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Hsu, Wei, Ph.D.

City University of New York, 1994

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REGULATION OF NUCLEAR FACTORS IN THE INTERLEUKIN-6 SIGNALING PATHWAY

by

WEI HSU

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.

1994

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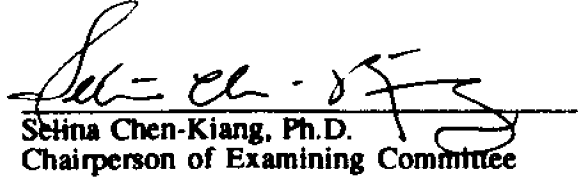

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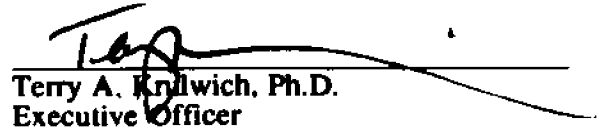

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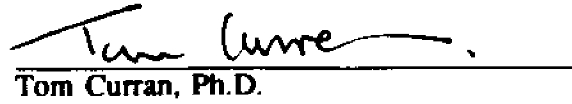

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Abstract

REGULATION OF NUCLEAR FACTORS IN THE INTERLEUKIN-6
SIGNALING PATHWAY

by

WEI HSU

Advisor: Dr. Selina Chen-Kiang

Regulation of nuclear factors in response to interleukin-6 (IL-6) has been investigated in order to understand the molecular mechanism underlying IL-6 signaling. Members of the NF-IL6 and AP-1 families, which share the common basic leucine zipper structure, are thought to mediate the nuclear signals of IL-6. The functional and physical interactions between NF-IL6 and AP-1 family proteins *in vitro and in vivo* have been demonstrated. The direct associations of NF-IL6 and AP-1 are independent of their DNA recognition elements. These associations lead to modifications of the DNA-binding specificity and transcriptional activity of NF-IL6 and AP-1 family proteins. Although the leucine zipper domain is necessary and sufficient to mediate the cross-family dimerization, regions outside the basic leucine zipper structure can enhance the binding of NF-IL6-Fos complexes to specific DNA sequences in the electrophoretic mobility shift assay. The coordinate elevations of NF-IL6 and Jun by IL-6 trigger their association upon macrophage differentiation. Regulated associations of these two family proteins may have implications for determining the promoter specific transcription by IL-6 induction. The octamer binding proteins are regulated by IL-6 suggesting that they represent a subset of DNA-binding proteins that respond to IL-6 signals. Oct-1 is downstream of NF-IL6 in the nuclear signaling cascade induced by IL-6. The abundance of Oct-1 and the ratio of NF-IL6 isoforms are convergently regulated by two diverse signals, IL-6 and retinoic acid. The enhanced synthesis of NF-IL6 and Oct-1 correlates with one of their functions: the

stimulation of adenovirus DNA replication. This result provides an example of possible functional consequences of IL-6 and retinoic acid signaling mediated through the regulation of NF-IL6 and Oct-1. Studies presented in this thesis contribute to understanding of the mechanism underlying the IL-6 signal transduction pathway, the IL-6 inducible transcription and the pleiotropic effect of IL-6.

FORMAT OF THESIS

This thesis was prepared in accordance with guidelines of the City University of New York. Chapter III contains results published as Hsu et al., *Molecular and Cellular Biology*, **14**, 268-276 (1994). Chapter IV contains results published as Hsu and Chen-Kiang, *Molecular and Cellular Biology*, **13**, 2515-2523 (1993). Each section contains an introduction and discussion with a general introduction and conclusion at the beginning and end of the thesis. To reduce redundancy, Materials and Methods have been consolidated in Chapter II, as have the Literature citations in Chapter VI.

To my girlfriend, Linda Chuang,
my dad, Tsangrong Jim Hsu,
and my mom, Yuchin Hsu,
with love and respect.

FOREWORD

On researching

Very much like the beginning of everything else, my graduate study started with excitement and humbleness. In the summer of the year 1989, I came to the Big Apple, an unknown destiny, after graduating from college in Formosa. I always want to continue my graduate training here. The opportunity of exposing myself to one of the world's greatest scientific environments has been a dream come true. Even though I never imagined I would become a scientist, I had never hesitated nor had second thoughts about my chosen profession--scientific research. During the course of my studies, I have often found myself in difficult situations. I am fortunate to have had my teachers, my girlfriend, my friends and my family to encourage me and to support me. Every trouble, stumble and frustration that I faced I was able to overcome. I was strengthened each time. I became more determined one after another. Everyday of the past four and a half years as a graduate student had the potential to affect the quality of my daily life. Encountering the learning and challenges has the capacity of influencing me day after day and taking me step by step to reveal the arts of science. Fortunately, I was able to remember to choose what I love and to love what I choose. Therefore, the journey began ..... in the land of dream.

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I would like to express my sincere gratification to Selina Chen-Kiang for my graduate training. I benefited greatly from her guidance through out these years.

I would also like to thank Tom Curran and Tom Kerppola for a very generous and supportive collaboration. Their helpful advice and fruitful discussions stimulated my graduate study.

Deep thanks are due as well to Manfred Frasch, Howard Worman, Francesco Ramirez, Victor Friedrich, Maurizio DiLiberto and many other faculty for giving me help and guidance during my graduate study.

Current and past members in the Chen-Kiang lab, as well as staff and fellows at Mount Sinai Medical Center, provided me friendship and assistance that I will never forget. Wonderful memories from our softball team and teammates will be cherished and remembered for years and years.

Special thanks to my sister's and girlfriend's families, whose encouragement and companionship are very much appreciated.

The deepest thanks from the bottom of my heart must be given to Linda Chuang, Tsangrong Jim Hsu and Yuchin Hsu. Their enormous love, support and encouragement are invaluable to me. I am very lucky to have them, and it is these three to whom this thesis is dedicated.

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Chapter I

INTRODUCTION

Cytokines or interleukins are molecules that serve as mediators of communication between cells of different lineages. These molecules exert their biological functions through specific membrane receptors on the surface of target cells. Molecular cloning and characterization have revealed that most cytokines function pleiotropically and exhibit biological effects in a redundant manner. Interleukin-6 (IL-6), a 26 Kd protein, is one of the multifunctional cytokines that regulates cell growth and differentiation depending on the nature of target cells (for a review, Kishimoto et al., 1992). IL-6 is produced by a wide variety of cell types and can exert many biological activities, including those in hematopoiesis, immunological reaction, inflammatory response, acute-phase reaction, and neural development. Abnormal production of IL-6 has been observed in patients with polyclonal B cell abnormalities, autoimmune diseases, proliferative diseases and malignancies (for a review, Hirano et al., 1990 and references therein). Understanding the IL-6 signaling pathway is therefore important from the physiological stand point.

IL-6 and lymphoid malignancy

Deregulated expression of IL-6 has been linked to plasma cell neoplasia. Plasmacytoma can be induced in BABL/c mice by injecting either paraffin oil or pristane (Potter, 1984), both of which have been found to induce IL-6 biosynthesis (Potter, 1984, Nordan and Potter, 1986). Transgenic mice which over expressed IL-6 in lymphoid tissues were shown to develop massive and severe plasmacytosis in the thymus, lymph nodes and spleen, and to exhibit elevated levels of IL-6 and polyclonal IgG1 in the sera (Suematsu et al., 1989, Suematsu et al., 1992). The plasma cells from the transgenic mice, however, did not contain chromosomal translocations of the *c-myc* proto-oncogene and were not transplantable to syngenic mice that are found in almost all pristane-induced plasmacytomas. These findings demonstrated that deregulated expression of IL-6 can lead to plasmacytosis.

The result suggested that additional genetic background might be required for the development of plasma cell neoplasia. This hypothesis was later found to be true when the genetic background of BABL/c mice was introduced into the transgenic animal by back-crossing. The F1 back-crossed transgenic mice generated monoclonal transplantable plasmacytomas with t(12;15) c-*myc* translocations (Suematsu et al., 1992). The crucial role of IL-6 in the development of lymphoid malignancy was therefore established.

The IL-6 signal transduction pathway

Studies on ligand-receptor interactions have revealed that many receptors consist of two components, a ligand-binding receptor and a signal transducer. IL-6 initiates the transduction of its signals by binding to IL-6 receptor (IL-6Rα) on the membranes of target cells (Figure 1) (Taga et al., 1989). The ligand-binding IL-6Rα, gp80, is a 80 Kd glycoprotein which does not contain an intracellular signal transducing domain (Yamasaki et al., 1988). The IL-6 signal is thought to be transduced by the signal transducing 130 Kd glycoprotein, gp130 (Hibi et al., 1990). Binding of IL-6 to IL6Rα triggers the homodimerization of gp130 through disulfide-linkage (Murakami et al., 1993). Expression of a soluble form of IL-6Rα, which lacks the transmembrane and cytoplasmic regions, can also mediate the IL-6 signals through gp130 upon IL-6 stimulation (Novick et al., 1989, Tamura et al., 1993). The signal transducer, gp130, is then rapidly and transiently phosphorylated by a cytoplasmic tyrosine kinase activity which is dependent on the dimerization of gp130 (Figure 1) (Murakami et al., 1993). This intracellular tyrosine kinase, Jak1, has recently been identified (Lütticken et al., 1994, Stahl et al., 1994). Jak 1 belongs to a newly described Jak-Tyk family of tyrosine kinases which includes Tyk2, Jak1, and Jak2 (Velazquez et al., 1992, Argetsinger et al., 1993, Silvennoinen et al., 1993, Witthuhn et al., 1993).

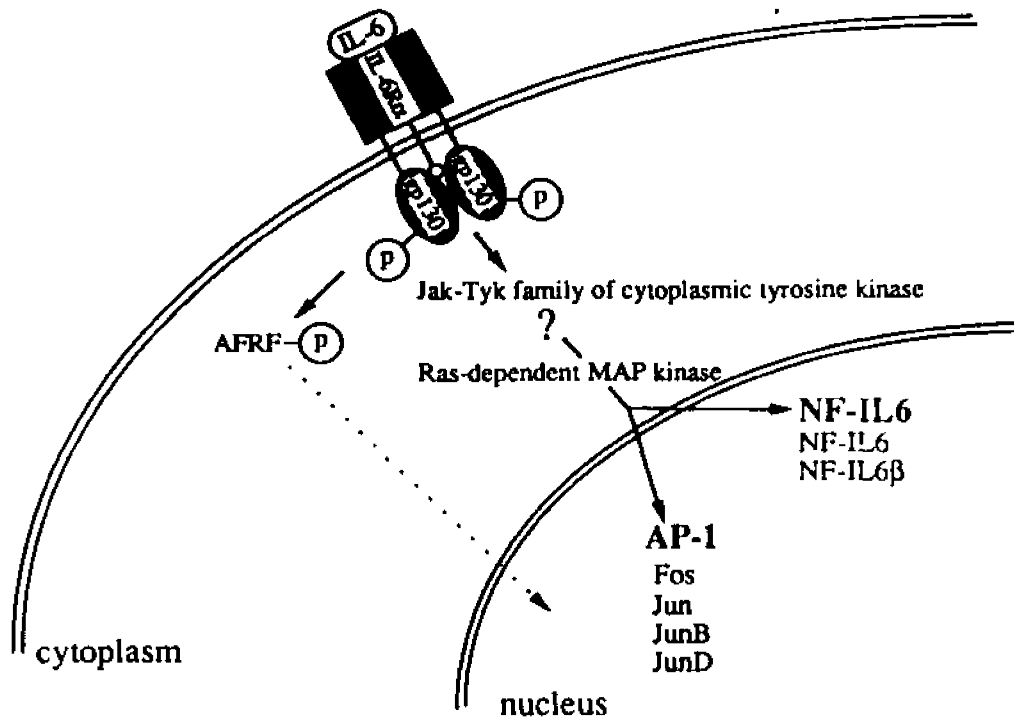


Figure 1. Diagram of the IL-6 signal transduction pathway. The activities of AP-1 family transcription factors, Fos, Jun, JunB, and JunD, NF-IL6 family transcription factors, NF-IL6 and NF-IL6β, and APRF are regulated by IL-6.

Studies on the interaction of leukemia inhibitory factor (LIF), oncostatin M (OSM), and ciliary neurotrophic factor (CNTF), and their receptor complexes have revealed that gp130 is essential for signaling by each of these cytokines (Figure 2) (Gearing et al., 1992, Ip et al., 1992). LIF was first shown to inhibit cell growth and induce macrophage differentiation of M1 myeloid leukemia cells (Hilton et al., 1988). OSM was initially characterized by its ability to inhibit growth of human myeloma cells (Zarling et al., 1986). CNTF was originally found to support the survival of ciliary neurons (Adler et al., 1979, Stöckli et al., 1989). These cytokines were later shown to function pleiotropically and exhibit overlapping biological properties (Table 1). Analogous to the gp130-gp130 homodimerization triggered by IL-6 induction, signaling by LIF and CNTF begins with stimulation of the gp130-LIFβ heterodimerization. The addition of the CNTFRα to the gp130-LIFRβ complex might be sufficient to convert the bipartite LIF receptor into a tripartite CNTF receptor (Davis et al., 1993). The limited cell type distribution of CNTFRα (predominantly in the nervous system) may restrict CNTF actions to the neuronal lineage. The sharing of the signal transducer among these cytokines may explain why they exhibit similar and redundant biological properties; however, the mechanism underlying their pleiotropic effects is not well understood.

The IL-6 signal is transduced into the nucleus by activation of nuclear proteins including members of AP-1 and C/EBP families in a cell type specific manner (Figure 1) (Chen-Kiang et al., 1993). In an alternative pathway, an acute-phase response factor (APRF), a protein antigenically related to the p91 subunit of the interferon-stimulated gene factor-3α (ISGF-3α), is rapidly phophorylated on tyrosine residues by the Jak1 kinase (Lütticken et al., 1994). Phosphorylated APRF apparently can translocate into the nucleus and bind to DNA, thus mediating the IL-6 signal. Regulation and interaction of these nuclear factors at multiple levels may be crucial for the lineage specific effects in IL-6 induction.

