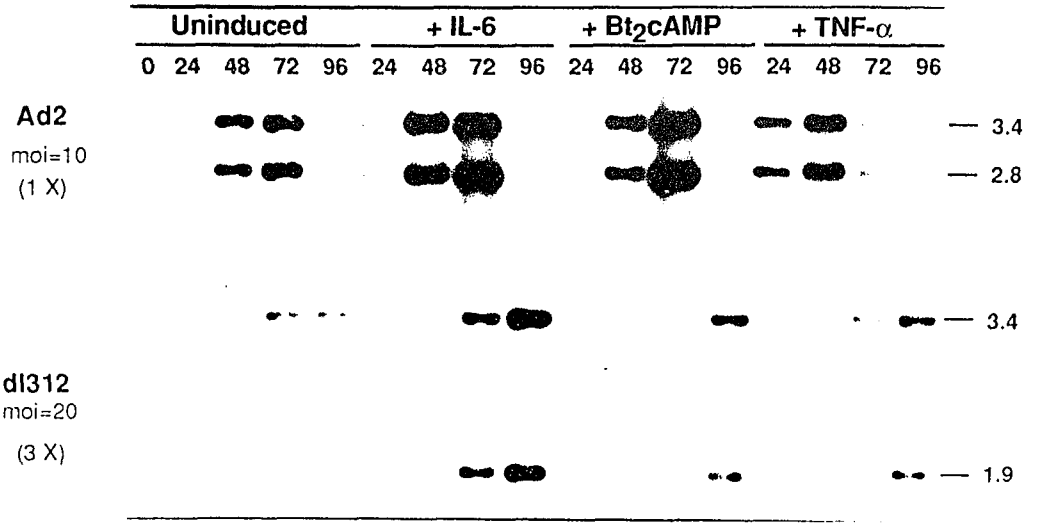
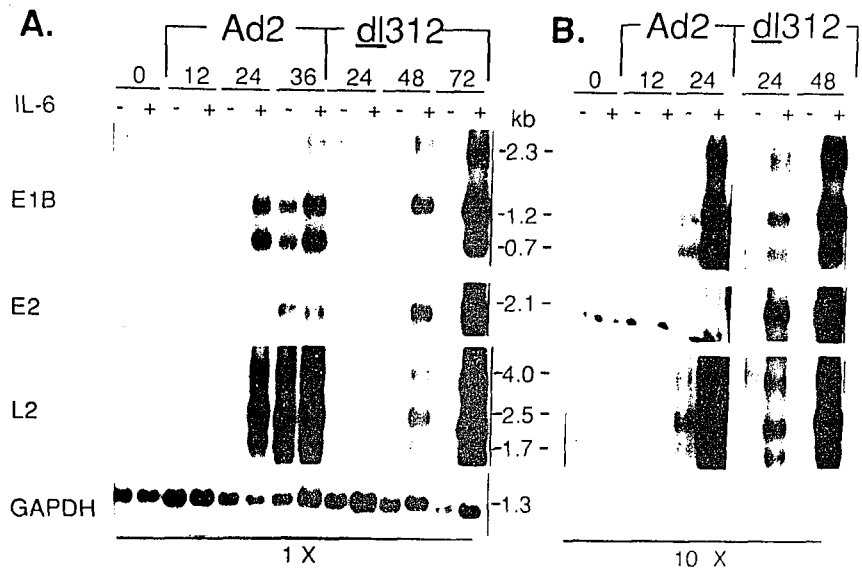


Figure 9: IL-6 enhances accumulation of mRNAs encoded by E1A-dependent viral genes. Poly(A)$^+$ RNA from 1.0 X 10^7 HepG2 cells was isolated at hours p.i. with Ad2 or *dl*312, with (+) or without (-) induction with IL-6 (500 units/ml) as indicated. The northern blot analysis with ^{32}P-labeled probe specific for GAPDH mRNA and adenovirus E1B, E2 and L2 mRNAs was performed as described (Lavery and Chen-Kiang, 1990). The size of the RNAs in kb is indicated. "X" represents the relative length of exposure time of the autoradiographs.



the absence of E1A, were even more dramatic to levels at least 5- to 10-fold higher than that of the wild-type Ad2 infection in the presence of E1A without IL-6 induction (Figs. 9 A and B, compare lane 24+ of *dl*312 with lane 24-of Ad-2). The mRNAs of a control cellular gene, *GAPDH* encoding glyceraldehyde-3-phosphate dehydrogenase, showed no appreciable difference after IL-6 induction. As expected, the mRNAs for the L2 family, transcribed from the major late promoter of adenovirus after viral DNA replication, were increased in proportional to the increases of E2ae and E1B mRNAs in IL-6 induction. As in the case of viral DNA replication, there appeared to be no synergy of E1A and IL-6 induction in viral early mRNA synthesis (Fig. 9A). Although IL-6 induced comparable increases of E1B mRNA levels in wild-type virus and in *dl*312 infected cells at 24 hr after infection, the IL-6 effect diminished markedly as viral infection progressed in Ad2 but not in *dl*312 (Fig. 9A, compare +/- lanes 24hr and 36hr of Ad-2).

IL-6 induction of early viral mRNA was confirmed with another E1A deletion virus infection, *dl*343, which contains a 2 base pair deletion (661-662) in the E1A codon region, resulting in a frame-shift in translation and no synthesis of functional E1A polypeptides (Figure 6B; Hearing and Shenk, 1985). The viral early mRNAs (E1B, E2, E3 and E4) were induced 10- to 15-fold by IL-6, a lesser degree than *dl*312 but greater stimulation than Ad2, wildtype (data not shown).

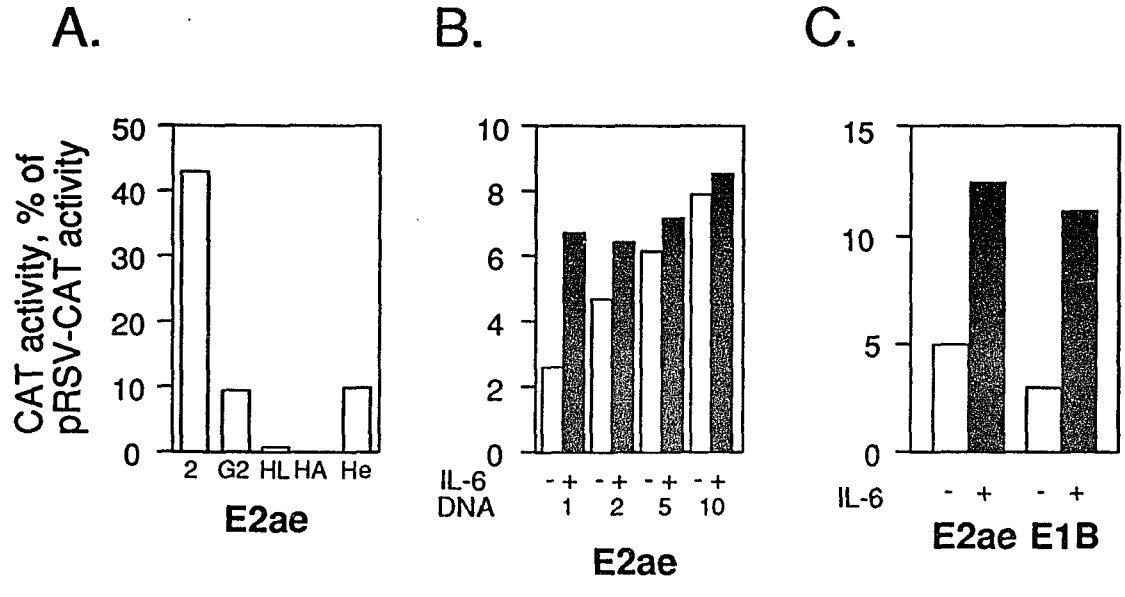
Together, these results provided strong evidence that, in their native chromosomal configuration, E1A-dependent viral early genes were activated for RNA synthesis by an endogenous activity present in HepG2 cells and that this activity was regulated in the IL-6 signal transduction pathway independent of E1A.

E1A-Substituting Activity Functions Transcriptionally. Increases in the accumulation of steady-state viral mRNAs by IL-6 induction was undoubtedly amplified by increases in the number of viral templates once viral replication began, even at the earliest times after infection consistent with yielding significant signals by Northern analysis. To address whether the endogenous E1A-substituting activity functions at the transcriptional level, promoters of *E2ae* and *E1B* were linked to the reporter gene encoding CAT and were assayed for their activity in cells with varying E1A-substituting activities. Correlating with their abilities to support the replication of *dl*312, HepG2 and HeLa cells activated pE2ae-CAT expression to 25% of that of 293 cells-human embryonic kidney cells transformed with and constitutively expressing the *E1A* and *E1B* genes (Fig. 10A). HA22T and HEL cells, in which *dl*312 does not replicate, did not support significant *E2ae* promoter activity (Fig. 10A). Induction of HepG2 cells with IL-6 resulted in a reproducible 2- to 3-fold increase in the *E2ae* promoter activity, and this increase was inversely related to the amount of pE2ae-CAT transfected (Fig. 10B and 10C). Likewise, the activity of the *E1B* promoter was increased 3- to 4-fold by IL-6 induction (Fig. 10C). Thus, the endogenous activity in HepG2 cells functionally substitutes for E1A by transcriptionally activating E1A-dependent early viral promoters.

Proteins in HepG2 Nuclear Extracts Form Distinct Complexes with E1A-Responsive Promoters. Transcriptional activation by the E1A-substituting activity in HepG2 cells suggested that nuclear factors in HepG2 cells may bind to E1A-responsive promoters and form DNA-protein complexes different in molar representation or composition from those formed by nuclear proteins in other cells.

Figure 10: Activation of *E2ae* and *E1B* promoters in HepG2 cells by IL-6 induction.

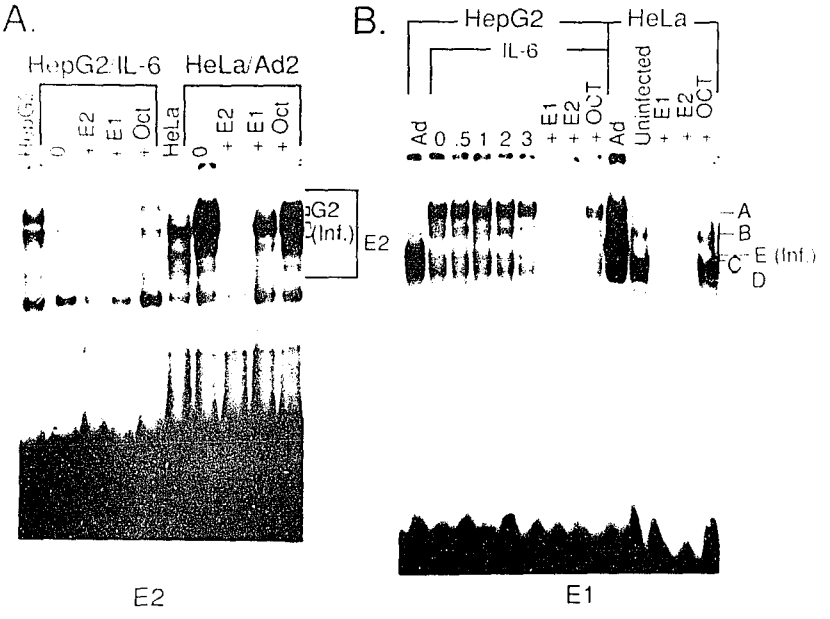
(A) 293 (2), HepG2 (G2), HEL (HL), HA22T (HA), or HeLa (He) cells were transfected with pE2ae-CAT DNA (6 μg per 6 x 10^5 cells). (B) HepG2 (6 x 10^5) cells were transfected with increasing micrograms of pE2ae-CAT DNA as indicated, with (+) or without (-) induction by IL-6 (500 units/ml). (C) HepG2 (1 x 10^6) cells were transfected with 1 μg of pE2ae-CAT (E2ae) or pE1B-CAT (E1B) DNA with (+) or without (-) IL-6 induction. The cells were harvested 48 hr after transfection and were assayed for CAT activity as described.



This was shown to be the case, at least in an in vitro binding assay (Fig. 11) in which binding of nuclear factors including E2F, thought to be important for the function of E1A-responsive *E2ae* promoter and the *E1A* promoter, was optimized (Hardy and Shenk, 1989). Regarding the *E2ae* promoter, as represented by the *E2* probe, which contains two E2F-binding sites, proteins present in HepG2 nuclear extracts formed complexes similar to those characterized in HeLa nuclear extracts (Fig. 11A, lane HeLa; Hardy and Shenk, 1989), except for a greater molar representation of a slowly migrating complex (Fig. 11A, G2 complexes in lanes HepG2 and HepG2/IL-6). These complexes were specific to the *E2*-probe and were not blocked by competition with a 100-molar excess of an E1A promoter probe, which contains two E2F binding sites (E1), or by a DNA probe containing the octamer sequence (lane Oct). Whether the G2 complexes formed in the presence of HepG2 and HeLa nuclear extracts are identical in composition remains to be examined. The G2 complex of HepG2 cells differed from the complexes formed in the presence of Ad-infected HeLa nuclear extracts (Figure 11, INF complexes in lanes HeLa/Ad; Hardy et al., 1989; Jansen-Durr et al., 1989; and Huang and Hearing, 1989) and disappeared upon IL-6 induction, with the concomitant appearance of a more slowly migrating complex (Figure 11A).

With respect to the promoter of the *E1A* gene, as represented by the E1 probe, which contains one E2F site, formation of complex A was favored in the binding of proteins in HepG2 nuclear extracts (Fig. 11B, lanes IL-6), as compared with the predominant presence of complexes C and D, which resulted from binding of proteins in HeLa nuclear extracts (Fig. 11B, lane uninduced; Hardy and Shenk,

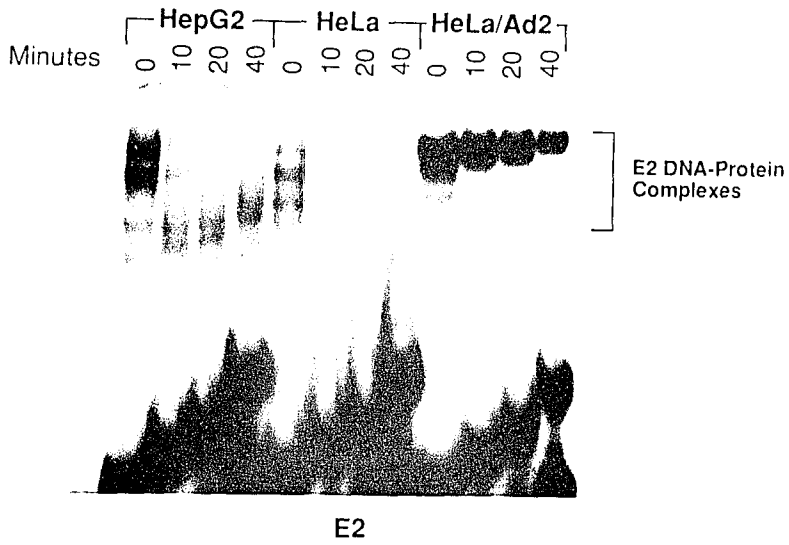
Figure 11: Proteins in HepG2 and HeLa nuclear extracts bind to E2ae and E1 promoters differently. (A) Nuclear extracts were prepared from HepG2 cells, without or with IL-6 induction (500 units/ml for 2 days) and from HeLa cells without or with Ad-2 infection (18 hr after infection with 10 pfu per cell). In lanes 0, extracts were not preincubated with competitors. (B) Nuclear extracts were prepared from HepG2 and HeLa cells 24 and 18 hr, respectively, after Ad-2 infection with 10 pfu per cell (lanes Ad), from uninfected HeLa cells (HeLa), from HepG2 cells induced with IL-6 (500 units/ml) for the number of day(s) indicated. The *E2* probe (A) and *E1* probe (B) used for binding and competition, as well as the octamer oligomer (Oct) used for competition were as described in Materials and Methods. "+" represents competition with oligomer as indicated. G2 and INF indicate HepG2 cell-specific and adenovirus infection-specific protein-DNA complexes, respectively. A-E, protein-DNA complexes.



1989). Infection of HepG2 cells with adenovirus resulted in the diminution of complex A (Fig. 11B, lane HepG2 Ad) and the appearance of complexes distinct from those in adenovirus infected HeLa cells (Fig. 11B, lane HeLa Ad; Hardy and Shenk, 1989). These results correlated transcriptional activation of E1A-responsive promoters by an endogenous activity in HepG2 cell with formation of specific protein-E1A-responsive promoter complexes by proteins in HepG2 nuclear extracts. Further studies were undertaken to differentiate the protein-DNA complexes seen in HepG2 cells and HeLa cells.

The stability of these protein-DNA complexes was examined by the dissociation of protein-DNA complexes (Figure 12). The dissociation rate for the protein-DNA complexes in HepG2 cells and HeLa cells for the E2 probe were nearly identical, while adenoviral infected DNA-protein complexes were significantly more stable, confirming previous reported results in HeLa and HeLa-adenovirus infected cells (Hardy et al., 1989; Marton et al., 1990, Leza and Hearing, 1989; and Babiss 1989). The differences in the mobility of the various samples is due to the variation of electrophoretic time of each sample. The increase in stability of the adenovirus infected protein-DNA complex has been identified to be from the interaction of E4 ORF6/7 gene product with E2F transcription factor (Marton et al., 1990, Leza and Hearing, 1988; and Babiss, 1989). Antibodies studies confirmed the previous published work of Marton et al., (1990) and others that E4 ORF6/7 was present in HeLa/Ad complexes and not present in uninfected cells (data not shown).

Figure 12. Dissociation rate of E2 promoter-complexes. Nuclear extracts were prepared from HepG2 cells, and from HeLa cells without or with Ad-2 infection (18 hr after infection with 10 PFU per cell). The DNA-protein complexes were formed on ^{32}P labelled probe, then 300-fold molar excess of unlabeled DNA identical to the probe was added. The dissociation rate was observed as samples were taken for analysis at the times indicated and loaded onto a running gel.



DISCUSSION

Two conclusions can be drawn from this study: (i) that an endogenous activity that can transcriptionally activate E1A-dependent viral promoters in the absence of E1A exists in HepG2 cells and (ii) that this activity is enhanced by IL-6. Transcriptional activation by this E1A-substituting activity leads to synthesis of viral early mRNAs and replication of viral DNA without the participation of *E1A* gene products. The >30-fold enhancement of accumulation of viral early mRNA and the 10- 15-fold increases in viral DNA replication elicited by IL-6 apparently have their molecular basis in the activation of transcription of E1A-dependent viral early genes. Thus, the E1A-dependence of viral early genes, which provides a severe constraint for expression of viral early genes and prevents the virus from replicating and entering the late cycle without E1A, can be abrogated by IL-6 signaling. This raises the question of the role of IL-6 in activating otherwise negligible expression of E1A-dependent and E1A-responsive genes physiologically. For example, in lymphoid cells, the most likely primary sites and carriers of adenovirus infection, the kinetics of adenovirus infection are markedly delayed relative to those in HeLa cells because of post-transcriptional regulation of *E1A* gene expression itself (Lavery et al., 1987; and Lavery and Chen-Kiang, 1990). The *E1A* gene product has been shown to transactivate other viral and cellular genes. In those lymphoid cells with sufficient IL-6 receptors on their surface, can IL-6 enhance and accelerate infections by adenovirus and by other viruses?

Although the cDNAs encoding the two polypeptides comprising the high-affinity IL-6 receptors have been cloned (Yamasaki et al., 1988; and Hibi et al.,

1990), the IL-6 signal transduction pathway is largely unknown. Signaling by IL-6 apparently leads to rapid and transient tyrosine phosphorylation of a 160kDa cellular protein and activation of transcription of *ts11* and *jun-B* genes in mouse B-cell lymphoma cells (Nakajima and Wall, 1991). Two results suggest that the E1A-substituting activity may be a nuclear component in the IL-6 signaling pathway: it functions transcriptionally and the patterns of protein-DNA complexes formed by HepG2 nuclear proteins with E1A-responsive promoters differ from those formed by HeLa nuclear proteins. The activity in HepG2 cells appears not to segregate with differentiated liver functions. However, it is enhanced by IL-6 induction, which also induces acute phase genes in the liver and in cultured cells of liver origin. Is the E1A-substituting activity expressed in HepG2 cells due to cellular transformation, since HepG2 cells are derived from a hepatoblastoma? Can IL-6 induce this activity in cells not expressing the E1A-substituting activity? These questions and the question of whether the activity in HepG2 cells is related to the E1A-substituting activities expressed in early mouse development and embryonal carcinoma cells remain to be investigated.