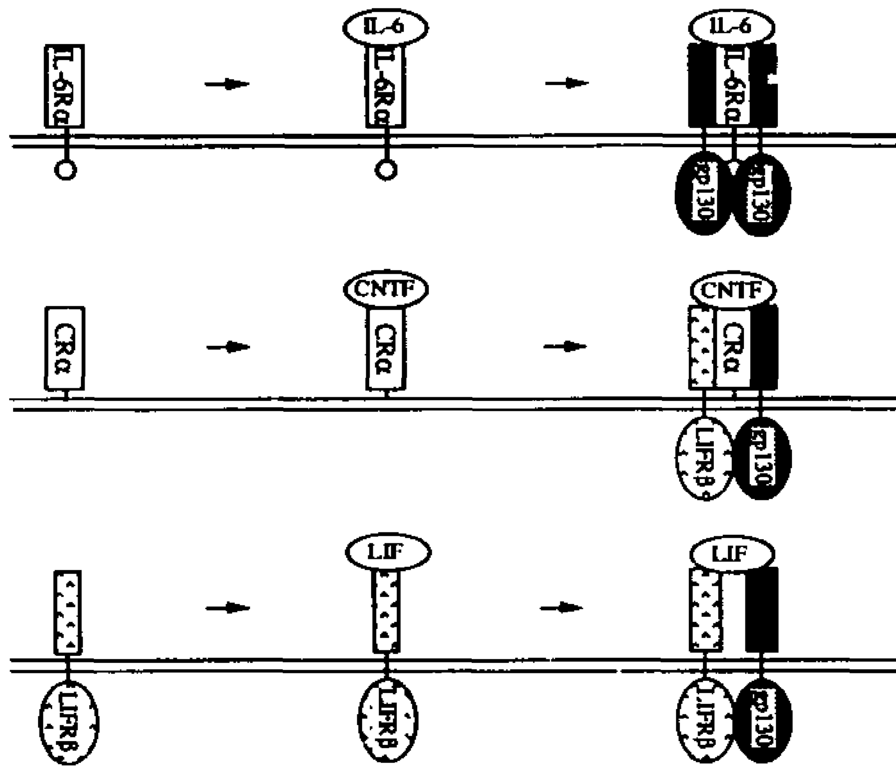


Figure 2. A model for ligand-induced receptor signaling complex formation (Adopted from Davis et al., 1993). IL-6 binds to IL-6Rα, which triggers the homodimerization of gp130 and transduces the signal downstream. For LIF, binding of LIF to LIFRβ leads to heterodimer formation of LIFRβ and gp130. CNTF initiates signaling by inducing gp130-LIFRβ heterodimerization that is mediated through the CNTFRα.

Function	IL-6	LIF	OSM	CNTF
Immunological reaction	Immunoglobulin production, T cell proliferation, Cytotoxic T cell differentiation			
Hematopoiesis	Macrophage differentiation of M1 cells, Stimulation of bone marrow progenitor cells, Platelet production, Proliferation of myeloma and plasmacytoma cells	Macrophage differentiation of M1 cells, Stimulation of bone marrow progenitor cells, Platelet production,	Macrophage differentiation of M1 cells,	
Inflammatory response	Acute-phase protein synthesis	Acute-phase protein synthesis	Acute-phase protein synthesis	
Neural Development	Neurite outgrowth of PC12 cells, Secretion of pituitary hormones, Survival of postnatal forebrain neurons	Survival and generation of sensory neurons, Survival of motor neurons, Switch from adrenergic to cholinergic phenotype		Survival of ciliary neurons, Survival of sympathetic, sensory, and motor neurons, Switch from adrenergic to cholinergic phenotype Differentiation of type-2 astrocytes
Others	Proliferation of AIDS-KS cells, keratinocytes, renal mesangial cells, smooth muscle cells, and myoblasts, Regulation of bone metabolism, Inhibition of melanoma and breast carcinoma cell growth	Maintenance of embryonic stem cells, Proliferation of myoblasts Regulation of bone metabolism, Inhibition of lipoprotein lipase in adipocytes	Maintenance of embryonic stem cells, Proliferation of AIDS-KS cells and fibroblasts, Inhibition of melanoma, breast, and lung carcinoma cells	

Table 1. Overlapping and pleiotropic function of cytokines that use gp130 as a signal transducer (Adopted from Kishimoto et al., 1992).

The C/EBP family transcription factors

The transcription factor C/EBP was first isolated from rat livers by its ability to bind to the "CCAAT" and the "enhancer core homologies" elements present in many promoters and enhancers. It was hence termed CCAAT/enhancer binding protein (C/EBP) (Landschulz et al., 1988a). C/EBP consists of a family of related transcription factors exhibiting a high degree of homology in their basic leucine zipper regions (bZIP) (Figure 3) (Akira et al., 1990, Descombes et al., 1990, Roman et al., 1990, Cao et al., 1991, Ron and Habener, 1992).

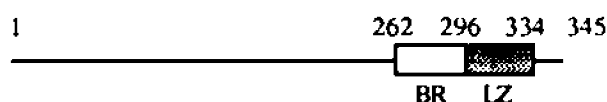


Figure 3. A schematic representation of NF-IL6, a member of the C/EBP family. BR, basic region; LZ, leucine zipper region.

Structural analyses have revealed three functionally distinct regions within the bZIP containing proteins (Figure 4). Near the carboxy-terminus, there exists a region consisting of four to five heptad leucine repeats, called the "leucine zipper domain" (Landschulz et al., 1988b). The leucine zipper domain can fold into an amphipathic α-helical structure (Abel and Maniatis, 1989). The hydrophobic face of the amphipathic structure would dictate the formation of either homodimers (two identical proteins) or heterodimers (two dissimilar proteins) through coiled-coil interaction (O'Shea et al., 1989). The region adjacent to the zipper domain is a highly basic region, which serves to directly interact with the acidic backbone of DNA through ionic forces (Landschulz et al., 1988b). The bipartite DNA-binding domain, the bZIP region, is necessary and sufficient for DNA-binding. Further detailed studies on AP-1 family proteins have shown that two bZIP proteins dimerize through the parallel interaction (Gentz et al., 1989). The "Scissors-Grip" model was

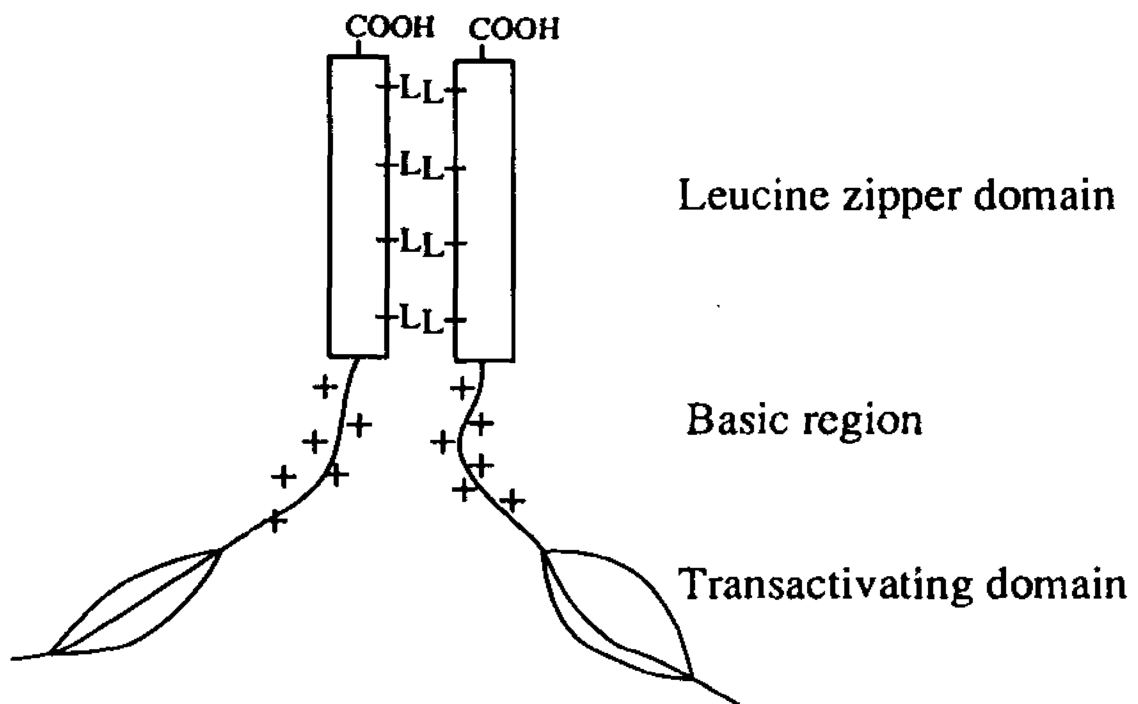


Figure 4. A schematic representation of the structure of bZIP containing proteins.

proposed for DNA recognition of bZIP proteins (Vinson et al., 1989).

The transcription activation regions of bZIP proteins have been localized to the amino-terminal portions of the molecules. Although deletion of the activation regions does not interfere with dimerization and DNA-binding, it abrogates the transactivating function (Descombes and Schibler, 1991, Umek et al., 1991). The biochemical features of the activation regions and the mechanism by which the activation regions mediate transcriptional activation are less well elucidated. One possibility is that the activation regions serve as surfaces to interact with regulatory or basal transcription factors in the transcription machinery.

The C/EBP family transcription factors are involved in terminal differentiation of adipocytes, hepatocytes, lymphocytes, and macrophages, as well as acute-phase responses. Several members of the C/EBP family have been identified (Table 2). They are C/EBPα, NF-IL6 (also known as LAP, IL6-DBP, AGP/EBP, C/EBPβ, and CRP2), Ig/EBP, C/EBPδ (NF-IL6β), CRP1, and CHOP-10 (Landschulz et al., 1988a, Akira et al., 1990, Descombes et al., 1990, Poli et al., 1990, Chang et al., 1991, Cao et al., 1991, Williams et al., 1991, Roman et al., 1990, Cao et al., 1991, Kinoshita et al., 1992, Williams et al., 1991, Ron and Habener, 1992). All of them can form homodimers or heterodimers. One intriguing feature of the genes in this family is that most of them do not contain introns.

Expression of C/EBP related genes was initially studied in terminally differentiated cells (Friedman et al., 1989, Descombes et al., 1990). During the course of adipogenesis, three C/EBP proteins are expressed temporally (Cao et al., 1991). They are thought to promote the differentiation process by activating adipocyte specific genes because activation of adipocyte specific genes are regulated by C/EBP related proteins in both *in vitro* transcription and *in vivo* transfection studies (Christy et al., 1989, Cheneval et al.,

Gene	Protein (Predicted number of amino acids)	bZIP homology	Reference
Human			
*NF-IL6**	345 322 147	83%	Akira et al., 1990
*NF-IL6β***	269	74%	Kinoshita et al., 1992
Rodent			
C/EBPα	358	100%	Landschulz et al., 1988
*LAP**	296	83%	Descombes et al., 1990
(*IL6-DBP*)			Poli et al., 1990
(*AGP/EBP*)			Chang et al., 1990
(*C/EBPβ*)			Cao et al., 1991
(*CRP2*)			Williams et al., 1991
(*rNFIL-6*)			Metz and Ziff, 1991
Ig/EBP	268	79%	Roman et al., 1990
*C/EBPδ*** (*CRP3*)	268	74%	Cao et al., 1991
CRP1	249	88%	Williams et al., 1991
CHOP-10	168	55%	Ron and Habener, 1992

Table 2 C/EBP family transcription factors (* and ** denote human and rodent homologues) (Adopted from Chen-Kiang et al., 1993).

1991, Smuelsson et al., 1991, Umek et al., 1991). Similar results have also been obtained in the regulation of liver specific genes in hepatocyte differentiation and the acute-phase response induced by IL-6 (Akira et al., 1990, Descombes et al., 1990).

Regulation of the C/EBP family appears to be controlled at multiple levels. At the mRNA level, members of the C/EBP family are expressed in a cell type and lineage specific fashion. The abundance of their mRNAs does not always correlate with protein levels, suggesting that the expression of the C/EBP family proteins may be regulated posttranscriptionally (Descombes et al., 1990). Indeed, the NF-IL6 (LAP) and C/EBPα mRNAs can potentially encode isoforms that are translated from different in-frame AUGs of the same mRNA species (Descombes and Schibler, 1991, Ossipow et al., 1993). These isoforms can function as either transcriptional activators (NF-IL6-1 and NF-IL6-2) or repressors (NF-IL6-3) depending on the presence or absence of the transactivating domains. At the protein level, the expression of these alternatively translated isoforms of NF-IL6 is dependent on the cell type, and the abundance and ratios of these isoforms are subject to regulation by external stimuli (Chapter VI, and Hsu and Chen-Kiang, 1993).

NF-IL6-3, which lacks the transactivating domains, sequesters the transcriptional activators, NF-IL6-1 and NF-IL6-2, by protein dimerization (Descombes and Schibler, 1991). Antagonism of the activator function can also be achieved by the dimerization of C/EBP related proteins with a dominant inhibitory protein, CHOP-10, which contains natural mutations in the basic DNA contact region that prevent DNA-binding by dimers (Ron and Habener, 1992). The formation of dimers can also be modulated by protein phosphorylation near and within the bZIP region (Wegner et al., 1992, Nakajima et al., 1993, Trautwein et al., 1993). The functions of C/EBP family transcription factors are subject to further regulation by subcellular compartmentalization (Metz and Ziff, 1991) and interaction with proteins outside the C/EBP family.

The AP-1 family transcription factors

The transcription factor activator protein-1 (AP-1) was originally identified as a sequence specific DNA-binding activity. AP-1 recognizes palindromic DNA sequences related to TGAC/GTCA present in the enhancer elements of SV40, human metalothionein IIA, human collagenase and stromelysin genes (Angel et al., 1987, Lee et al., 1987a, Lee et al., 1987b). Since its identification, the DNA-binding sites for AP-1 have been located in the positive as well as negative regulatory regions in many genes.