Other cytokines such as cAMP and phorbol 12-myristate 13-acetate (PMA) have been shown to function in synergy with E1A (Engel et al., 1988; Müller et al., 1989; and Buckbinder et al., 1989) or independent of E1A (Leza and Hearing, 1989) in a highly cell type-dependent manner. However, the participation of E1A is indispensable for the activity of E1A-dependent promoters during cAMP induction (Engel et al., 1988; and Müller et al., 1989). While PMA has been shown to replace the requirement of E1A in activating one of the E1A-responsive viral promoters, the

EIII promoter, the TPA action is synergistic with E1A (Buckbinder et al., 1989). In this regard, the magnitude of enhancement of mRNA synthesis from E1A-dependent viral early genes (Fig. 9), the full complementation of an E1A deletion mutant in viral replication by IL-6, (Fig. 7), and the lack of synergy with E1A in IL-6 induction (Fig. 10) suggest that there is an IL-6 regulated activity present in HepG2 cells that has not yet been described before.

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

Portion of this chapter was published in Journal of Virology, (1992) **66**: 1021-1030.

ABSTRACT

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 recently described (Spergel and Chen-Kiang, 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 concentration 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 *dl*312 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.

INTRODUCTION

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 Berk, 1986; Flint and Shenk, 1989; and Rochette-Egly et al., 1990). Although structural analyses have yielded insights into domains important for the multiple functions of the E1A gene products (Moran and Mathews, 1987), the molecular mechanism of E1A action has remained elusive. It has been suggested that E1A proteins function in synergy with cyclic AMP (cAMP) (Engel et al., 1988; Lin and Green, 1988; and Müller et al., 1989); through TFIID (Wu et al., 1987, and Kao et al., 1990) or ATF-2 (Liu and Green, 1990); or by sequestering repressors in the cell (Bagchi et al., 1990). E1A also enhances RNA polymerase III transcription by activating transcription factor IIIC (Hoeffler et al., 1988). 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 (Hen et al., 1985; and Borrelli et al., 1986). Although many cellular proteins have been shown to be associated with the E1A polypeptides, most notably the retinoblastoma gene products (Whyte et al., 1988) and cyclins (Giordano et al., 1989), 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 (Imperiale et al., 1984; and La Thangue and Rigby, 1987), in oocytes, and during early development in preimplantation embryos (Dooley et al., 1990). 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 also 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 IL-6, resulting in full complementation of infection of E1A deletion viruses (Spergel and Chen-Kiang, 1991).

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 Hirano and Kishimoto, 1990). Among its many differentiating 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 (Raynal et al., 1989). 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 (Akira et al., 1990). It recognizes a consensus sequence

present in nearly all IL-6-inducible genes, 5'-T[T/G]NNGNAA[T/G]-3', and transactivates promoters linked to this sequence in transfection. Here, I 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.

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 (Spergel and Chen-Kiang, 1991) and the lack of this activity in other human cells, including lymphoid cells which require very low levels of E1A for productive infection (Lavery and Chen-Kiang, 1990), 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 15-20 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. 13A). Jurkat cells do not express the 80kDa IL-6 receptor (Yamasaki et al., 1988) or an E1A-substituting activity, but can support productive adenovirus infection in the presence of E1A (Lavery et al., 1987).

In agreement with regulation of both NF-IL6 (Akira et al., 1990) and an E1A-substituting activity in HepG2 cells by IL-6 (Spergel and Chen-Kiang, 1991), the

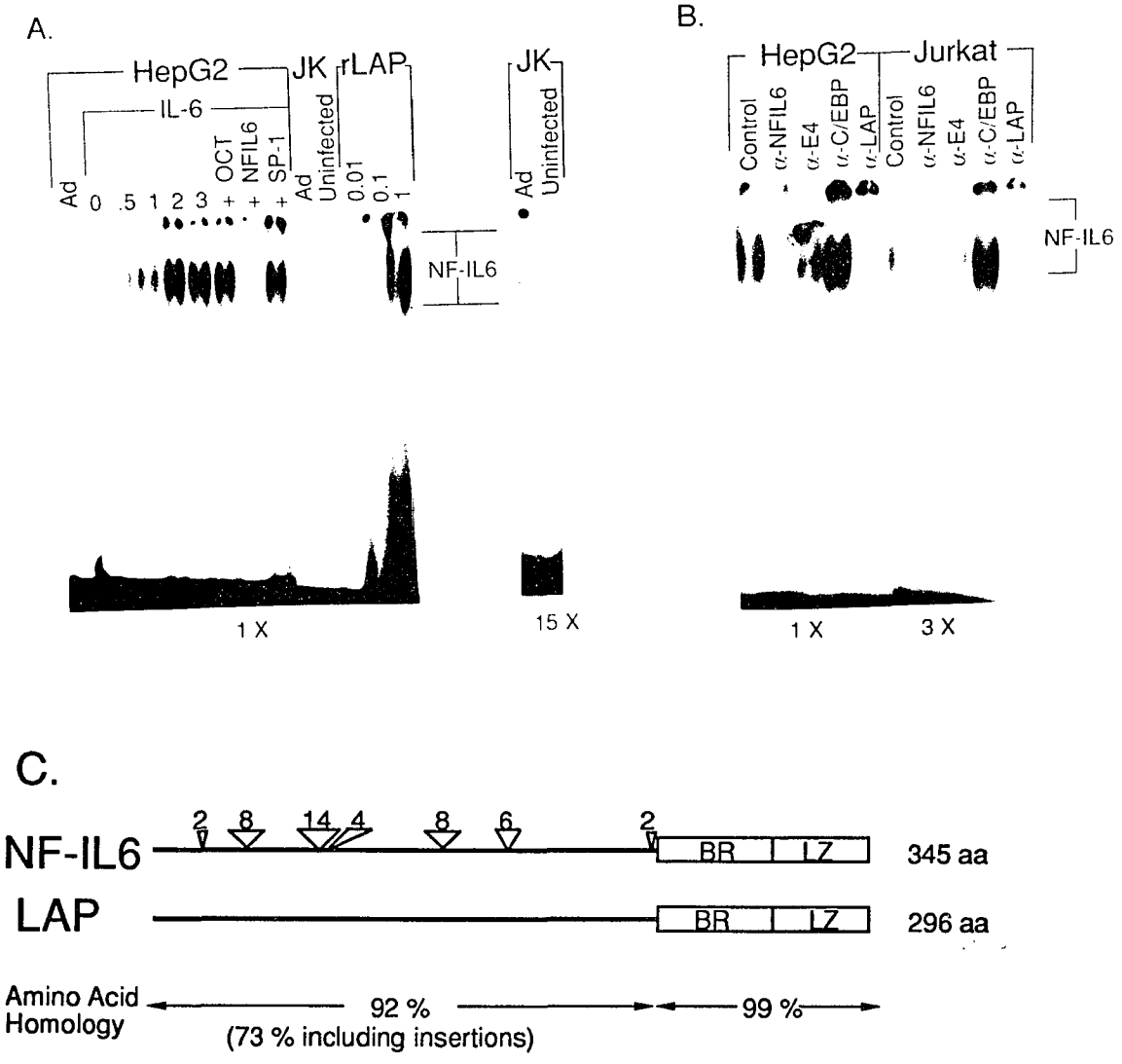
formation of DNA-protein complexes was increased by two to threefold in extracts prepared after IL-6 induction. The complexes formed were specific, since they were not inhibited by DNA of unrelated sequences, and they comigrated with those formed with recombinant LAP (rLAP), a reference protein, on the same NF-IL6 probe. LAP (Descombes et al., 1990), also known as IL-6 DBP and AGP/EBP (Chang et al., 1990; and Poli et al., 1990), is a rodent transcription factor of the C/EBP family that is inducible by IL-6 (Poli et al., 1990). Highly homologous in its amino acid sequence and nearly identical in its DNA-binding and leucine zipper domains to NF-IL6, it is most probably the rodent homolog of human NF-IL6 (Fig. 13C).

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 19kDa polypeptide product of the adenovirus E4 gene, and α-C/EBP, a peptide antiserum specific for the rat C/EBP, did not. In fact, α-C/EBP reproducibly enhanced protein-NF-IL6 complex formation (Fig. 13B). Although the significance of this enhancement requires further investigation, C/EBP appeared not to be a component of protein-NF-IL6 complexes at equilibrium. Whether these results suggest that C/EBP dimerizes with NF-IL6, and that α14 inhibits this dimerization, thereby allowing more NF-IL6 to bind to the NF-IL6 DNA probe, remains to be determined. 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.

Further characterization of the protein-DNA complex involved examination

Figure 13: 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 days indicated post-induction, as well as from Ad-2 infected HepG2 and Jurkat cells (10 pfu per cell) at 24 and 48 hr post-infection, respectively. The oligomer probes used for binding and competition were as described in Materials and Methods. 3 μg of nuclear extracts or 0.01, 0.1 or 1 μg of purified rLAP was used 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 time of the autoradiographs. (C) Schematic diagram of NF-IL6 and LAP protein structures. Abbreviations: BR, basic region; LZ, the Leucine zipper domain; aa, amino acids.

Figure 13

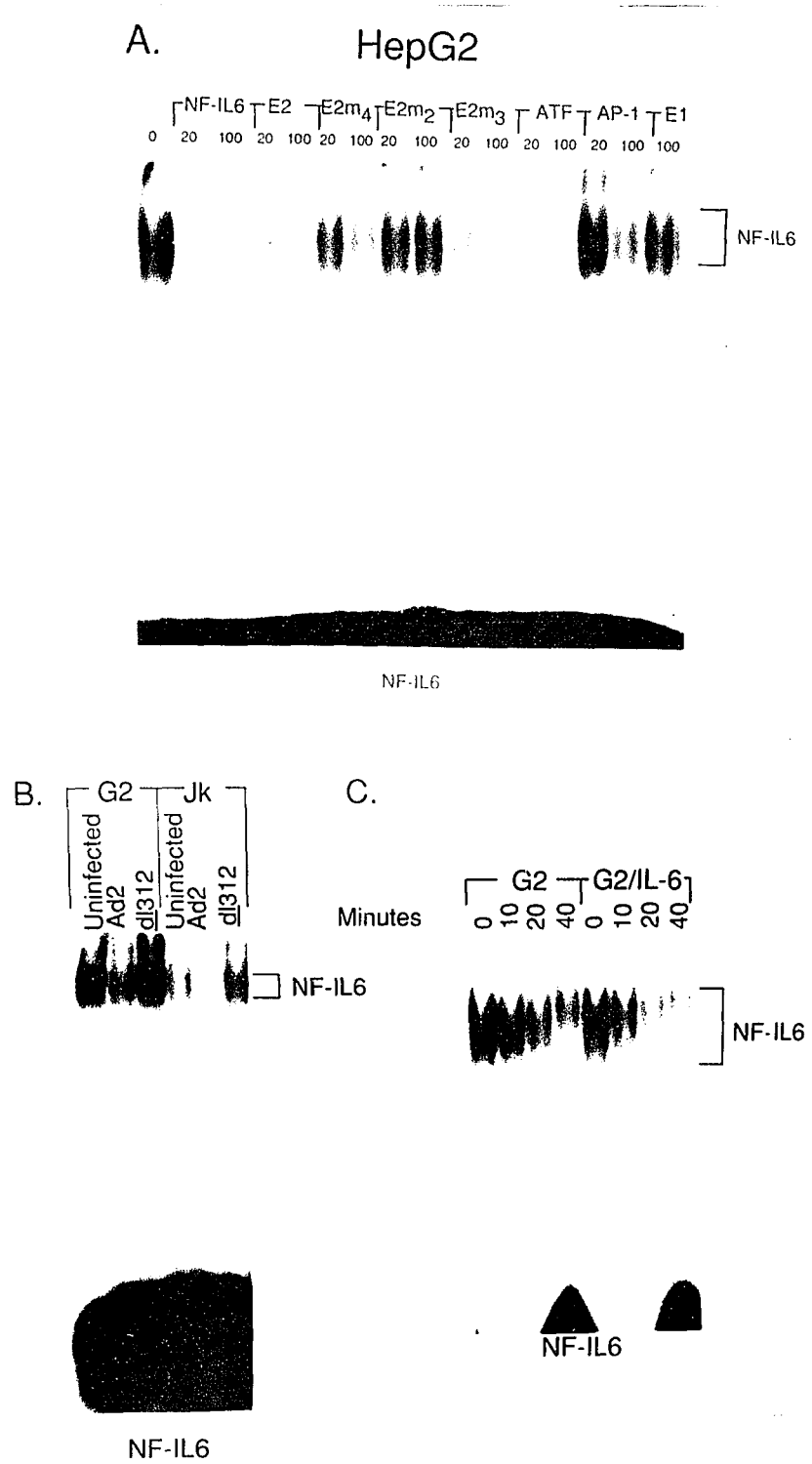

A.
HepG2
JK
rLAP
JK
IL-6
Ad
0 .5 1 2 3
OCT
NFIL6
SP-1
Ad
Uninfected
0.01
0.1
1
Ad
Uninfected
NF-IL6
1 X
15 X
B.
HepG2
Jurkat
Control
α-NFIL6
α-E4
α-C/EBP
α-LAP
Control
α-NFIL6
α-E4
α-C/EBP
α-LAP
NF-IL6
1 X
3 X
C.
NF-IL6
2 8 14 4 8 6 2
BR
LZ
345 aa
LAP
BR
LZ
296 aa
Amino Acid Homology
92 %
(73 % including insertions)
99 %

of the specificity of the protein-DNA complex, in particular in reference to the *E2ae* promoter. Different oligonucleotide probes compete with varying affinity to the NF-IL6 protein. The identical oligomer, NF-IL6, had the highest affinity for the NF-IL6 DNA protein complexes competing at 20-fold molar excess, while the oligomers for E1[E2F], OCT and SP-1 consensus sequences did not complete with NF-IL6 DNA-protein complex at 100 molar excess of oligomers (Figures 13A and 14A). The NF-IL6 site on the E2 probe (E2) also competes but to a lesser degree than the NF-IL6 consensus probe. NF-IL6* site is a 9/10 match with the consensus NF-IL6 motif (TGACGTAGT: mismatch nucleotide is underlined). Interestingly, the NF-IL6* site ($E2m_3$) has a higher affinity to the NF-IL6 protein than the NF-IL6 site ($E2m_4$) even though the NF-IL6 site has higher homology to the consensus recognition sequence. The binding was specific to the NF-IL6 site as $E2m_2$, a probe with the NF-IL6 site mutated did not compete with the NF-IL6 probe at 100-fold molar excess (Figure 14A). It was interesting to note that both AP-1 site from the collagenase gene and ATF/CREB site from the fibronectin promoter can also complete with NF-IL6 site with varying affinity for the NF-IL6 probe. NF-IL6* site overlaps extensively with the ATF/CREB site in the *E2* promoter to which both AP-1 and ATF can bind. A possible explantation for the higher affinity of $E2m_3$ (NF-IL6*) probe for the NF-IL6 versus $E2m_4$ (NF-IL6) probe could be the presence of ATF sites in the $E2m_3$ (NF-IL6*) probe. These results predict that NF-IL6 binds to either the ATF or AP-1 consensus sequence or that ATF and AP-1 are part of the NF-IL6 DNA-protein complex.

Since wildtype infection lead to a decrease in DNA-binding of NF-IL6 protein

Figure 14. Binding of proteins in nuclear extracts to NF-IL6 consensus sequence, (see page 33). (A) Nuclear extracts were prepared from HepG2 as described in Material and Methods. Competition by preincubating the extracts with the oligonucleotides for ten minutes prior to incubation with [γ-P^{32}]NF-IL6 probe at the indicated molar excess of oligonucleotides. (B) Nuclear extracts were prepared from wildtype-infected HepG2 (G2) cells or Jurkat (JK) cells, (10 pfu/cell) at 12 hr pi and 24 hr pi, *dl*312-infected HepG2 (G2) cells or Jurkat (JK) cells, (20 pfu/cell) at 48 hr pi and 72 hr pi, respectively. (C) Nuclear extracts were prepared from IL-6 induced HepG2 cells, at 500 units/ml at 48 hours post-induction. The dissociation rate constant for protein-DNA complexes were measured at minutes after addition of unlabeled NF-IL6 probe at 300 molar excess as indicated as described in Materials and Methods.

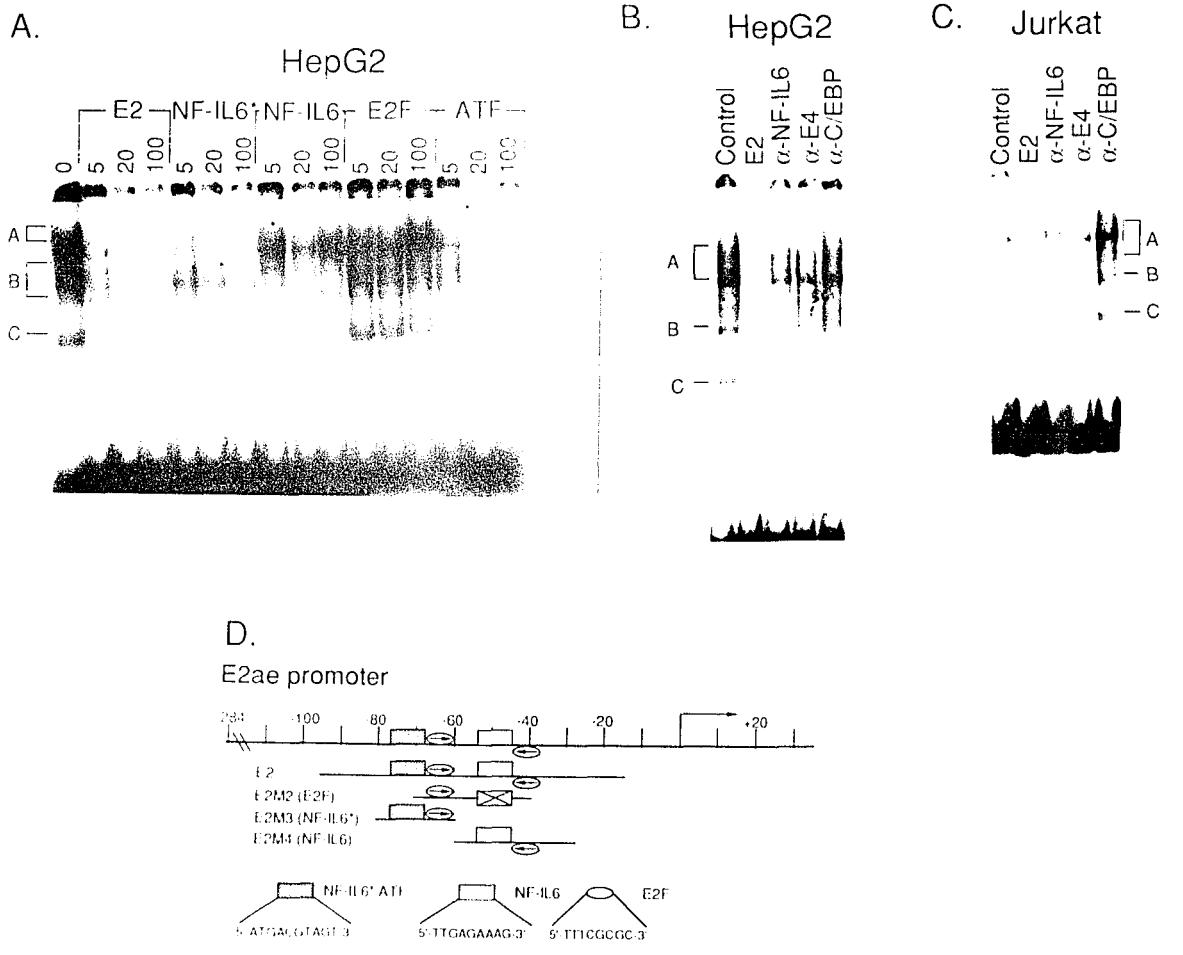
Figure 14


A.
HepG2
NF-IL6
E2
E2m4
E2m2
E2m3
ATF
AP-1
E1
0 20 100 20 100 20 100 20 100 20 100 20 100 20 100 100
NF-IL6
NF-IL6
B.
G2
Jk
Uninfected
Ad2
dl312
Uninfected
Ad2
dl312
NF-IL6
C.
Minutes
G2
G2/IL-6
0 10 20 40 0 10 20 40
NF-IL6
NF-IL6
NF-IL6

complex in HepG2, HeLa and Jurkat cells (Figures 13 and data not shown), the question was raised if this action was due to E1A. Therefore, HepG2 and Jurkat cells were infected with *dl*312 or wildtype virus [Ad2] and the formation of protein-NF-IL6 probe complexes was assayed. The wildtype infected nuclear extracts prepared from HepG2 and Jurkat cells showed a decrease in the intensity of the protein-DNA complex (Figure 14B), while *dl*312-infected nuclear extracts from either Jurkat or HepG2 cells did not diminish protein-DNA complex under optimal NF-IL6 binding conditions. This result is suggestive that E1A is responsible for diminution in binding to the NF-IL6 probe after infection through either indirect or direct actions. Examination of the dissociation rate of the NF-IL6 DNA complex revealed no significant difference in the off rate for the protein-DNA complex generated by the nuclear extracts from HepG2 cells: uninduced or induced with IL-6 (Fig. 14C).