The composition of AP-1 has been deduced by a series of biochemical experiments. The product of the proto-oncogene, *c-jun*, was first shown to display structural and functional properties similar to those of AP-1 (Bohmann et al., 1987a). An independent line of investigation revealed that the product of another proto-oncogene, *c-fos*, and Fos related antigens, participated in AP-1 activity (Distel et al., 1987, Franza et al., 1988). The participation of both Fos and Jun in AP-1 activity was based on the discovery that the Fos-associated protein (FAP) p39 is the product of *c-jun* (Chiu et al., 1988, Rauscher et al., 1988a, Sassone-Corsi et al., 1988). The composition of AP-1 was later confirmed by the reconstitution of direct physical association between Fos and Jun and the binding of Fos-Jun dimers to the AP-1 site *in vitro* (Rauscher et al., 1989).

The *fos* gene was originally characterized as an oncogene carried by the FBJ (Finkel-Biskis-Jinkins) and FBR (Finkel-Biskis-Reilly) murine osteogenic sarcoma viruses (Curran and Teich, 1982). The cellular homologue of the oncogene, *c-fos*, encodes a 62 Kd phosphoprotein localized in the nucleus (Figure 5) (Curran et al., 1984). Nucleotide sequence analysis revealed that *v-fos* underwent an out of frame deletion of 104 bp (Van Beveren et al., 1983, Van Straaten et al., 1983). As a result, the carboxy-terminal 48 amino acids were replaced by an unrelated 49 amino acids. This difference prevents posttranslational modification of $p55^{v\text{-}fos}$ but does not alter the transforming potential of Fos proteins (Miller et al., 1984).

The *jun* oncogene is the transforming gene carried by avian sarcoma virus 17, which induces fibrosarcomas in chickens and transforms chicken embryo fibroblasts into spindle-shape neoplastic cells (Maki et al., 1987). The product of *v-jun* differs from that of *c-jun* in the deletion of negative transcriptional regulatory regions. Both *fos* and *jun* are capable of inducing cellular transformation *in vitro* and tumors *in vivo* (Curran and Vogt, 1992). Due to the amino acid sequence similarity with the carboxy-terminal DNA-binding domain of the yeast transcription factor GCN4, Jun was speculated to function as a transcription factor (Figure 5) (Vogt et al., 1987). This was shown to be the case when Fos and Jun were described to exhibit AP-1 activity. Three Fos related proteins, FosB, Fra-1 and Fra-2 (Zerial et al., 1989, Cohen and Curran, 1988, Nishina et al., 1990), and two Jun related proteins, JunB and JunD (Ryder et al., 1988, Nakabeppu et al., 1988), were later identified as transcription factors that shared homology in the bZIP regions with Fos and Jun.

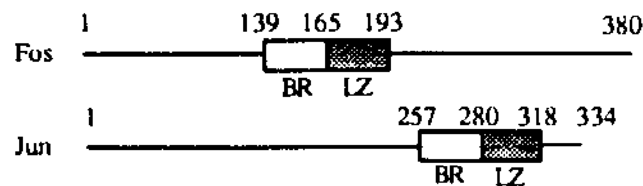


Figure 5. A schematic representation of Fos and Jun. BR, basic region; LZ, leucine zipper region.

Since their discovery, the mechanisms of Fos and Jun action have been analyzed extensively. Dimerization through leucine zipper regions was the first mechanism of action of the bZIP proteins to be elucidated. Fos proteins do not form stable homodimers under physiological conditions (O'Shea et al., 1992), whereas Jun family transcription factors form dimers and bind to specific DNA recognition elements (Rauscher et al., 1989). Fos-Jun heterodimers are more stable than Jun-Jun homodimers, and bind to DNA recognition

elements with higher affinity (O'Shea et al., 1992). The Fos molecule appeared to be a more potent activator than Jun in transcriptional activation (Abate et al., 1990a). Therefore, by two criteria, Fos-Jun heterodimers are favored in AP-1 site mediated transcriptional regulation. Fos-Jun and Jun-Jun dimers can also induce DNA bends of different magnitudes and orientations (Kerppola and Curran, 1991b). This result implies that the interplay of Fos and Jun with other transcription factors may lead to distinct functional consequences due to changes in DNA conformation (Kerppola and Curran, 1991a).

AP-1 activity is shown to be expressed at a low level in many cell types. AP-1 activity can be transiently and dramatically induced by a number of extracellular stimuli (Franza et al., 1988, Rauscher et al., 1988b). Seizures were found to induce AP-1 activity in the hippocampus (Sonnenberg et al., 1989). Light and circadian rhythms also induce AP-1 activity in the suprachiasmatic nuclei (SCN) of the hypothalamus (Kornhauser et al., 1992).

Modification of AP-1 family transcription factors by phosphorylation, reduction/oxidation (redox) of a conserved cysteine residue in the DNA-binding region, and interactions with proteins within and outside the AP-1 family can regulate their DNA-binding, transactivating, and transforming activities (Abate et al., 1990c, Binetruy et al., 1991, Dobrazanski et al., 1991, Mumberg et al., 1991, Nakabeppu and Nathans, 1991, Yen et al., 1991, Minor and Yamamoto, 1992, Jain et al., 1993, Kerppola et al., 1993). Although the regulation of AP-1 family proteins by phosphorylation has been extensively studied, the implications are not completely understood. The DNA-binding activity of Jun was observed to be negatively regulated by phosphorylation at serine/threonine residues near the basic region. The DNA-binding activity of Jun is enhanced by dephosphorylation near the basic region as a result of protein kinase C signaling pathway in response to phorbol esters (Boyle et al., 1991). MAP kinase phosphorylates serine/threonine residues within the amino-terminal transactivation domain of Jun. This phosphorylation leads to an increase in Jun transcriptional activity (Binetruy et al., 1991, Pulverer et al., 1991, Smeal et al., 1991).

Dimerization and DNA-binding can also influence the phosphorylation of Fos and Jun by protein kinases (Abate et al., 1993).

IL-6 and virus interactions

IL-6 markedly enhances the infection of human cells by adenovirus (Spergel and Chen-Kiang, 1992). The "cellular E1A-like activity" was originally defined as a cellular activity that substitutes for E1A in transactivating E1A-responsive viral and cellular genes. Three cellular E1A-like activities had been reported. At high multiplicity of infection, HeLa cells can support a low level of replication of *dl* 312, an E1A-deletion mutant virus (Shenk et al., 1979). This activity in HeLa cells has not been identified and may be attributed to the E7 gene product of human papilloma virus type 18. The product of E7 gene is constitutively expressed in HeLa cells and is able to activate E1A-responsive genes in transfected cells (Schneider-Gadicke and Schwarz, 1986, Phelps et al., 1988). A second activity, which is developmentally regulated, has been described in the mouse embryonal carcinoma F9 cells (Imperiale et al., 1984, La Thangue and Rigby, 1987), oocyte (Dooley et al., 1989), and preimplantation embryos (Suemori et al., 1988). The third cellular E1A-substituting activity has been found in human hepatoblastoma HepG2 cells. This activity can transactivate all E1A-responsive viral early genes and support replication of an E1A-deletion mutant virus *dl* 312 in the absence of E1A (Spergel and Chen-Kiang, 1991). In addition, this activity is stimulated by IL-6 induction of HepG2 cells, resulting in full complementation of *dl* 312 replication (Spergel and Chen-Kiang, 1991). These data implicate a cellular component regulated in the IL-6 signal transduction pathway that can substitute for E1A in transactivation.

The protein encoded by the adenovirus transforming gene E1A is capable of transactivating all the adenoviral early genes, as well as a subset of cellular genes (Berk, 1986, Flint and Shenk, 1989). The mechanism of E1A action is difficult to delineate for two reasons. First, E1A protein can function as an activator or repressor in a promoter specific

and cell type specific manner. Second, E1A protein has a very low affinity for DNA and no apparent E1A-responsive DNA sequence has been identified. Based on experimental evidence, three models of E1A action that are not mutually exclusive have been proposed. In the first model, E1A stimulates the transcriptional activity of a number of transcription factors, including E2F, E4F, TFIIIC, and AP-1 (Hoeffler et al., 1988, Bagchi et al., 1989, Müller et al., 1989, Raychaudhuri et al., 1989), which activate the transcription of viral early genes. In the second model, E1A functions as a cofactor by forming a molecular bridge between ATF-2/CREBP-1 and TFIID that results in stimulating the initiation of transcription (Kao et al., 1990, Liu and Green, 1990, Lee et al., 1991, Scholer et al., 1991). In the third model, E1A transactivates by sequestering cellular repressors such as E2F and YY-1. Derepression is thought to be accomplished by either modifying a repressor or replacing a repressor that masks the function of the activator (Bagchi et al., 1990, Shi et al., 1991).

Activation of the viral early genes by E1A allows viruses to enter the late-phase of the infection cycle which is defined by the synthesis of the viral genome and the production of virion structural proteins in great abundance. Replication of adenovirus requires participation of the virus-encoded E2 gene products [terminal protein precursor (pTP), adenovirus DNA polymerase (Ad pol) and single-strand DNA-binding protein] and three cellular factors [NF-I/CTF, Oct-1/NF-III and a cellular topoisomerase activity (NF-II)] (for a review, Challberg and Kelly, 1989). Adenovirus replication is unidirectional and is initiated at one of the 5'-inverted terminal repeats of the linear viral genome (for a review, Stillman, 1983). During each round of replication, only one new DNA strand is synthesized. Initiation of adenovirus DNA replication occurs by a "protein priming" mechanism. Each 5'-end of the genome is covalently linked to the 55 Kd terminal protein (the mature product of pTP). This linkage occurs during package of the viral genome into virion. For optimal initiation of adenovirus DNA replication *in vitro*, at least four proteins are required. They are pTP, Ad pol, NF-I/CTF, and Oct-1/NF-III (Enomoto et al., 1981, Lichy et al., 1982, Stillman et al., 1982, Nagata et al., 1983, Ostrove et al., 1983, Pruijn et al., 1986, Rosenfeld

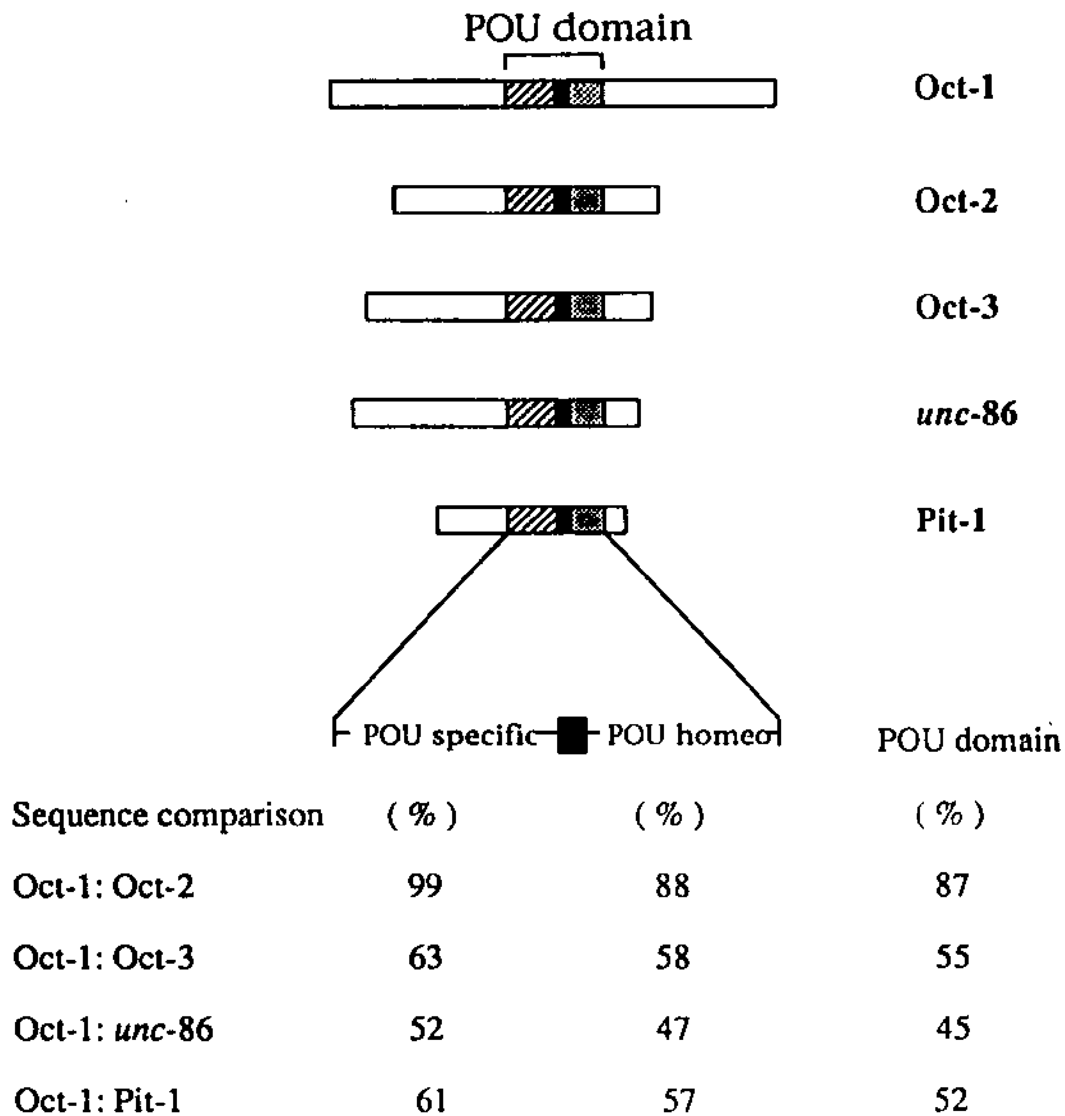
and Kelly, 1986, Rosenfeld et al., 1987). The initiation of adenovirus replication appears to take place in a two-step reaction. First, the terminal region is unwound and second, the pTP is covalently linked to the first nucleotide residue dCMP. Direct association of NF-I/CTF and pTP-Ad pol stimulates the initiation process by enhancing pTP-Ad pol binding to the origin of replication (Chen et al., 1990). In contrast, Oct-1/NF-III stimulates the initiation by unwinding DNA in the origin of replication (Mul et al., 1990, Verrijzer et al., 1990a).