To investigate the participation of the two NF-IL6 sites in the formation of E2ae promoter-protein complexes, the formation of *E2ae*-promoter complexes was analyzed by bandshift assay using HepG2 nuclear extracts and the E2 probe as shown in Figure 15. Specific oligonucleotides were used to compete for binding (Figure 15). Competition with an oligonucleotide containing a mutated NF-IL6 site (E2F/E2m$_2$), indicated that it did not compete and E2F, thought to be essential for promoter function, was not part of the complex (Figure 15A). Furthermore, competition with NF-IL6* and NF-IL6 site-specific oligonucleotides (E2m$_3$ and E2m$_4$, respectively) suggested that complex C was formed preferentially with the NF-IL6 site while complex A preferentially involved the NF-IL6* site (Figure 15A). Competition with an ATF oligonucleotide abolished the formation of these complexes, suggesting that

Figure 15: NF-IL6 forms specific protein-DNA complexes with the E2ae promoter. (A) 2 μg of nuclear extracts prepared from HepG2 cells were incubated with the E2 probe in the absence (0) or in the presence of unlabeled oligomers at 5, 20 or 100-fold molar excess as indicated. The protein-DNA complexes were arbitrarily marked as A, B, and C. (B and C) 2 μg of nuclear extracts prepared from HepG2 cells (B) or Jurkat cells (C) were preincubated with antisera as indicated, prior to binding to the E2 DNA probe. (D) A schematic diagram of the E2 probe used for binding and oligomers used for competition in (A). The arrow indicates the initiation site of transcription, the crossed box indicates mutation of the NF-IL6 site in the E2M2 probe.



ATF or the related transcription factor AP-1 may also participate in these complexes (Figure 15A). This result is consistent with competition of ATF for NF-IL6* site due to overlapping recognition sequences. It can not be excluded, however, that the ATFprobe in molar excess may sequester NF-IL6 protein and prevent it from binding to the E2 promoter at equilibrium. The lack of reduction of complexes A and B in the presence of α-C/EBP, or α-NF-IL6 suggested that neither NF-IL6 nor C/EBP was essential for their formation (Fig. 15B and C). However, antisera directed against c-fos caused a slight retardation of migration of Jurkat- and HepG2-specific complex A (data not shown) suggesting that AP-1 may be part of complex A.

It is apparent that α-NF-IL6 abolished the formation of the complex C indicating that NF-IL6 is part of this complex (Figures 15B and 15C). Consistent with the results obtained with the NF-IL6 DNA probe (Figure 13), the control serum α-C/EBP or α-E4 did not inhibit this complex. The pattern of the binding of proteins in Jurkat nuclear extracts to the *E2ae* promoter probe differs from that derived from HepG2 cells (Figures 15B and 15C). Complex B was absent or below the level of detection in nuclear extracts derived from Jurkat cells (Figure 15C). Although no functional correlation can be drawn at the present time, the results indicate a cell-type difference in the formation of *E2ae*-promoter complexes.

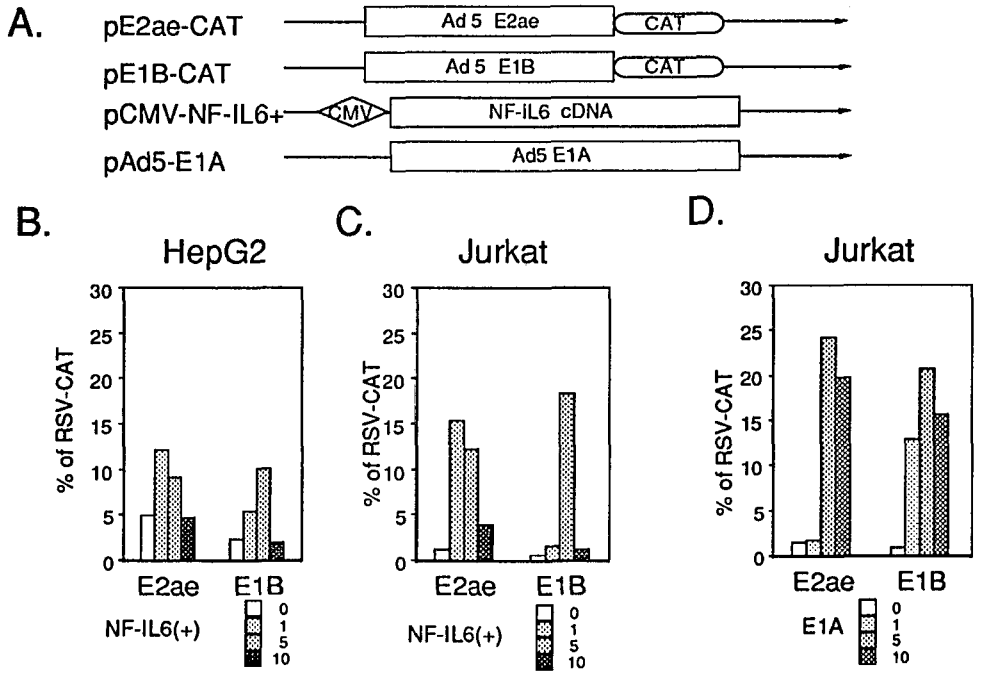
NF-IL6 transactivates E1A-responsive promoters in a concentration-dependent manner. There is no sequence homology between E1A and NF-IL6. Although E1A activates genes without apparent sequence specificity, NF-IL6 and LAP bind to specific sequences in the promoters and activate target cellular genes (Akira et al., 1990, and Descombes et al., 1990). The NF-IL6 recognition sequence is found to be

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. 13A), 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-NF-IL6(+), 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. 16). It is clear that when expressed at lower concentrations [1 to 5 μg of pCMV-NF-IL6(+) transfected into 10^6 cells], NF-IL6 transactivated *E2ae* and *E1B* promoters in both cell lines (Fig. 16B 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. 16C 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. 16C and D).

Thus, although the magnitude of transactivation by NF-IL6 is cell type dependent, NF-IL6 can transactivate E1A-responsive promoters in transfection,

Figure 16: 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 μg of pE2ae-CAT or pE1B-CAT DNA per 10^6 cells and varying amounts of pCMV-NF-IL6(+) DNA as indicated. In panel B the calcium phosphate precipitation method (Graham and van der Eb, 1973) was used to transfect HepG2 cells and in panel C the electroporation method was used for Jurkat cells (Potter et al., 1984). (D) pAd5E1A DNA was cotransfected, at varying amounts as indicated, with 1 μg of pE2ae-CAT or pE1B-CAT per 10^6 cells into Jurkat cells as in panel C. The cells were harvested at 48 hours after transfection and assayed for CAT activity as described in Material and Methods.

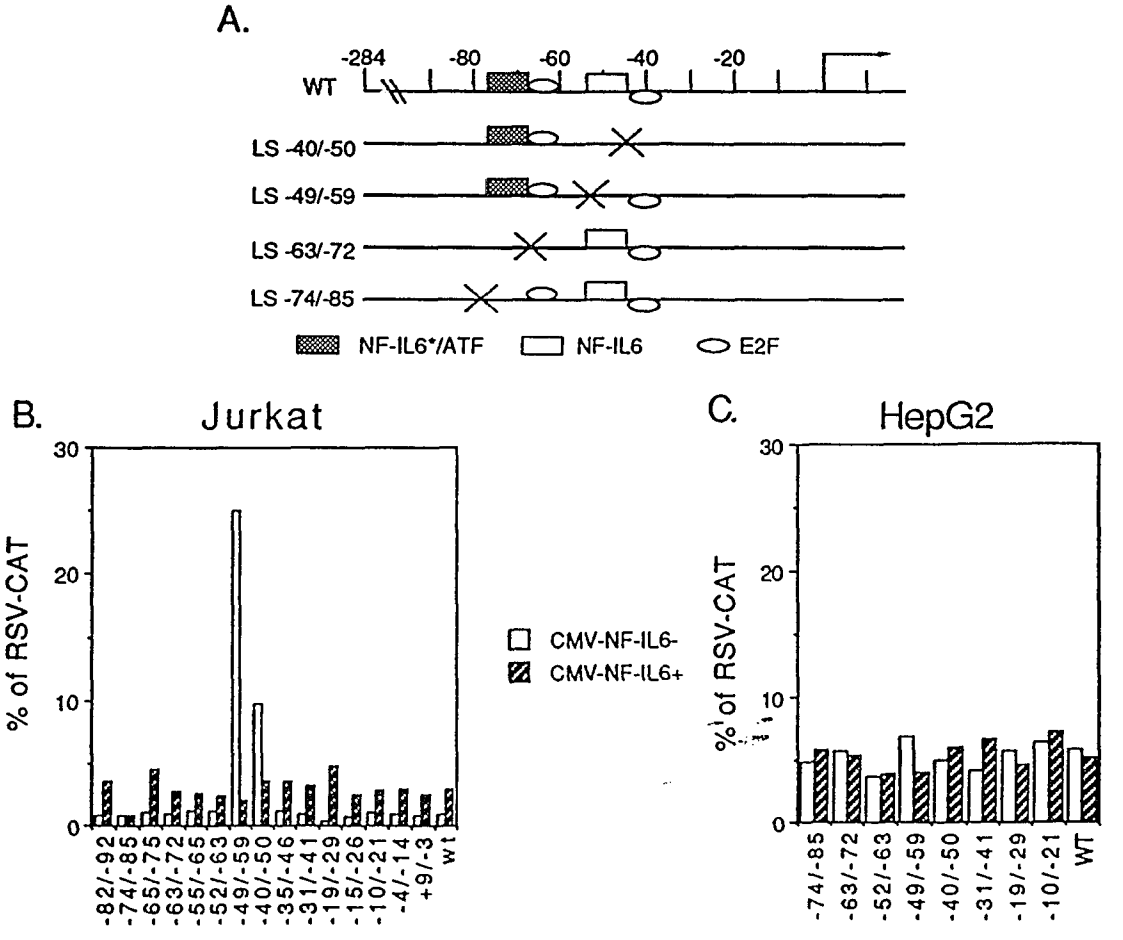


contingent upon its concentration and to levels comparable to those for transactivation by E1A proteins.