The octamer binding proteins

The octamer binding proteins (Octs) bind to consensus octamer sequences related to 5'-ATGCAAAT-3'. This octamer motif is present in the regulatory regions of many promoters and enhancers, such as SV40, heavy and light chain immunoglobulin, and small nuclear RNA (snRNA) (Sive et al., 1986, Bohmann et al., 1987b, Nomiyama et al., 1987, Ondek et al., 1987). Amino acid sequence comparison of four transcription factors, Pit-1, Oct-1, Oct-2, and *unc*-86 revealed a structurally conserved 150-160 amino acid POU domain, consisting of a 75-82 amino acid POU specific domain, a short variable linker region and a 60 amino acid POU homeodomain (Figure 6) (Herr et al., 1988). The POU specific domain is an unique feature of proteins in this family, whereas the POU homeodomain is related to the homeodomain first identified in *Drosophila* homeotic gene products (Gehring, 1987).

Both POU specific domain and POU homeodomain of the octamer binding proteins contribute to sequence specific and high affinity DNA-binding (Verrijzer et al., 1990b). The POU specific domain and the adjacent POU homeodomain cooperatively recognize an asymmetrical DNA-binding sequence (Aurora and Herr, 1992). The POU homeodomain recognizes the 3'-half of the octamer motif (Verrijzer et al., 1990b), whereas the POU specific domain recognizes the 5'-half (Verrijzer et al., 1992). These interactions involve the direct contact of both domains with the major groove of DNA molecule and trigger DNA bending (Aurora and Herr, 1992, Verrijzer et al., 1991).

POU domain containing proteins



Sequence comparison	POU specific (%)	POU homeo (%)	POU domain (%)
Oct-1: Oct-2	99	88	87
Oct-1: Oct-3	63	58	55
Oct-1: *unc*-86	52	47	45
Oct-1: Pit-1	61	57	52

Figure 6. A diagram of POU domain containing proteins. Oct-1, Oct-2, Oct-3, *unc*-86 and Pit-1 contain the conserved 150-160 amino acid POU specific and POU homeodomains. The percentages of identical amino acids between two proteins are indicated.

The homeodomain is composed of three well defined helices, with helices 2 and 3 forming a helix-turn-helix motif which is the DNA-binding domain (Otting et al., 1988). The third helix is predicted to contact the major groove of DNA and has been termed the recognition helix (Kissinger et al., 1990). Recent studies further demonstrate that the POU specific domain also folds into four α-helices connected by a short conserved hydrophobic loop (Assa-Munt et al., 1993, Dekker et al., 1993). The structure is similar to the DNA-binding domains of the bacteriophage λ and 434 repressors and 434 cor (Aggarwal et al., 1988, Jordan and Pabo, 1988, Wolberger et al., 1988, Beamer and Pabo, 1992). The third helix is proposed to mediate the DNA-binding (Assa-Munt et al., 1993, Dekker et al., 1993).

Many members of the Oct family have been identified (Table 3). Almost all members of this family have been suggested to play roles in cellular differentiation and development (for a review, Scholer, 1991). Most of the octamer binding proteins are expressed in a cell type specific and tissue specific fashion, with the exception of Oct-1. The expression pattern of their target genes appears to correlate with the cell type specific distribution of the octamer binding proteins. Oct-1 is through to activate the transcription of ubiquitously expressed histone H2B and snRNA genes, whereas Oct-2 activates the transcription of B cell specific immunoglobulin genes (Müller et al., 1988, Scheidereit et al., 1988, Staudt et al., 1988, Sturm et al., 1988).

Although the POU domains of Oct-1 and Oct-2 are 87% identical, they can activate distinct target genes through the same octamer site. The nature of this discrimination is dependent on the protein-protein interaction. Studies on the herpes simplex virus gene product VP16 suggest that it forms a complex with Oct-1 to activate transcription of viral early genes (Gerster and Roeder, 1988, O'Hare and Goding, 1988, Preston et al., 1988). Association of VP16 with Oct-1 supplies a strong acidic transactivation domain present in VP16 to the transcriptional complex (Goding and O'Hare, 1989). The seven amino acids

Octamer proteins	Alternative spliced species	Expression	
		Embryo	Adult
Oct-1 *(OTF-1)* *(NF-A1)* *(NFIII)* *(OBP100)*	*Oct-1-A-* *Oct-1-C*	Ubiquitous	Ubiquitous
Oct-2 *(OTF-2)* *(NF-A2)*	*Oct-2.1-* *Oct-2.6* *mini Oct-2*	Neural tube, entire brain except telencephalon	Lymphoid cells nervous system, intestine, testis, kidney
Skn-1a/i *(Oct-11)*	*Skn-1a* *Skn-1i*	developing epidermis	Epidermis (suprabasal cells, hair follicles)
Brn-2 *(N-Oct3)* *(N-Oct5)*		Developing nervous system	central nervous system, glioblastoma, neuroblastoma
Brn-4 *(RHS2)* *(N-Oct4)*		Neural tube	central nervous system (forebrain)
Tst1 *(SCIP)* *(Oct-6)*		Blastocyst, ES cells, EC cells,	Nervous system (neurons, myelinating glia), testis
Oct-3/4 *(Oct-5)* *(NF-3A)*	*Oct-3A* *Oct-3B*	ES and EC cells, embryonic ectoderm, primordial germ cells, testis and ovary	Oocyte

Table 3 Octamer binding proteins and their tissue distribution (Adopted from Scholer et al., 1991).

where Oct-1 and Oct-2 homeodomains differ allow VP16 to discriminate between them (Sturm et al., 1988). Among the seven amino acids, the glutamic acid residue at position 22 in the first α-helix of Oct-1 homeodomain is the major determinant for selective VP16 association *in vitro* and VP16 mediated transactivation *in vivo* (Lai et al., 1992, Pomerantz et al., 1992). Single amino acid substitution of the alanine residue at position 22 of the Oct-2 homeodomain with the glutamic acid residue is sufficient to confer the VP16 accessibility and cooperativity (Lai et al., 1992, Pomerantz et al., 1992). Therefore, the cooperation between VP16 and Oct-1 in transcriptional activation serves as a possible mechanism to explain how the homeodomain containing proteins activate differential gene expression. The differences in the homeodomains that allow octamer binding proteins to associate with different cofactors lead to activation of distinct subclasses of genes. In this respect, many factors have been shown to associate with Oct-1 activator complex. These factors include OCA-B (the B cell specific coactivator), HCF/C1 (a cellular factor in the VP16-Oct-1 complex), Jun and JunD (Kristie et al., 1989, Katan et al., 1990, Luo et al., 1992, Ullman et al., 1993).

Specific aims

C/EBP and AP-1 are two major families of transcription factors that are regulated by IL-6 according to cell type. NF-IL6 and AP-1 family proteins bind to DNA as dimers through the bZIP region. Emerging evidence has suggested that protein-protein interactions play a key role in determining the promoter and cell type specificity in gene transcription and signal transduction. The first and major objective of this investigation is to study the interactions of NF-IL6 and AP-1 family proteins and their functional consequences in IL-6 signal transduction pathway. The IL-6 nuclear signaling pathway is not well understood. IL-6 regulates the replication of adenovirus, suggesting that components in the IL-6 nuclear signaling pathway are important for cytokine-virus interactions. The second objective is to

study the regulation of two transcription factors, NF-IL6 and Oct-1, that potentially link cytokine and viral signaling pathways. The goal of this thesis is:

(1) To determine if a direct physical interaction between C/EBP and AP-1 family transcription factors occurs *in vitro*.

(2) To deduce the regions in C/EBP and AP-1 family proteins that mediate their interactions, and to elucidate the mechanism(s) that underlies this protein-protein interactions.

(3) To elucidate the functional consequences and the physiological implications of NF-IL6 and AP-1 cross-family association *in vivo*.

(4) To investigate the nuclear cascade of IL-6 signal transduction pathway, focusing on the regulation of NF-IL6 and Oct-1.

Chapter II

MATERIALS AND METHODS

Recombina..t plasmids (Table 4)

General plasmids pBS(SK)NF-IL6-1 was constructed by isolating and inserting the 1.8 kb *Hin*dIII-*Bam*HI fragment of pCMV-NF-IL6 (+) (Akira et al., 1990), which contains the coding DNA sequences of human NF-IL6, into the *Hin*dIII and *Bam*HI restriction sites of pBluescript (SK). pGEM5-NF-IL6-2 was constructed by isolating and inserting the 1.6 kb *Nco*I-*Spe*I fragment of pBS(SK)NF-IL6-1 into the corresponding restriction sites of pGEM5. pBS(SK)NF-IL6-3 was constructed by excision of the 1.5 kb *Sma*I-*Bam*HI fragment of pGEM5-NF-IL6-2 and insertion into the corresponding restriction sites of pBluescript(SK). pBS(SK)NF-IL6-4 was constructed by isolating and inserting the 1.1 kb *Hin*dIII-*Pst*I fragment of pBS(SK)NF-IL6-1 into the corresponding restriction sites of pBluescript(SK). pEB-55/106(oct+) and pEB-44/106(oct-) (Hartfield and Hearing, 1991) are plasmids containing adenovirus inverted terminal repeats (ITR), with or without octamer binding sequence, respectively (provided by P. Hearing). pBSOct-1 (Sturm et al., 1988) is a plasmid containing a partial human *oct-1* cDNA clone (provided by W. Herr). pGAPDH (Fort et al., 1985) is a plasmid containing the glyceradehyde phosphate dehydrogenase. p3Wt18 (Ruether et al., 1986) is a plasmid containing the left end 5.8 kb *Xho*I-C fragment of the adenovirus type 5 genome. pE1A/E1B (Marton et al., 1990) is a plasmid containing the 1,833 bp of the adenovirus left end cloned into pGEM3 (provided by T. Shenk). pSP65-*fos* (Curran et al., 1987) is a plasmid containing the full length *fos* cDNA isolated from rat, which was cloned into the *Eco*RI site of pSP65 (provided by T. Curran). pGEM4-*jun* (Rauscher et al., 1989) is a plasmid containing the rat *jun* cDNA which is inserted into the *Eco*RI site of pGEM4 (provided by T. Curran). pIBIE2ae (Spergel and Chen-Kiang, 1991) contains the 346 bp (-284 to +62) of the adenovirus type 5 E2ae promoter. pBlue6W (Schreiber et al., 1988) is a plasmid containing 6 tandemly repeated copies of the 51 bp *Dde*I-*Hin*fI fragment of the mouse Ig heavy chain enhancer (provided by Walter Schaffner).

p4xNF-IL6-CAT is a plasmid containing four tandemly repeated copies of NF-IL6 binding sites from the IL-6 promoter linked to a chloramphenicol acetyltransferase (CAT) gene (provided by T. Kishimoto). p2xAP-1-CAT is a plasmid containing two tandemly repeated copies of AP-1 binding sites from the promoter of the TSG-6 gene, a TNF-α and IL-1 inducible gene (Lee et al., 1993), linked to a CAT gene (provided by Jan Vilcek).

Eukaryotic expression plasmids pCMV-NF-IL6 (+) and pCMV-NF-IL6 (-) (Akira et al., 1990) contain a human NF-IL6 cDNA encoded in the sense (+) and anti-sense (-) orientation, respectively, under the control of the cytomegalovirus promoter (provided by T. Kishimoto). The pCMV-NF-IL6-2 (Hsu et al., 1994) was constructed by inserting the *Nco*I-*Bam*HI fragment of pBS(SK)NF-IL6, after treatment with the Klenow fragment of DNA polymerase to obtain even ends of DNA, into the *Hind*III site of pCMV (obtained from F. Rauscher). The pCMV-NF-IL6-3 (Hsu et al., 1994) was constructed by inserting the *Xma*I-*Bam*HI fragment of pBS(SK)NF-IL6 into the even ended *Hin*dIII and *Bam*HI sites of a pCMV. pCMV-*fos* and pCMV-*jun* (Sonnenberg et al., 1989) are as described (provided by T. Curran).

Prokaryotic expression plasmids pGST-NF-IL6-1 (Hsu et al., 1994) was constructed by inserting the *Hin*dIII-*Bam*HI fragment of pBS(SK)NF-IL6 into the corresponding restriction sites of pGEX-2T (obtained from P.-L. Chen). The pGST-NF-IL6-2 (Hsu et al., 1994) was constructed by inserting the *Nco*I-*Bam*HI fragment of pBS(SK)NF-IL6, after treatment with a Klenow fragment of DNA polymerase to obtain even ends of DNA, into the *Sma*I site of pGEX-2T (obtained from P.-L. Chen). To construct 3'-deletion mutants of pGST-NF-IL6-2 (Hsu et al., 1994), the plasmid was linearized with *Eco*RI, treated with a Klenow fragment of DNA polymerase, and partially digested with *Rsa*I. pGST-NF-IL6-3 (Hsu et al., 1994) was constructed by inserting the partially digested *Sma*I-even ended *Bam*HI fragment of pBS(SK)NF-IL6 into the *Sma*I site of pGEX-3X (obtained from P.-L. Chen). pGST-Myc (Goodrich and Lee, 1992) has been described previously (provided by W.-H. Lee).