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 (Landschulz et al., 1989, Descombes et al., 1990, and Roman et al., 1990) and LAP can dimerize with C/EBP (Descombes et al., 1990). Since dimerization is concentration-dependent, it raises the possibility that formation of specific NF-IL6-promoter complexes and the promoter activity may depend 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. 17A]). 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 (Nakabeppu et al., 1988, and Rauscher et al., 1988). One of them, *jun*B, has been shown to be regulated by IL-6 signaling (Nakajima and Wall, 1991).

The functional significance of NF-IL6 and NF-IL6* sites in the *E2ae* promoter was explored by co-transfecting linker scanning (LS) mutants of the *E2ae* promoter with the NF-IL6 expression vector, pCMV-NF-IL6(+) or its anti-sense derivative, pCMV-NF-IL6(-), into Jurkat and HepG2 cells (Fig. 17). 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 of the wild-type *E2ae* promoter (Fig. 17B). The magnitude of

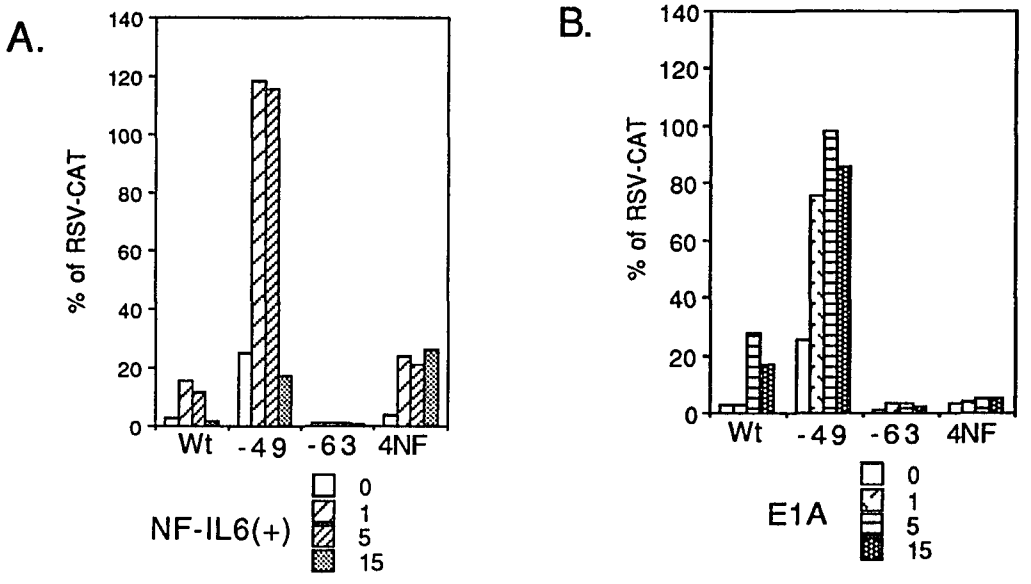
Figure 17: NF-IL6 functions as a negative or positive regulator in a site-specific manner. (A) Schematic diagram of representative LS of the E2ae promoter used in panels B and C. The NF-IL6 and NF-IL6* sites are represented by rectangles and the two E2F sites are represented by ovals. The E2F sites are binding sites for the E2F factors (Kovesdi et al., 1986, Hardy and Shenk, 1988). (B and C) Jurkat cells (panel B) and HepG2 cells (panel C) were cotransfected with pE2ae-CAT (WT) or its LS mutants (Murthy et al., 1985) at 1 μg DNA per 10^6 cells and the NF-IL6 expression vector pCMV-NF-IL6 (+) or its antisense derivative, pCMV-NF-IL6 (-), at 10 μg DNA per 10^6 cells as indicated.



derepression 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. 17B and 16A). These results are reminiscent of an earlier observation in HeLa cells, in which LS -49/-59 exhibited a fivefold-greater promoter activity than the wild-type promoter (Murthy et al., 1985). Derepression was not exhibited by LS mutant -63/ -72 or -74/-85 in which the NF-IL6* site was mutated (Fig. 17B). 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. 13) and form additional *E2ae* promoter complexes in reference to Jurkat cells in vitro (Figure 15). When expressed at a high level by transfection with pCMV-NF-IL6(+) at 10 μg or greater per 10^6 cells, NF-IL6 abrogates the phenotype of the LS mutants in Jurkat cells (Fig. 17B and 18A). 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. The loss of transcription activation was *E2* promoter specific, since at high concentration (15 μg/ 10^6 cells), NF-IL6 significantly transactivates 4xNF-IL6pA_{10}-CAT, a reference plasmid containing four copies of the NF-IL6 recognition element of the IL-6 promoter linked to the reporter gene (Fig. 18A). It was not due to sequestering of

Figure 18: 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 mutants -49/-59 (-49), or -63/-72 (-63), or 4XNF-IL6pA$_{10}$-CAT (4NF) which contains four copies of the NF-IL6 responsive element and serves as a control, at 1 μg plasmid per 10^6 cells; and pCMV-NF-IL6 (+) (A) or pAd5E1A (B) at 1, 5, or 10 $\mu g/10^6$ cells as indicated. Transfection and assay of CAT activity were as described in Material and Methods.



transcription factors by the strong cytomegalovirus promoter used for expressing NF-IL6, since transfecting pCMV-NF-IL6(-) at a concentration of 10 $\mu g/10^6$ cells did not interfere with the promoter activity of either LS mutant -49/-59 or -40/-50 (Fig. 17B). Moreover, the loss of transcriptional activation was NF-IL6-specific, since E1A proteins, which did not activate the control promoter in 4XNF-IL6pA_{10}-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 μg of pAd5E1A per 10^6 cells (Fig. 18B).

The NF-IL6* site certainly also serves as a positive regulatory site in response to NF-IL6: LS mutant -49/-59 was transactivated by NF-IL6 when it was expressed at a lower concentration [1 to 5 μg of pCMV-NF-IL6(+) per 10^6 Jurkat cells] (Fig. 18A). 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. 18A). 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. 18A). Together, these results provided compelling evidence that NF-IL6 transactivates 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, I transfected pCMV-NF-IL6(+) into

human Jurkat T cells prior to their infection with an E1A deletion mutant virus *dl*312. *dl*312 carries a 902-bp deletion within the *E1A* gene, rendering it incapable of E1A expression (Jones and Shenk, 1979).

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. 19). 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 *dl*312, was comparable at two days after viral infection prior to the onset of viral replication, in approximately 15% of cells (Fig. 19). In cells infected with *dl*312 alone or transfected with the antisense derivative of NF-IL6, pCMV-NF-IL6(-) prior to infection with *dl*312, the 72K protein was undetectable.

Similarly, NF-IL6 activates the *E1B* transcription unit, 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 one day after viral infection (Figure 20). 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 *dl*312 infected cells. Likewise, control cells infected with *dl*312, with or without transfection of the antisense construct of NF-IL6, showed no synthesis of E1A and E1B mRNAs (Figure

Figure 19: NF-IL6 activates the *E2ae* gene in infection by an E1A deletion mutant virus. Jurkat cells were infected with Ad2 (Wild Type) or *dl*312 at 20 PFU per cell, with or without transfection with pCMV-NF-IL6(+) [NF-IL6+/*dl*312] or pCMV-NF-IL6(-) [NF-IL6-/*dl*312] at 5 $\mu g/10^6$ cells, 8 hours prior to infection. Infected and uninfected (Control) cells were subjected to indirect immunofluorescence staining at two days post infection as described in Materials and Methods. Abbreviations: IF, immunofluorescent; Phase, phase contrast microscopy.