Plasmid	DNA insert (size)	Insertion sites	Vector	Reference
pBS(SK)NF-IL6-1	NF-IL6(1.8 kb)	*Hin*dIII/*Bam*HI	pBluescript	Hsu et al., 1994
pGEM5-NF-IL6-2	NF-IL6(1.6 kb)	*Nco*I/*Spe*I	pGEM5	Hsu, unpublished data
pBS(SK)NF-IL6-3	NF-IL6(1.5 kb)	*Sma*I/*Bam*HI	pBluescript	Hsu, unpublished data
pBS(SK)NF-IL6-4	NF-IL6(1.1 kb)	*Hin*dIII/*Pst*I	pBluescript	Hsu, unpublished data
pSP65-*fos*	*c-fos*(2.1 kb)	*Eco*RI	pSP65	Curran et al., 1987
pGEM4-*jun*	*c-jun*(1.7 kb)	*Eco*RI	pGEM4	Rauscher et al., 1989
pBSOct-1	*oct-1*(2.4 kb)	*Xba*I/*Pst*I	pBluescript	Sturm et al., 1988
pGAPDH	*gapdh*(1.3 kb)	*Pst*I	pBluescript	Fort et al., 1985
p3Wt18	Ad 5'*Xho*I-C(5.8 kb)	*Cla*I/*Sal*I	pBR322	Ruether et al., 1986
pE1A/E1B	Ad 5'(1,833 bp)	*Pst*I	pGEM3	Marton et al., 1990
pEB-55/106(oct+)	Ad ITR(1.3 kb)	*Xba*I	pBR322	Hartfield et al., 1991
pEB-44/106(oct-)	Ad ITR(1.3 kb)	*Xba*I	pBR322	Hartfield et al., 1991
pIBIE2ae	Ad E2ae (346 bp)	*Xho*I/*Bgl*II	pIBI30	Spergel et al., 1992
pBlue6W	Ig μ enhancer(51 bp)	*Sal*I	pBluescript	Schreiber et al., 1988
p4xNF-IL6-CAT	4x(NF-IL6 site)			Akira et al., 1990
p2xAP-1-CAT	2x(AP-1 site)			Klampfer, unpublished data
pCMV-NF-IL6-1(+)	NF-IL6(sense, 1.8 kb)	*Hin*dIII/*Bam*HI	pCMV	Akira et al., 1990
pCMV-NF-IL6-1(-)	NF-IL6(antisense, 1.8 kb)	*Hin*dIII/*Bam*HI	pCMV	Akira et al., 1990
pCMV-NF-IL6-2	NF-IL6(1.6 kb)	*Hin*dIII	pCMV	Hsu et al., 1994
pCMV-NF-IL6-3	NF-IL6(1.5 kb)	*Xma*I/*Bam*HI	pCMV	Hsu et al., 1994
pCMV-*fos*	*c-fos*(2.1 kb)	*Hin*dIII/*Bam*HI	pCMV-IE	Sonnenberg et al., 1989
pCMV-*jun*	*c-jun*(1.7 kb)	*Pvu*II	pCMV-IE	Sonnenberg et al., 1989
pGST-NF-IL6	NF-IL6(1.8 kb)	*Hin*dIII/*Bam*HI	pGEX-2T	Hsu et al., 1994
pGST-NF-IL6(24-345) (pGST-NF-IL6-2)	NF-IL6(1.7 kb)	*Sma*I	pGEX-2T	Hsu et al., 1994
pGST-NF-IL6(199-345) (pGST-NF-IL6-3)	NF-IL6(1.2 kb)	*Sma*I	pGEX-3X	Hsu et al., 1994
pGST-NF-IL6(24-273)	NF-IL6(1.5 kb)	*Sma*I	pGEX-2T	Hsu et al., 1994
pGST-NF-IL6(24-203)	NF-IL6(1.3 kb)	*Sma*I	pGEX-2T	Hsu et al., 1994
pGST-NF-IL6(24-78)	NF-IL6(0.9 kb)	*Sma*I	pGEX-2T	Hsu et al., 1994
pGST-NF-IL6(24-39)	NF-IL6(0.8 kb)	*Sma*I	pGEX-2T	Hsu et al., 1994
pGST-Myc	*c-myc*(1.3 kb)	*Sma*I	pGEX-2T	Goodrich & Lee, 1992

Table 4 List of recombinant plasmids

Antibodies (Table 5)

α-P3 (Hsu and Chen-Kiang, 1993) is a rabbit antiserum directed against P3, a synthetic 17 amino acid peptide (SKAKKTVDKHSDEYKIR) present in the basic region of all three NF-IL6 proteins. α-P4 (Hsu and Chen-Kiang, 1993) is a rabbit antiserum directed against a 13 amino acid peptide (PAARPGPRPPAGE) present in the amino-terminal region of NF-IL6-1 and NF-IL6-2, but not NF-IL6-3. α-LAP (Descombes and Schibler, 1991) is a rabbit antiserum directed against the amino-terminal region of recombinant LAP (provided by U. Shibler). α-Fos (Rauscher et al., 1989) is a rabbit antiserum directed against the full length Fos. α-Alu (Cohen and Curran, 1990) is a rabbit antiserum directed against the amino-terminal part (Fos1-131) of Fos. α-wbF (Abate et al., 1990b) is a rabbit antiserum directed against the bZIP region of Fos (F118-211). α-M (Cohen and Curran, 1990) is a rabbit antiserum directed against Fos(127-152). α-Jun (Rauscher et al., 1989) is a rabbit antiserum directed against the full length Jun. α-wbJ (Cohen and Curran, 1990) is a rabbit antiserum directed against the bZIP region (J225-334) of Jun. All antisera directed against different regions of Fos and Jun were provided by T. Curran. α-C/EBPα (Cao et al., 1991) is a rabbit antiserum directed against a 14-amino-acid unique peptide of C/EBPα (provided by S. McKnight). α-Oct-1 (Pruijn et al., 1989) is a rabbit antiserum directed against Oct-1 protein (provided by I. W. Mattaj). α–POU is a monoclonal antibody directed against the highly conserved POU domain of Oct-1 and Oct-2 (provided by T. Gerster). α-E4 (Marton et al., 1990) is a monoclonal antibody directed against the adenovirus E4 ORF6/7 polypeptide (provided by T. Shenk).

Antibody	Antigen	Species ; Poly(P)- or Mono(M)-clonal	Reference
α-P3(NF-IL6)	17 amino acid	rabbit; P	Hsu and Chen-Kiang, 1993
α-P4(NF-IL6)	13 amino acid	rabbit; P	Hsu and Chen-Kiang, 1993
α-LAP	N-terminal LAP	rabbit; P	Descombes and Schibler, 1991
α-C/EBPα	14 amino acid	rabbit; P	Cao et al., 1991
α-Fos	full length Fos	rabbit; P	Rauscher et al., 1989
α-Alu	Fos(1-131)	rabbit; P	Cohen and Curran, 1990
α-M	Fos(127-152)	rabbit; P	Cohen and Curran, 1990
α-wbF	Fos(118-211)	rabbit; P	Abate et al., 1990
α-Jun	full length Jun	rabbit; P	Rauscher et al., 1989
α-wbJ	Jun(241-334)	rabbit; P	Cohen and Curran, 1990
α-Oct-1	Oct-1	rabbit; P	Pruijn et al., 1989
α-POU	Oct-POU domain	mouse; M	Gerster (unpublished)
α-E4	Ad E4 ORF6/7	mouse; M	Marton et al., 1990

Table 5. List of antibodies.

In-gel ligation of recombinant DNA

Plasmid DNA (usually 1 to 2 μg) was digested with the desired endonucleases and separated by electrophoresis on a low gelling/melting temperature agarose gel (1 to 2 %; NuSieve GTG agarose, FMC) in a buffer of 50 mM Tris-acetate (pH 8.2). The DNA fragments, visualized by long-wave ultraviolet light after staining with ethidium bromide, were excised from the gel with a clean razor blade in a volume as small as possible. Sliced gels containing the desired DNA fragments were melted at 70°C for 10 min, and combined with 10 times excess of vector plasmid into a final volume of 10 μl. The mixture was incubated at 37°C for 10 min, to which 10 μl of ice cold 2x ligation buffer (0.1 M Tris-HCl, pH 7.6, 20 mM $MgCl_2$, 20 mM DTT, and 100 μg/ml BSA) containing 1 μl of T4 DNA ligase (Promega) was added and mixed quickly. The mixture was then incubated at 16°C for 12 to 24 hours. To introduce the ligated products in to *E. coli* cells, the reaction mixture was heated at 70°C for 10 min and diluted with 10 volumns of ice-cold TCM (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 10 mM $CaCl_2$) prior to the standard transformation procedure.

Purified recombinant proteins

GST (glutathione S-transferase) and GST fusion proteins were expressed in *E. coli* BL21(DE3) cells by using the T7 expression system (Studier et al., 1990). To express soluble fusion proteins, overnight cultures of bacteria were inoculated with single colonies that were newly transformed with recombinant plasmids, and were diluted with 10 volumns of medium. After an additional 2 hours incubation (optical density at 600 nm of 0.4), the culture was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG, Sigma) at 37°C for 2 hours. For small scale preparation, cells from 3 ml of culture were harvested by centrifugation at 4°C in the Eppendorf centrifuge (14,000 rpm for 5 min) and resuspended in 0.5 ml of phosphate-buffered-saline (PBS) containing 1% Triton X-100 (PBS-Triton). The lysates were sonicated 3 times with 15-sec pulses (Vibra Cell Sonicator at 40% duty cycle) and the debris were pelleted by centrifugation (14,000 rpm, Eppendorf, for 5 min).

The supernatants (10 μl) were mixed with 50 μl of glutathione-Sepharose (Sigma) equilibrated and suspended in PBS-Triton according to the manufactures' specification, and incubated at 4°C for 30 min. The Sepharose was then collected by centrifugation (5,000 rpm, Eppendorf, for 2 min), washed three time with PBS-Triton, resuspended in 10 μl of SDS sample loading buffer, which contains 50 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM DTT, 0.1% bromophenol blue, 10% glycerol, boiled for 5 min and analyzed by SDS-PAGE. GST fusion proteins were purified to greater than 90% homogeneity by this single-step procedure, as determined by silver staining or coomassie blue staining. To elute the fusion proteins, the fusion protein-glutathione Sepharose complexes were washed with a buffer containing 5 mM Tris-HCl (pH 8.0), 5 mM glutathione, 1 mM bezamidine (Sigma), 50 μg/ml of soybean trypsin inhibitor (Sigma), 5 μg/ml of aprotinin (Sigma), 1 μg/ml of bacitracin (Sigma), 0.3 μg/ml of Antipain (Boehringer Mannhein), 1 μg/ml of Leupeptin (Boehringer Mannhein), 1 mM PMSF (Sigma), and 5 mM DTT. Purified human Oct-1 protein from HeLa cells (provided by Claus Scheidereit), purified recombinant LAP (provided by U. Schibler), purified truncated form of recombinant C/EBPα (provided by S. McKnight), and purified recombinant Fos, Jun, and their derivatives (Fos, F59-380, F2-321, F2-270, F139-321, F139-270, F139-289, F139-255, F139-245, F139-200, F118-211, F118-211D186, F188-211D139-144, Jun, J225-334, and J241-334) (provided by T. Curran, T. Kerppola and C. Abate) were used as indicated.

***In vitro* transcription and translation**

RNA was transcribed *in vitro* from plasmids, pSP65-*fos*, pGEM4-*jun*, and pBSOct-1 by SP6 or T7 RNA polymerase in a 100 μl of transcription buffer containing 200 mM Tris-HCl (pH 7.5), 50 mM NaCl, 30 mM $MgCl_2$, 10 mM spermidine, 10 mM DTT, 120 units of RNase inhibitor RNasin (Promega), 0.5 mM nucleoside triphosphate (NTP) (Boehringer Mannhein), 0.5 mM ^{7}GpppG (New England Biolab.), and 2 μg of linearized DNA templates at 37°C for 60 minutes. Following digestion of the plasmid DNA with 5 μl of RNase-free

DNase I (Promega) at 37°C for 15 min, the transcribed RNA (5-10 μg) was precipitated with ethanol. The proteins were translated *in vitro* from 0.1 to 1 μg of RNA in 40 μl of nuclease-treated, messenger-dependent rabbit reticulocyte lysates (Promega) at 30°C for 60 min. Each reaction mixture was supplemented with 1 mM amino acid mixture without methionine and 20 μCi of ^{35}S-methionine for synthesis of labeled proteins, or 1 mM of amino acid mixture with methionine for synthesis of unlabeled proteins.

Association of proteins *in vitro*

Fos, Jun, and Oct-1 (0.3 fmol) translated *in vitro* and labeled with ^{35}S-methionine were incubated with 2 pmol of bacterially expressed GST or GST fusion proteins in 300 μl of buffer A, which contained 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 5 mM DTT, 2 mg/ml Leupeptin, 2 mg/ml Antipain, and 0.5% NP-40; or in RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 2 mg/ml of Leupeptin, 2 mg/ml of Antipain, 0.1 mM PMSF, 1% Triton X-100, 1% SDS, and 1% Sodium deoxycholate). The reactions were incubated at 37°C for 10 min and then at 4°C for 10 min. The protein complexes were precipitated with 50 μl glutathione-Sepharose, washed three times with the same incubation buffer, and analyzed by 12% SDS-PAGE and autoradiography.

Cells and cytokine induction

NT2/D1 cells, a clonal human embryonal carcinoma cell line (Andrews, 1984), were cultured in Dulbecco's modified essential medium. HepG2 cells, a human hepatoblastoma cell line (Aden et al., 1979), were cultured in Eagle's minimal essential medium. Jurkat cells, a human T lymphoblastoid cell line, NJBC-8 cells, a stable transforment of Jurkat cells, which constitutively express the human IL-6 receptor-α, gp80, and M1 cells, a mouse myeloid leukemia cell line, were cultured in RPMI 1640 medium. All media contained 10% heat-inactivated fetal calf serum (expect that serum used to culture HepG2 was not heat-inactivated), L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and

nonessential amino acids (GIBCO) as supplements. Induction of NT2/D1, NJBC-8, and M1 cells by recombinant IL-6 was performed as described previously (Raynal et al., 1989). NT2/D1, NJBC-8, and M1 cells were induced with 200 units of IL-6 per ml. One unit of recombinant IL-6 was defined as the activity that induces 50% of the maximum response of IgM production in 10^4 SKW6-C14 cells (Hirano et al., 1986). Induction of NT2/D1 cells with retinoic acid (RA) at 10^{-5} M was performed as described previously (Lee and Andrews, 1986).