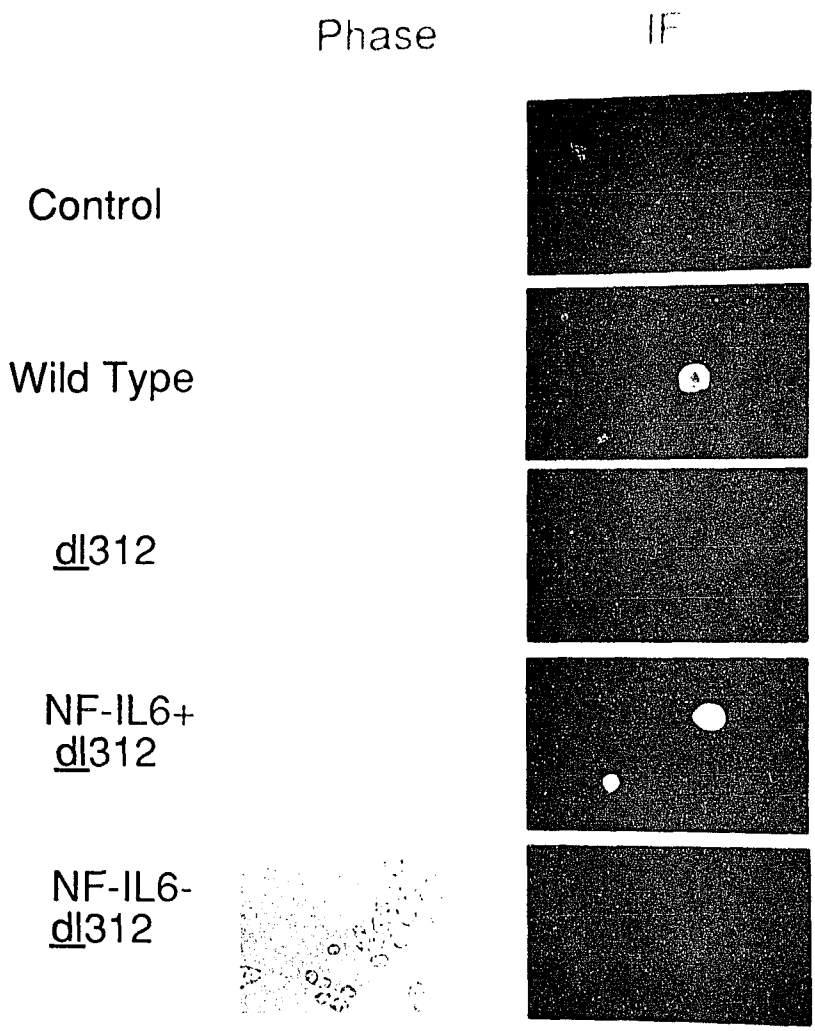
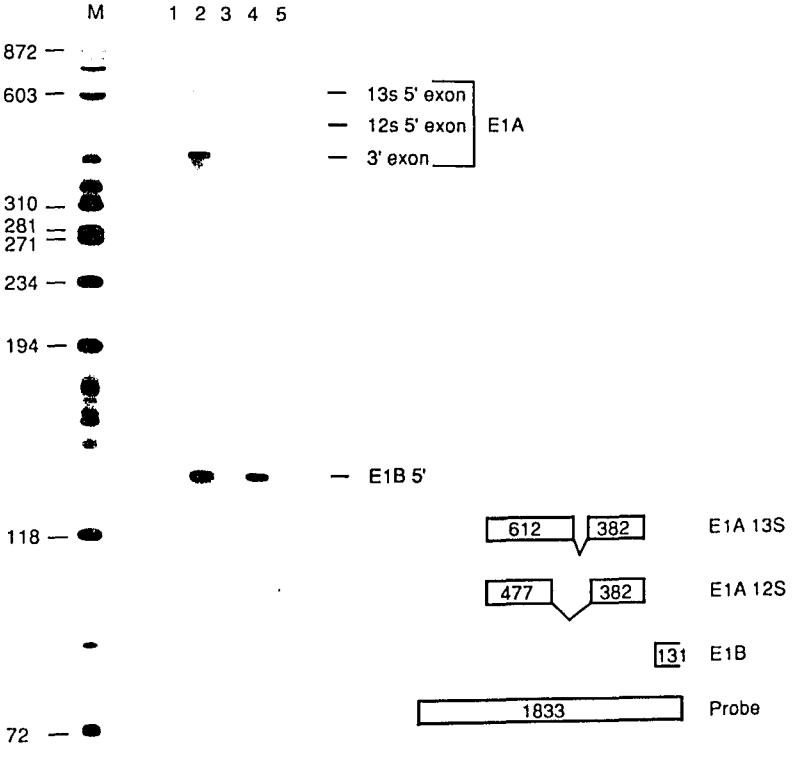


Figure 20: NF-IL6 activates the synthesis of E1B mRNA in infection by an E1A deletion mutant virus. Jurkat cells were infected with Ad2 or *dl*312 with or without transfection as described in the legend in Figure 19. In the left panel, RNA was isolated at 48 hours or 72 hours after infection with Ad2 or *dl*312, respectively. The poly $(A)^+$ RNA was selected from 5 x 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), *dl*312-infected (lane 3), pCMV-NF-IL6(+) transfected and *dl*312-infected (lane 4), and pCMV-NF-IL6(-) transfected and *dl*312-infected (lane 5), cells and DNA size markers (lane M). In the right panel is a schematic diagram of the probe (Marton et al., 1990) used in the assay and the predicted protected E1A and E1B specific RNAs.



20).

Previously, I 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 mRNAs in viral infection in the absence of E1A (Spergel and Chen-Kiang, 1991). 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 (Spergel and Chen-Kiang, 1991).

Although other members of the C/EBP family, LAP and C/EBP, are enriched in terminally differentiated hepatocytes and adipocytes (Christy et al., 1989; and Descombes et al., 1990), the NF-IL6 activity is not restricted to specific cell types. However, its level, as measured in any in vitro binding assay (Fig. 13) and by Western immunoblotting (Hsu, McCully and Chen-Kiang, unpublished data), 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 (Imperiale et al., 1984; La Thangue and Rigby, 1987; and Dooley et al., 1990) 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 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 (Hsu and Chen-Kiang, unpublished data). In a cell type-dependent manner, NF-IL6 appears to complex with members of the AP-1 family (Hsu, McCully and Chen-Kiang, unpublished data) which are known to bind to DNA in dimerized form (Curran and Franza, 1988).

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., (1990). Friedman and McKnight (1990) suggested that regions outside the DNA binding and leucine zipper domains are important for the activating function of C/EBP. We have observed that by Western immunoblotting, 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 (Hsu, McCully and Chen-Kiang, unpublished). 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 (Descombes et al., 1990), 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, *jun*B, has been shown to be regulated by IL-6 signaling (Nakajima and Wall, 1991).

Why is an E1A-responsive promoter not active in most cells? The NF-IL6 binding activity is detectable by electrophoretic mobility shift assay 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 those binding sites might serve as a repressor site. Since the E1B gene is shown to be 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 (Fig. 13A). This effect is most probably due to modification or regulation of NF-IL6 by E1A, since it occurs early after infection, before viral induced host protein synthesis shut-off takes place (Fig. 13A). 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 genes 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 (Cao et al., 1991) and in mouse liver after injection of the mouse with IL-6 (Isshiki

et al., 1991).

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. 17B and 18A). Together with the possibility that NF-IL6 may cooperate with members of the AP-1 family, and possibly also of the ATF family, in forming specific *E2* promoter complexes (Hsu and Chen-Kiang, unpublished), 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. (1989) who showed that cAMP functions synergistically with E1A in transactivating E1A-responsive promoters during viral infection, possibly through c-*fos* and *jun*B, whose syntheses are regulated by E1A (Müller et al., 1989; and van Dam et al., 1990). 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 (SivaRaman

et al., 1986; Hurst and Jones, 1987; Lee and Green, 1987; Hardy and Shenk, 1988; Lin and Green, 1988; Buckbinder et al., 1989; Liu and Green, 1990; and Rooney et al., 1990). Bagchi et al. (1990) recently reported that E1A proteins can dissociate heterodimer complexes involving the E2F transcription factor in vitro, suggesting that E1A may function by sequestering cellular repressors. Can NF-IL6 at high concentration act as the repressor proposed by Bagchi et al. (1990)? 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. 16 and 18; Spergel and Chen-Kiang, 1991). 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 (Hen et al., 1985; and Borrelli et al., 1986). Since immunoglobulin genes are regulated at the level of transcription during IL-6 induced terminal differentiation of B cells

(Raynal et al., 1989), 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.

V. Discussion

I have found that E1A-deletion viruses replicate in HepG2 cells and that this activity is inducible by the cytokine IL-6. Also, NF-IL6 can transactivate the adenovirus E2 and E1B promoters in transfection assays (see Chapter 3 and 4). These results raised some important questions: (1) What is the mechanism for the transcriptional activation and repression by NF-IL6? (2) What is the mechanism for adenovirus mediated down-regulation of NF-IL6? (3) What role, if any, does NF-IL6 play in transformation and the cell cycle? (4) What is the biological significance of IL-6-responsive elements in the adenovirus genome?

Transcriptional Activation and Repression by NF-IL6

Low concentrations of NF-IL6 activates the adenovirus E2 and E1B promoters, yet at high concentrations, NF-IL6 is inactive in transactivating the same promoters in co-transfection experiments. NF-IL6 interacts with the *E2* promoter activity at two different sites (Figure 16 -18). Similarly, C-EBP-α, a related protein, regulates the hepatitis B virus core/promoter in a concentration-dependent fashion, being active at low concentration of C/EBP and inactive at high concentration of C/EBP (Lopez-Cabrera et al., 1990). However, C/EBP transcripitional activation was only 3- to 5-fold, and which of the five putative C/EBP binding motifs are responsible for the activity was not examined.

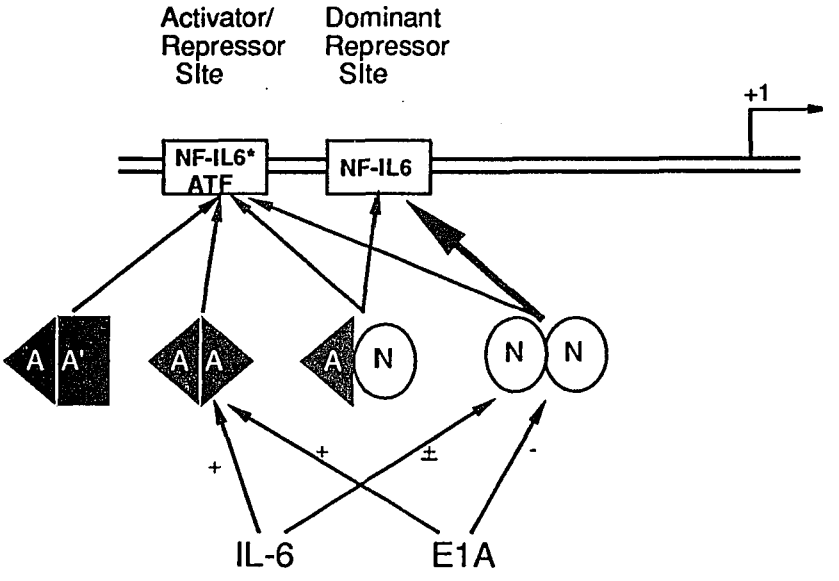
A possible mechanism for the activation and repression of the C/EBP family is provided by recent work from Schibler's laboratory. They found that LAP, the rodent NF-IL6, is regulated at the post-transcriptional level, as a single LAP mRNA encodes for three proteins, LIP, LAP, and LAP*. These three proteins can

heterodimerize or homodimerize. LIP, which utilizes the third methionine for translation initiation, acts as a repressor when co-transfected with a reporter plasmid containing nine tandemly repeated LAP-responsive elements (D element of the albumin promoter). LAP, which contains a longer amino terminus, acts as an activator with the same reporter construct (Descombes et al., 1991).