Viruses and virus infection

Infections and propagation of adenovirus type 2 (Ad 2) and *dl* 312, an E1A-deletion mutant of adenovirus type 5, were as described previously (Lavery and Chen-Kiang, 1990). Cells were resuspended in serum-free media containing 10 to 20 PFU (plaque forming unit) per cell of virus after washing twice with serum free media. Following adsorption of virus at 37°C for 30 min, the infected cells were resuspened and diluted in complete media (3×10^5 cells/ml).

Nuclear extract preparation

Nuclear extracts were prepared essentially as described previously (Mattila et al., 1990). Cells ($3\text{-}5\times10^7$) were collected and washed twice with PBS (without Mg^{2+} or Ca^{2+} ions) before being resuspended in 1 ml of buffer A containing 10 mM Na-Hepes (pH 7.8), 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 μg/ml of Antipain, 0.3 μg/ml of Leupeptin, and 1% milk. After dropwise addition of 100 μl of 2% NP-40 to the cell suspension with constant mixing, the nuclei were separated from the cytoplasmic fraction by overloading the mixtures onto 5 ml of 10% sucrose-buffer A and centrifuging at 2,000 rpm (RT6000B) for 5 min at 4°C. The nuclei were resuspended in 100 ul of buffer B containing 50 mM Na-Hepes (pH 7.8), 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 μg/ml of Antipain, 0.3 μg/ml of Leupeptin, and 10% glycerol. Ammonium sulfate

was added to a final concentration of 0.32 M. After mixed gently at 4°C for 30 min, the lysates were clarified by centrifuging at 70,000 rpm for 15 min (Beckman ultracentrifuge TLA100). The supernatant was transfer to a new microtube and the ammonium sulfate concentration was brought up to 2.4 M. After centrifugation at 50,000 rpm for 10 min (Beckman ultracentrifuge TLA100), the pellet was resuspended in buffer B and incubated at 4°C for 15 min. Whole cell extracts were prepared similarly to the nuclear extracts. The cell pellet was lysed in buffer B followed by ammonium sulfate precipitations of proteins. For small scale nuclear extracts, 0.5-1×10^6 cells were collected, resuspended in 1 ml PBS and transferred to a new Eppendorf tube. The cells were pelleted, resuspened in 400 μl of buffer of 10 mM Na-Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 5 mM DTT, and 0.5 mM PMSF, and incubated on ice for 15 min. Before centrifugation at 14,000 rpm (Eppendorf) for 3 min, 25 μl of 10% NP-40 was added to the lysates. The pellet was resuspended in 50 μl of ice-cold 20 mM Na-Hepes (pH 7.9), 0.25 M NaCl, 2 mM EDTA, 1 mM EGTA, 5 mM DTT, and 1 mM PMSF, incubated at 4°C for 15 min, and clarified by centrifugation at 14,000 rpm (Beckman ultracentrifuge TLA100) for 5 min. The protein concentrations of extracts were determined by the Bradford assay (Bradford, 1976) with a protein assay kit (Bethesda Research Laboratory).

Immunoblotting analysis

Immunoblotting analysis was performed as described (Harlow and Lane, 1989). Whole cell or nuclear extracts (20 μg) were diluted in an equal volumn of 2x SDS sample loading buffer and boiled for 5 min before fractionation on a 12% denaturing polyacrylamide gel. The proteins were electrotransferred (40 V, at 4°C for 16 hours) onto nitrocellulose membranes in a transfer buffer (0.24 M Tris base, 0.18 M glycine, 3.5 mM SDS, and 800 ml methanol). After blocking with a buffer containing 4% BSA, 1 mM Tris-HCl (pH 8.0), 5 mM NaCl, and 0.05% Tween-20, the blots were incubated with the primary antibodies diluted in a buffer containing 0.5% BSA, 1 mM Tris-HCl (pH 8.0), 5 mM NaCl,

and 0.05% Tween-20 at room temperature for 1 hour. After washing three times with the same buffer for 5 min, the bolt was then incubated with an alkaline phosphatase-conjugated secondary antibody (3:10,000 dilution) (Promega) at room temperature for 30 min. The bound secondary antibodies were detected and visualized by a chromogenic substrate reaction (0.1 µg/ml of nitro blue tetrazolium (NBT) and 0.05 µg/ml of 5'-bromo-4-chloro-3-indolyl phosphate (BCIP)).

Immunoprecipitation-immunoblotting assay

Nuclear or whole cell extracts (100 µg) were incubated with or without 2 pmol of purified recombinant Fos or Jun, in 300 µl of buffer A containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 5 mM DTT, 2 mg/ml Leupeptin, 2 mg/ml Antipain, and 0.5% NP-40 at 37°C for 10 min. For detecting the presence of the NF-IL6-Jun complex in M1 cells, whole cell lysates were extracted with buffer containing 5 mM Tris-HCl, 10 mM Na-Hepes (pH 7.6), 250 mM NaCl, 0.5 mM EDTA, and 0.1% NP-40. Protein complexes were recovered by the addition of anti-Fos or anti-Jun (1:333 dilution) and incubated at 4°C for 1 hour. Protein A-Sepharose (75 µl of 40 mg/ml suspension, Sigma) was added. After incubation at 4°C for 30 min, the immune complexes were precipitated by centrifugation (5,000 rpm, Eppendorf centrifuge for 3 min), washed 3 times with buffer A and resuspended in 20 µl SDS sample loading buffer. After being boiled for 5 min, the supernatant was analyzed by SDS-PAGE (12% gel). The proteins were transferred onto nitrocellulose membranes by electrophoresis at 4°C followed by immunoblotting analysis as described.

Electrophoretic mobility shift analysis

Synthetic oligonucleotides were ^{32}P-labeled by kinase reaction and DNA fragments were ^{32}P-labeled by filled-in reaction (Hsu and Chen-Kiang, 1993). The probes were separated from unincorporated isotopes by electrophoresis on a 7% polyacrylamide gel. Autoradiography of the wet gel was obtained for locating the probes. The regions of the gel

corresponding to the position of the labeled probes were excised. Probes were electroeluted from the sliced gel in dialysis tubings (3,000 MW cut off) at 250 V for 30 min. For the analysis of DNA binding activities of octamer proteins, nuclear extracts (0.6 μg) or Oct-1 protein (1 ng) purified from HeLa cells was incubated with 65 pmol of DNA probe in a binding buffer containing 20 mM Na-Hepes (pH 7.9), 50 mM KCl, 1 mM EDTA, 1 mM DTT, 4% Ficoll, and 1 μg/μl poly(dIdC) at room temperature for 20 min. Confirmation of the presence of Oct-1 in protein-DNA complexes was performed by incubating anti-Oct-1, and anti-POU antisera for 10 min at room temperature prior to the addition of probes. The DNA fragments containing the octamer site from the adenovirus ITR, pEB-55/106(oct+) and Ig heavy chain enhancer, pBlue6W, are as follows (the octamer core sequences are underlined):

pEB-55/106(oct+)

5'ATTTTGGATTGAAGCCAATATGATAATGAGGGGGTGGAGTTTGTGACGTGGCGCGGGGCGTGGGAACGGGGCGGGTGACGTAG
3'TAAAACCTAACTTCGGTTATACTATTACTCCCCCACCTCAAACACTGCACCGCGCCCCGCACCCTTGCCCCGCCCACTGCATC

pEB-44/106(oct-)

5'ATTTTGGATTGAAGCCAATATGATGGAGTTTGTGACGTGGCGCGGGGCGTGGGAACGGGGCGGGTGACGTAG
3'TAAAACCTAACTTCGGTTATACTACCTCAAACACTGCACCGCGCCCCGCACCCTTGCCCCGCCCACTGCATC

Ig heavy chain enhancer

5'AGCAAAACACCACCTGGGTAATTTGCATTTCTAAAATAAGTTGAGGATTC
3'TCGTTTTGTGGTGGACCCATTAAACGTAAAGATTTTATTCAACTCCTAAG

For the analysis of DNA binding activities of NF-IL6 and Fos-Jun, nuclear extracts (1 μg) or *in vitro* translated Fos and Jun (0.3 pmol) were incubated with 65 pmol of probe in a buffer containing 5 mM Tris-HCl, 10 mM Na-Hepes (pH 7.6), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% glycerol, 1 μg/μl of poly(dIdC) at room temperature for 30 minutes. For the binding analysis of purified recombinant proteins, proteins were incubated in 20 μl of binding buffer containing 5 mM Tris-HCl, 10 mM Na-Hepes (pH 7.6), 50 mM NaCl, 15 mM EDTA, 5 mM DTT, 10% glycerol at 37^{0}C for 15 min. The presence of EDTA is not essential for NF-IL6 binding, however, the presence of Mg^{2+} disturbed the formation of

stable protein-DNA complex. To each reaction mixture, poly(dIdC) (1 μg/μl) was added, and incubation was continued at room temperature for 5 min before the addition of ^{32}P-labeled probe (65 pmol). After incubation at room temperature for 10 min, the protein-DNA complexes formed were separated from free probes by electrophoresis on a 6% native polyacrylamide gel in 0.25x TBE buffer (22 mM Tris-borate and 0.625 mM EDTA) at 250 volts at 4°C for 3 hours. Confirmation of the presence of Fos and Jun was performed by incubating anti-Fos, anti-Jun, anti-wbF, anti-wbJ, anti-M, and anti-Alu antisera for 10 min at room temperature prior to the addition of probes. The oligonucleotide probes used are as follows (the core recognition sequence of each DNA probe is undrerlined):

NF-IL6(IL-6)

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

NF-IL6(E2)

5'-TTAAATTTGAGAAAGGGCGCGAAACTAGTCCTT-3'
3'-AATTTAAACTCTTTCCCGCGCTTTGATCAGGAA-5'

ATF/NF-IL6*(E2)

5'-GAGATTGAGTAGTTTTCGCGCTT-3'
3'-CTCTAACTCATCAAAAGCGCGAA-5'

AP-1

5'-GAATTCTAAAGCATGAGTCAGACACCTC-3'
3'-CTTAAGATTTCGTACTCAGTCTGTGGAG-5'

DNase I footprint analysis

The analysis was performed basically as described previously (DiLiberto et al., 1989). Single stranded, end-labeled (10,000 cpm, 65 pmol) DNA fragments were used for binding reactions with increasing amounts of proteins as described in electrophoretic mobility-shift analyses (excepting only 1 mM EDTA was present in the binding buffer). Following the binding reaction, sample was brought to 5 mM $MgCl_2$ and digested with 3 μg of DNase I (Phamacia) for 90 seconds on ice. The reaction was stopped by the addition of the 35 μl of termination buffer containing 0.6 M NaCl, 0.2% SDS and 10 mM EDTA,

followed by phenol/chloroform extraction and ethanol precipitation of DNA. The digested end-labeled DNA fragments were then analyzed on a denaturing 8% polyacrylamide sequencing gel.

Metabolic labeling and immunoprecipitation

M1 cells ($3x10^6$ cells) were washed twice with methionine-free RPMI medium. To deplete the endogenous methionine, the cultured cells were incubated with methionine-free medium and 10% dialyzed fetel calf serum at a concentration of $2x10^6$ cells/ml at 37°C for 30 min. ^{35}S-methionine (100 μCi) was added to continue the incubation for 30 min. The cells were collected by centrifugation (5,000 rpm, RT6000B for 1 min), washed with PBS, and lysed in 1 ml of RIPA buffer. The lysate was clarified by ultracentrifugation at 40,000 rpm (TLA100), at 4°C for 40 min. The supernatant was transferred to a new microtube, to which the desired antibody was added and incubated at 4°C for 2 hours. The antigen-antibody complexes were collected by the addition of 20 μl Staph A cells (10% suspension in PBS, 0.1% NaN_3, Calbiochem), incubation at 4°C for 30 min, and centrifugation at 5,000 rpm for 1 min. The pellet was then resuspended in denaturing SDS sample loading buffer, and boiled for 5 min, before analysis on SDS-PAGE (12%)

Transfection

NT2/D1 cells was transfected by the modified calcium phosphate-mediated transfection method (Sambrook et al., 1989). Cells (10^6 cells per 90-mm dish) were plated 24 hours before transfection. Superhelical plasmid DNA (30 μg, including the carrier DNA) was incubated in 0.5 ml of 2.5 M $CaCl_2$, to which 2x BES (N,N-bis{2-hydroxyethyl}-2-aminoethanesulfonic acid) buffer (50 mM Na-BES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.0) was added, at room temperature for 30 min. The $CaCl_2$/DNA/BES buffered solution was then added dropwise to NT2/D1 cells. The transfected cultures were incubated at 37°C in an atmosphere of 3% CO_2 humidified incubator for 15-24 hours and in an atmosphere of 5%

CO_2 for an additional 24 hours. Jurkat cells were transfected by electroporation (Spergel et al., 1992) using a Gene-Pulser (Bio-Rad) at 1.0 kV and 25 μF. For each transfection, 10^6 cells were transfected with 5 μg of expression plasmid DNA in a total of 20 μg DNA (including carrier, salmon sperm or pBluescript DNA) and incubated at 37°C in the presence of 5% CO_2 for 48 hours. The chloramphenicol acetyltransferase (CAT) activity expressed by the reporter plasmids in transfected cells was measured as described previously (Spergel et al., 1992). The cell extracts were prepared 48 hours after transfection. Cells were harvested by centrifugation at 1,000 rpm (Eppendorf) for 5 min and washed twice with PBS, resuspended in 100 μl of 0.25 M Tris-HCl (pH 7.8). After freezing and thawing three times, the debris were pelleted, and the supernatant was collected for CAT activity analyses. The CAT activity was determined by the diffusion of ^3H acetyl coenzyme A into organic-miscible scintillation fluid (Sambrook et al., 1989). The slope of the organic-miscible radioactivity as a function of time was calculated and used to represent the CAT activity, with the slope of RSV-CAT as a positive control and that of JYM-CAT as a negative control.

RNA isolation and analysis

Total RNA isolation, selection of polyadenylated RNA, and Northern blot analysis were performed essentially as described previously (Lavery and Chen-Kiang, 1990). Total RNAs were isolated by homogenization in 4 M guanadinium thiocyanate (GTC). Polyadenylated RNAs were selected by poly(U)-Sepharose chromatography (Chen-Kiang and Lavery, 1989) and analyzed by Northern blotting. RNAs were fractionated by denaturing formaldehyde-agarose gel electrophoresis in MOPS buffer containing 20 mM morpholino-propanesulfonic acid (Sigma), 5 mM sodium acetate, 1 mM EDTA (pH 7.0), transferred onto nitrocellulose membranes (Schleicher and Schuell), and baked at 80°C for 2-3 hours. DNA probes were hybridized to membranes at 42°C in the presence of 50% formamide. The membranes were then washed four times with buffer A (pH 7.0) containing, 150 mM NaCl, 15 mM sodium citrate, and 0.2% SDS, for 10 min at 42°C, and

twice with buffer B containing 7.5 mM NaCl, 0.75 mM sodium citrate (pH 7.0), and 0.2% SDS, for 30 min each at 55°C.

Southern blot analysis

Total DNA from adenovirus-infected NT2/D1 cells were isolated and analyzed by Southern blotting as described previously (Lavery and Chen-Kiang, 1990). After infection with adenovirus type 2 or *dl* 312 at 10 PFU per cells, 10^5 cells from each cell culture were withdrawn at various times postinfection as indicated. Total DNA was then isolated, digested with *Hind*III, separated by 1% agarose gel electrophoresis, and blotted onto nitrocellulose membranes. Adenoviral DNA was detected by hybridization, using a ^{32}P-labeled probe, the left end 5.7 kb of the adenovirus 5 genome from p3Wt18 plasmid.

Chapter III

CROSS-FAMILY INTERACTION BETWEEN NF-IL6 AND AP-1

Portions of results contained in this chapter have been published (Hsu et al., 1994, *Molecular and Cellular Biology*, 14, 268-276).

INTRODUCTION

The IL-6 signals mediated through the IL-6 receptor complex on the cell surface lead to regulation of genes that are specific to the differentiated phenotype. This implies that different transcription factors are selectively and coordinately regulated in cells responding to IL-6. NF-IL6 and AP-1 family transcription factors are induced by IL-6 in a cell type specific manner. In B cells, *junB* is activated by IL-6 (Nakajima and Wall, 1991). In myeloid leukemia cells, *jun, junB and junD*, but not *fos* are activated after IL-6 induction (Lord et al., 1993). In PC12 cells, only *fos* is induced upon neuronal differentiation by IL-6 (Satoh et al., 1988).

NF-IL6 and AP-1 family proteins have been shown to interact with proteins outside their families. Association of NF-IL6 with the *c-rel* domain of NFκB *in vitro* implies that their transcriptional activities are subject to regulation by this association (LeClair et al., 1992). Transcription activity of the glucocorticoid receptor is regulated by association with Fos and Jun positively and negatively, depending on the composition of Fos and Jun (Diamond et al., 1990, Kerppola et al., 1993, Minor and Yamamoto, 1992). Similarly, transcription activation by helix-loop-helix proteins, such as MyoD and myogenine, is antagonized upon association with Fos and Jun (Bengal et al., 1992, Li et al., 1992). Recent studies on the early events of T cell activation further demonstrate that Fos and Jun are components of a complex that is important for activation of transcription of the IL-2 gene (Jain et al., 1993, Ullman et al., 1993). Investigation of the physical and functional interactions between NF-IL6 and AP-1 family transcription factors may give new insights

into interplay of these IL-6 regulated nuclear proteins. The objectives of experiments presented in this chapter are to investigate the interactions between NF-IL6 and AP-1 family proteins *in vitro* and *in vivo*.

RESULTS

NF-IL6 associates with Jun and with Fos

NF-IL6 and members of AP-1 family have been shown to be coordinately regulated by IL-6. These data suggest that NF-IL6 may associate with Fos or with Jun via the common bZIP region in the absence of DNA (Figure 7). To test the physical interaction between NF-IL6 and AP-1 family proteins *in vitro*, (i) bacterially expressed GST fusion proteins of NF-IL6 and Myc and (ii) Jun, Fos and Oct-1 proteins that were translated *in vitro* in the presence of ^{35}S-methionine were used in the coprecipitation assays (Figure 8A). In these assays, the ^{35}S-labeled protein was incubated with GST fusion protein and the protein complexes were precipitated with glutathione-Sepharose and analyzed by SDS-PAGE analysis. Analysis of protein complexes precipitated by glutathione-Sepharose showed that NF-IL6 directly associated with both Jun and Fos but not with the POU domain containing Oct-1 (Figure 8B, lanes 7 to 9). The association is specific to NF-IL6 because none of the three proteins precipitated with GST or with a GST-Myc fusion protein which contains a basic helix-loop-helix zipper structure (Figure 8B, lanes 4 to 6 and Figure 8C, lanes 3 and 4).

The NF-IL6 mRNA encodes two transcriptional activators (NF-IL6-1 and NF-IL6-2) and a repressor (NF-IL6-3). I therefore examined whether Fos and Jun can associate with any of the three NF-IL6 proteins selectively. NF-IL6-3 was found to associate efficiently with Jun and with Fos (Figures 8C, lane 5 to 8). The interaction took place even in the presence of 1% deoxycholate and 1% Triton X-100 (Figure 8C, lanes 9 and 10). These results indicate that the carboxy-terminal DNA-binding region that is shared by the three alternatively translated NF-IL6 proteins mediates cross-family association with Fos and with Jun.

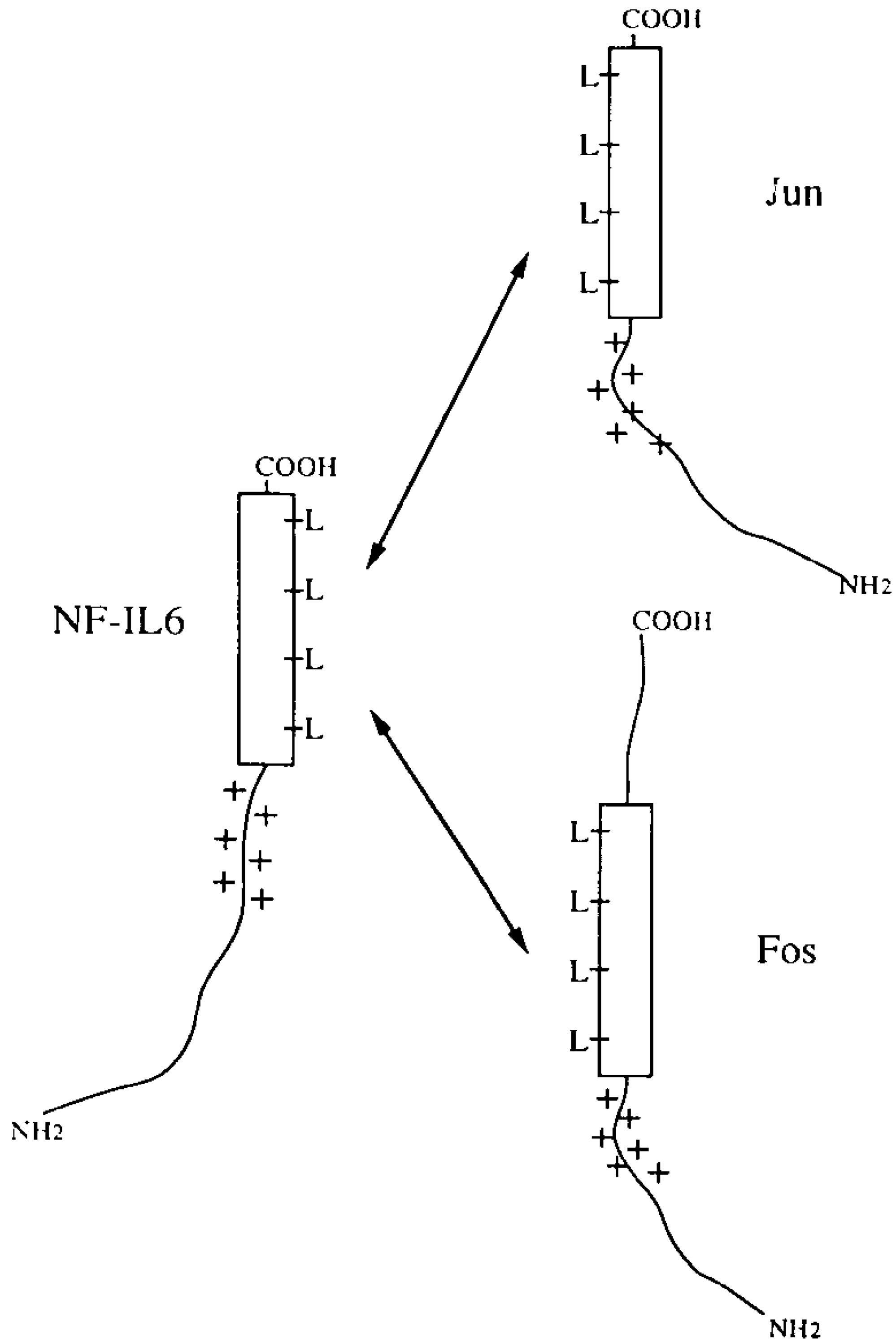


Figure 7. A diagram illustrating the potential interactions between NF-IL6 and AP-1 family proteins.

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

A

^{35}S-Protein
GST or GST-fusion proteins
Incubation
Precipitation by Glutathione-Sepharose
SDS-PAGE

B

Input GST GST-NF-IL6-1
Jun Fos Oct-1 Jun Fos Oct-1 Jun Fos Oct-1
— Oct-1
— Fos
— Jun
1 2 3 4 5 6 7 8 9

C

Input GST-Myc GST-NF-IL6-1 GST-NF-IL6-3 GST-NF-IL6-3
Jun Fos Jun Fos Jun Fos Jun Fos Jun Fos
— Fos
— Jun
1 2 3 4 5 6 7 8 9 10

D

1 24 199
AUG AUG AUG
LZ
(A)n
NF-IL6-1
NF-IL6-2
NF-IL6-3

The bZIP regions of NF-IL6 and AP-1 are necessary and sufficient for their association

The domain(s) of NF-IL6 that mediates association with Fos and with Jun was analyzed further by serial deletions of the carboxy-terminus of NF-IL6-2, using a GST fusion construct encoding residues 24 to 345 of NF-IL6 [GST-NF-IL6(24-345)] (Figure 9A). Deletion of the bZIP region in GST-NF-IL6(24-273) completely abolished its ability to associate with Jun (Figure 9B, lane 5). Progressive deletions of the carboxy-terminal part of NF-IL6-2 confirmed that the bZIP region is required for interaction with Jun (Figure 9B, lanes 6 to 8).

The association of NF-IL6 and Fos or NF-IL6 and Jun was investigated with an independent line of analysis. Immunoprecipitations were performed with anti-Jun or anti-Fos antibodies followed by immunoblotting with anti-NF-IL6 (α-P4). The α-P4 antibody specifically recognizes the amino-terminal portion of NF-IL6-1 and NF-IL6-2 and does not cross-react with other members of the C/EBP family (Figure 10, lane 11). NF-IL6 is expressed at a detectable level in HepG2 cells by direct immunoblotting analysis. In contrast, Jurkat cells do not express detectable amounts of NF-IL6 by immunoblotting analysis (Figure 10, lane 10). NF-IL6 was not detected by immunoblotting with anti-P4 antibody following immunoprecipitation of HepG2 nuclear extracts with an anti-Jun antibody, suggesting that either NF-IL6 does not associate with Jun in such experimental conditions or the abundance of NF-IL6-Jun complexes formed *in vivo* does not permit detection by this method (Figure 10, lane 4). Preincubation of the HepG2 nuclear extracts with either a nearly full length Jun (Figure 10, lane 8) or a truncated form of Jun containing the bZIP region (Figure 10, lane 9) resulted in the formation of NF-IL6-Jun complexes that could be precipitated by anti-Jun but not by anti-Oct-1 antibodies (Figure 10, lane 3). This association of NF-IL6 and Jun is attributed to NF-IL6 in the nuclear extracts, since preincubation of Jun proteins with Jurkat nuclear extracts did not give rise to any NF-IL6-Jun complexes (Figure 10, lane 5 to 7). Similarly, the bZIP region of Fos is sufficient for association with NF-IL6 in the HepG2 nuclear extracts (Figure 10, lane 12 to 14). Thus, by

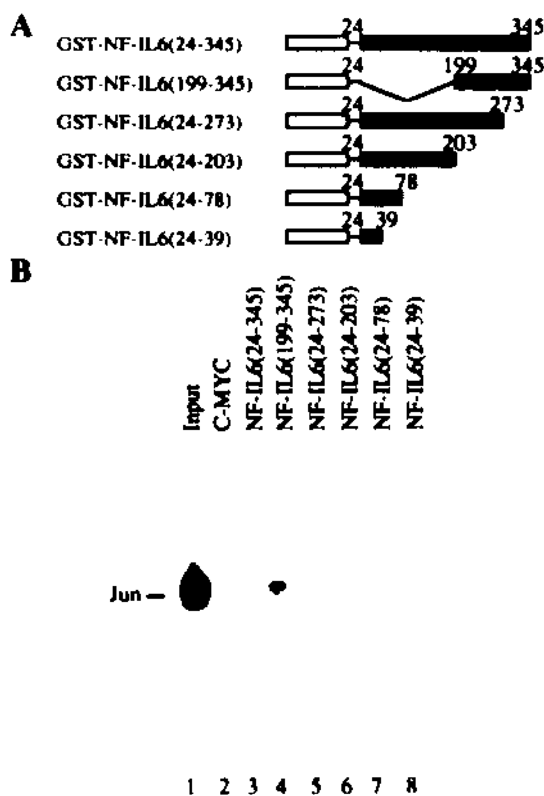


Figure 9. The bZIP region of NF-IL6 mediates association with Jun. **(A)** Schematic representation of the GST-NF-IL6-2 fusion protein [GST-NF-IL6(24-345)] and the deletion mutants. The open boxes represent GSTs, and the shaded boxes represent regions of NF-IL6 indicated by the amino acid residues of its coding region. **(B)** Analysis of the association of ^{35}S-methionine-labeled Jun with GST-NF-IL6 proteins diagrammed in Fig. 8A.