NF-IL6 contains methionine residues at +1, +24, and +197, suggesting three possible protein products as described above. The amino terminus is designated as +1 and the amino acids are numbered consecutively from amino terminus to carboxyl terminus. C/EBP contains methionine residues at +1, +14,+105, +208,+221, +318 (Landschulz et al., 1988A). LAP, NF-IL6 and C/EBP-α all have an internal methionine for translation initiation before the DNA binding region. Therefore, a putative repressor using the internal methionine at +197 (NF-IL6) or +208 (C/EBP), similar to LIP, is predicted to exist for NF-IL6 and C/EBP. Since one of the two transcriptional activating domains is located in the amino terminus (Friedman and McKnight, 1990), the truncated proteins may have different activities. Therefore, the positive and negative transcriptional regulation by NF-IL6 could occur if (1) at low concentration in transfection, the full length form would predominate, activating transcription, and (2), at high concentration, the shorter form would predominate, making the promoter inactive.

A simplistic model for the positive and negative transcriptional regulation of the *E2* promoter when E2F is non-limiting is shown in Figure 21. In this model, N represents ΔNF-IL6, a truncated NF-IL6 which functions as a repressor similar to LIP or other repressors. A and A* represent activator proteins: for example, the full

Figure 21. A model for regulation of the E2ae promoter by NF-IL6. The model represents a schematic representation of the possible interactions between the putative transcription factors and the *E2* promoter. N represents repressor proteins, while A and A* represent activator proteins (See text for details). In this model, E2F is non-limiting and is not diagrammed.



length NF-IL6 protein, the C/EBP family, the AP-1 family or the ATF/CREB family. AA are the homodimers (e.g. NF-IL6/NF-IL6), while AA* are the heterodimers between the transcription families mentioned above. The activity of the promoter is dependent on the balance between the homodimers and heterodimers of N and A, and their binding affinities to the specific recognition sequences.

Activation occurs when the ΔNF-IL6 level is below a threshold level, or when the level of A is increased by IL-6, increasing the probability of AA or AA* dimer formation. The model predicts that A would be regulated by IL-6 since IL-6 stimulates NF-IL6 and LAP DNA binding activity and *jun-B* mRNA expression (Fig. 13, Akira et al., 1990; Poli et al., 1990; Nakajima and Wall, 1991). Therefore, the IL-6 stimulation of the *E2* promoter (Figure 10) would occur by the above mechanism. Also, consistent with this model is the positive regulation by cAMP of the adenovirus promoters seen by Shenk and colleagues and other laboratories (Engel et al., 1988; Lin and Green, 1988; Leza and Hearing, 1989). cAMP activates ATF/CREB (Montminy et al., 1986; Hai et al., 1988), AP-1 (Müller et al., 1990) and members of the C/EBP family,(rNF-IL6: Metz and Ziff, 1991), all of which are potential AA or AA* forming protein complexes that can activate transcription. These transcription factors, (fos/jun, ATF and C/EBP) can interact with a similar sequence motif, the cyclic AMP responsive element (Hai and Curran, 1991).

Repression of the promoter occurs when N is high: NN dimers form and bind to the NF-IL6* site. Potential repressors, N, are ΔNF-IL6, truncated NF-IL6, IP-1, an inhibitor of fos and jun (Auwerx and Sassone-Corsi, 1991), truncated fos (Δfos; Nakabeppu and Nathans, 1991), or as-yet undiscovered nuclear factors. CHOP, a

member of the C/EBP family, contains mutations in the carboxyl terminus decreasing its DNA-binding activity and represents another repressor in the C/EBP family. Also, in transfection, expression of CHOP attenuates the expression of C/EBP- and LAP-driven CAT promoters (Ron and Habener, 1992). The repressor complex could be NN dimers, or a single repressor sequestering the activator factors in the heterodimer complex, NA, such as the described CHOP-LAP dimers (Ron and Habener, 1992). These complexes could interact at either the NF-IL6 or NF-IL6* site preventing the AA or AA* complexes from binding to DNA and activating transcription. Consistent with this model is the hypothesis that NF-IL6 activates a repressor complex at high concentration of a NF-IL6 expression plasmid.

A test for this model would involve the creation of vectors that express only one species of NF-IL6 protein. These expression vectors could then be used for similar transfection experiments performed in this thesis for the *E2* and *E1B* promoter. The vector that expresses only the full-length NF-IL6 would activate the promoters at all concentrations according to this model. The vector that expresses the truncated NF-IL6 protein would repress the promoter at all concentrations if ΔNF-IL6 is proven to be a negative regulator. Also, examination of potential repressor complexes stimulated by NF-IL6 can be investigated by Western blot analysis with specific antisera. *In vitro* experiments using recombinant NF-IL6 and ΔNF-IL6 would definitely determine if any function existed for the NF-IL6 proteins.

Also, the IL-6 effect on the expression of these various forms of NF-IL6 could be examined. The model predicts that IL-6 would activate expression by altering the ratio of NF-IL6 to ΔNF-IL6, in favor of NF-IL6. IL-6 may increase NF-IL6 in the

nucleus by translocation of the protein from the cytoplasm. A precedent for this mechanism for rNF-IL6 (rat NF-IL6, LAP) is cAMP induced translocation after forskolin treatment (Metz and Ziff, 1991). Western blot analysis using nuclear extracts from uninduced and IL-6 stimulated cells would confirm this theory. The IL-6 stimulated extracts would be expected to have a higher NF-IL6/ ΔNF-IL6 ratio than unstimulated extracts.

Interaction of NF-IL6 and Adenovirus

The total NF-IL6 binding activity was significantly reduced after adenovirus infection (Figure 13). This effect is most likely due to modification or regulation by E1A, since it occurs early after infection, before adenoviral-induced shut-off of host-protein synthesis has begun (Smith and Chen-Kiang, unpublished). Also, *dl*312 infected extracts did not produce the diminution of NF-IL6 binding activity (Figure 14). E1A may down-regulate the repressor form, N, specifically, while up-regulating the activator form, A. Consistent with this theory, E1A has been shown to up-regulate jun-B (Müller et al., 1990; and de Groot et al., 1991), a possible component of A. E1A appears to interact with members of ATF family selectively. For example, E1A will activate transcription in Gal4 assays with ATF-2 but not with ATF-1 (Liu and Green, 1990).

E1A may also influence the NF-IL6 concentration by altering the location of the protein similar to cAMP's action on rNF-IL6 (LAP, Metz and Ziff, 1991). rNF-IL6 also associates with two proteins, 43 kDa and 64 kDa, upon treatment with forskolin. An interesting possibility for the decrease in the NF-IL6 binding activity after adenovirus interaction is that E1A alters the affinity of these protein complexes.

If E1A altered the formation of the rNF-IL6-p43 or rNF-IL6-p64 complexes, could NF-IL6/LAP still localize to the nucleus? One way to test this hypothesis is to prepare nuclear and cytoplasmic extracts from uninfected and infected cells. If the infected cells have a higher amount of LAP in the cytoplasm with a concomitant decrease in the nucleus, it would suggest that E1A could be modifying the nuclear localization signal for LAP.

A Role for NF-IL6 in Transformation and Cell-Cycle Control?

E1A can transform cells with the cooperation of a second oncogene and regulate the cell cycle, in addition to its role as transcriptional activator and repressor. It is interesting to speculate that NF-IL6 may have some of these additional properties of E1A. Three lines of evidence suggest that some overlap might exist. First, overproduction of IL-6 has been correlated with the growth of multiple myeloma, T cell lymphoma, renal and ovarian cell carcinomas and Kaposi's sarcoma (reviewed in Hirano et al., 1990; Sehgal, 1990). Second, IL-6 alters the expression of proto-oncogenes by repressing *c-myb* and *c-myc*, while inducing *c-jun* and *jun-B* mRNA expression (Hoffmann-Liebermann and Liebermann, 1991; Nakajima and Wall, 1991). Finally, transgenic mice with a human IL-6 cDNA under the transcriptional control of the Ig enhancer developed a massive plasmacytosis (Suematsu et al., 1989). Deregulated expression of IL-6 in an appropriate genetic background, BALB/c mice, resulted in plasmacytomas and a chromosomal translocation t(12;15) (Suematsu et al., 1992).

These results strongly implicate a role for IL-6 in oncogenesis. Could the aberrant production of an IL-6-inducible transcription factor, NF-IL6, alter

transcription of oncogenes, promoting tumorigenesis? Could NF-IL6 be a proto-oncogene? An interesting experiment that would begin to address these questions would be the creation of a transgenic mouse with aberrant NF-IL6 production. Could NF-IL6 be interacting with an anti-oncogene? Western blot analysis could be used to examine the products co-precipitated using Rb and/or NF-IL6 antisera.

IL-6 Responsive Elements in Adenovirus

What is the biological reason for adenovirus to have multiple IL-6 responsive elements? The virus uses IL-6 to up-regulate its own transcription and replication, allowing it to accomplish its most basic function, which is to produce more virus. Why does the virus need these additional mechanisms of stimulation? The virus would want multiple transcription factors to interact with a single promoter creating optimal and efficient viral transcription. Other adenoviral promoters have also been shown to contain this type of redundancy. For example, the major late promoter (MLP) has at least four transcription motifs binding cognate factors: upstream stimulating factor (USF), CAAT (CP1), downstream element factor (DEF) and TFIID factor. These elements are functionally redundant for transcriptional activation in mutational analysis (Reach et al., 1991). Also, the *E4* promoter has multiple pathways for E1A activation through the E4F factor and another unidentified pathway (Jones and Lee, 1991). Additional examples of overlapping pathways are the regulation of the *E4* and the *E3* promoter by cAMP and TPA, respectively (Müller et al., 1989; Buckbinder et al., 1989). Therefore, adenovirus promoters have redundant pathways for activation. Cellular promoters also contain this type of redundancy. For example, the *IL-6* promoter can be activated by IL-1,

TNF-α, TPA, cAMP and serum through different motifs (Shimizu et al., 1990).

What is the purpose of so much redundancy? The virus or the cell must respond to multiple stimuli or conditions and still retain proper function. The virus can infect many types of cells, and each of these cells contains a different set of transcription factors. The virus uses the correct transcription factors available in the cell to allow for optimal transcription. Then why does each cell type contain its particular collection of transcription factors? To understand this question, we must examine the regulation of the transcription factors in a developmental fashion and continue to decipher the cascade of factors and their regulation that makes each cell different.

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