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

	1	2	3	4	5	6	7	8	9	10	11		12	13	14
NE	-	G2	G2	G2	JK	JK	JK	G2	G2	JK	G2		G2	G2	G2
Protein	J(2-334)	J(2-334)	J(2-334)	-	-	J(2-334)	J(225-334)	J(2-334)	J(225-334)	-	-		-	F(1-380)	F(118-211)
α-Jun	+	-	-	+	+	+	+	+	+	-	-	α-Fos	+	+	+
α-Oct-1	-	-	+	-	-	-	-	-	-	-	-		-	-	-

(Kd)

98 —
69 —
46 —
30 —
22 —
18 —

←NF-IL6→

1 2 3 4 5 6 7 8 9 10 11 12 13 14

two criteria, the bZIP regions of NF-IL6 and AP-1 family proteins mediate their direct physical interactions.

NF-IL6 binds to an ATF site, but Fos and Jun do not bind to an NF-IL6 site

The bZIP regions mediate the binding of NF-IL6 to consensus DNA sequences of 5'-T(T/G)NNGNAA(T/G)-3' (Akira et al., 1990) and the binding of AP-1 proteins to 5'-TGACTCA-3' (Lee et al., 1987a). Fos and Jun also bind to the ATF binding sites with reduced efficiency (Hai and Curran, 1991). The interactions of NF-IL6 with NF-IL6 and AP-1 recognition sequences were analyzed by electrophoretic mobility shift assays. The bacterially expressed recombinant GST-NF-IL6-3 protein was purified by a single-step purification procedure using glutathione-Sepharose (Smith et al., 1986, Johnson et al., 1989). All three NF-IL6 fusion proteins could be precipitated by glutathione-Sepharose as determined by silver staining or coomassie blue staining. Initial attempts to elute NF-IL6-1, NF-IL6-2, and NF-IL6-3 fusion proteins from glutathione-Sepharose gave very poor yields, possibly due to degradation of proteins. After the addition of several protease inhibitors in the elution buffer (Chapter II Materials and Methods), NF-IL6-3 fusion protein could be eluted and purified to greater than 90% homogeneity (Figure 11, lanes 4 and 5). However, elution of NF-IL6-1 and NF-IL6-2 by the same protocol was not successful, suggesting that the presence of the amino-terminal portion of NF-IL6 renders it more labile under these experimental conditions (data not shown). Recombinant NF-IL6-3 bound to two NF-IL6 sites present in the adenovirus E2 promoter [NF-IL6(E2); 5'-TTGAGAAAG-3'] and the IL-6 promoter, [NF-IL6(IL-6); 5'-TTGTGCAAT-3'] with comparable efficiency (Figure 12, lanes 1 to 5 and 11 to 14). NF-IL6 proteins also bound to an ATF/AP-1 site present in the adenovirus E2 promoter (termed ATF/NF-IL6*; 5'-ATGACGTAGT-3'), but less efficiently (Figure 12, lane 6 to 10).

Figure 11. Purification of recombinant GST-NF-IL6 fusion protein. Fractions from each step of the purification of GST-NF-IL6-3 were analyzed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue. Lane 1: Protein markers whose corresponding molecular masses (Kd) are indicated at their left side. Lane 2: IPTG-induced crude bacterial extracts. Lane 3: unbound fraction of glutathione-Sepharose. Lanes 4 and 5: Purified GST-NF-IL6-3 contained in the eluted fraction from glutathione-Sepharose. Lane 6: 0.1 μg BSA. Lane 7: 0.5 μg BSA. Lane 8: 1 μg BSA. Lane 9: 5 μg BSA.

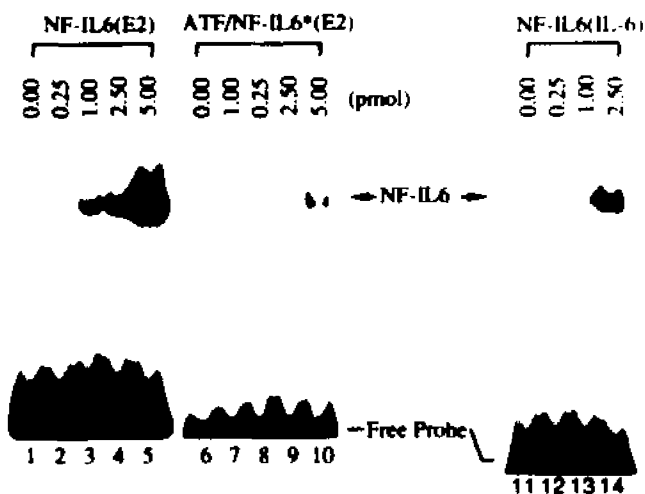


Figure 12. NF-IL6 binds to an ATF/NF-IL6* site with reduced efficiency. Electrophoretic mobility shift assay of NF-IL6-3 binding to the NF-IL6 sites of adenovirus E2 promoter [NF-IL6(E2)] and IL-6 promoter [NF-IL6(IL-6)], and the ATF/AP-1 site of adenovirus E2 promoter [ATF/NF-IL6*] was performed in increasing protein concentrations, as indicated. In each reaction, 70 pmol of oligonucleotide probes labeled with ^{32}P to comparable specific activity (1.3×10^5 cpm/nmol) was used.

DNase I footprint analysis showed that both the recombinant NF-IL6-2 (LAP) and a truncated form of recombinant C/EBPα (88 amino acids) containing the bZIP region recognized the NF-IL6 (N) and ATF/NF-IL6* (A) sites of the adenovirus E2 promoter (Figure 13). The nucleotides (sites) protected from DNase I digestion (indicated by bracket) by NF-IL6-2 and C/EBPα were extended beyond their predicted core sequences (open boxes) on the NF-IL6 and ATF/NF-IL6* sites of both coding and noncoding DNA strands. Compared with NF-IL6-2, the bZIP region of C/EBPα appeared to generate two DNase I hypersensitive sites at the 5'-boundary of the ATF/NF-IL6* (A) site on the noncoding strand (Figure 13, marked with an arrow head). On the coding strand, the bZIP region of C/EBPα also generated DNase I hypersensitive sites at the 5'-boundary of the ATF/NF-IL6* (A) and NF-IL6 (N) sites (Figure 13, marked with arrow heads). Different footprints generated from binding of NF-IL6-2 and the truncated form of C/EBPα containing the bZIP region imply that the amino-terminal region of NF-IL6 may contribute to DNA recognition. These results are in agreement with those obtained by electrophoretic mobility shift assays (Figure 12) and confirm that NF-IL6 can bind to both the NF-IL6 and ATF/NF-IL6* sites of the E2 promoter.

Binding of Fos and Jun to these two sites was examined by the use of truncated recombinant Fos and Jun containing their bZIP regions and full length Fos and Jun translated *in vitro*. Fos-Jun heterodimers and Jun-Jun homodimers were able to bind to the ATF/NF-IL6* site (Figure 14A, lanes 2 and 5). The participation of Fos and Jun in the complexes was confirmed by the inhibition of DNA-protein complex formation by anti-Fos and anti-Jun antibodies (Figure 14A, lanes 6 and 7). In contrast, Fos and Jun did not bind to the NF-IL6 site (Figure 14A, lane 8 to 11). Identical results were obtained with full length Fos and Jun proteins translated *in vitro* (Figure 14B). The inability of Fos and Jun to bind to the NF-IL6 sites is therefore an intrinsic property of this protein-DNA interaction.

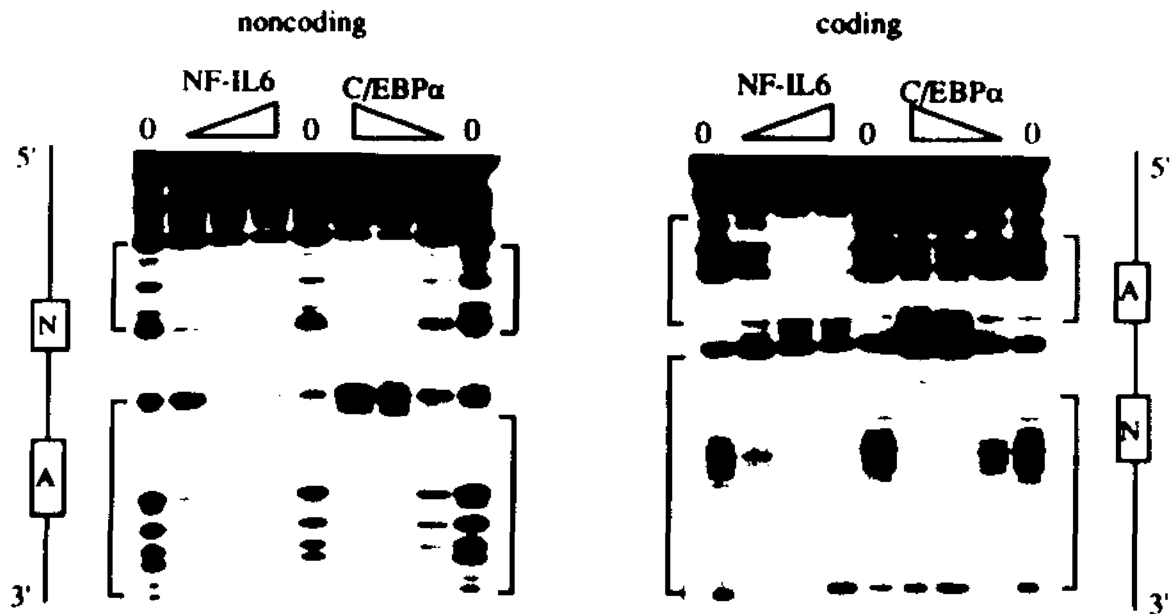


Figure 13. DNase I footprint analysis of the adenovirus E2 promoter. Protection of the E2 promoter from DNase I digestion was performed with purified recombinant LAP (NF-IL6-2; obtained from U. Schibler), or a truncated form of C/EBPα containing the bZIP region (obtained from S. McKnight), in increasing protein concentrations as indicated by triangles. Both the coding and noncoding DNA strands were analyzed. Regions protected by NF-IL6 and C/EBPα are indicated by brackets, and the predicted NF-IL6 (N) and ATF/NF-IL6* (A) binding sites are shown in boxes.

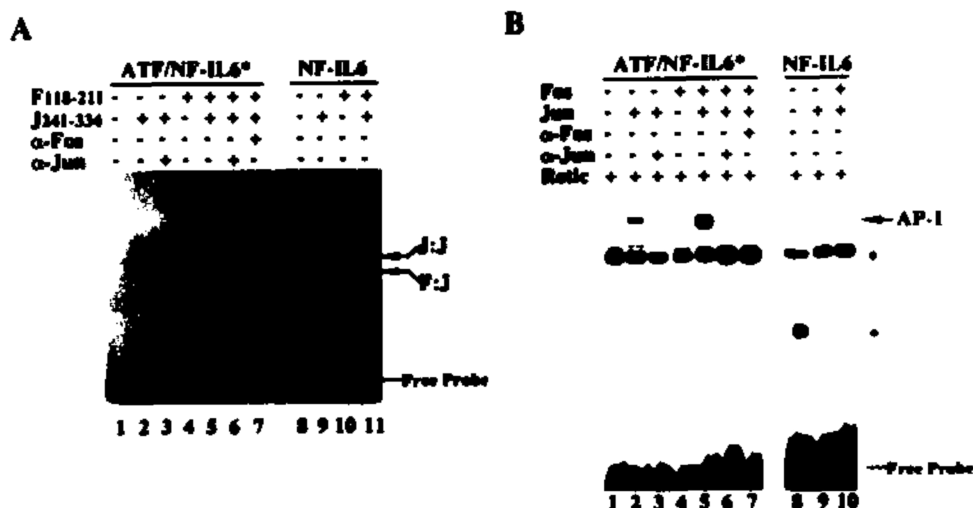


Figure 14. Fos and Jun bind to the ATF/NF-IL6* site but not to the NF-IL6 site. **(A)** Electrophoretic mobility shift assay of binding of recombinant Fos and Jun proteins (2.5 pmol) to the ATF/NF-IL6* and NF-IL6 sites of the E2 promoter. F118-211 and J241-334 are the truncated forms of Fos and Jun proteins containing their bZIP regions. Oligonucleotide probes and α-Fos and α-Jun antisera are described in Chapter II Materials and Methods. The positions corresponding to the mobilities of Jun-Jun homodimers and Fos-Jun heterodimers in the native gel are marked as J:J or F:J, respectively. **(B)** Electrophoretic mobility shift assay of binding of full length Fos and Jun proteins translated *in vitro* (0.3 fmol) to the DNA probes used for panel A. The mobilities of Jun-Jun and Fos-Jun dimers are indistinguishable in the gel and marked as AP-1. The nonspecific binding activities from reticulocyte lysates (Retic) are indicated as asterisks (*).

Alteration of NF-IL6 binding specificity by interaction with Fos and Jun

Cross-family dimerization of Fos and Jun with ATF family proteins generated a novel DNA-binding specificity (Hai and Curran, 1991). This suggests that the DNA-binding activity of NF-IL6 may be altered by association with Fos and with Jun. Binding of NF-IL6-3 to two NF-IL6 sites, to which Fos and Jun can not bind, was reduced in the presence of recombinant truncated Fos and Jun containing their bZIP regions in a concentration dependent manner (Figure 15, lanes 2 to 6 and 16 to 20). Consistent with dimerization with NF-IL6 via the leucine zipper region, Fos and Jun mutants containing deletions in the basic region were as effective as the wild-type proteins in reducing the NF-IL6 DNA-binding activity (Figure 15, lanes 7 to 11 and 21 to 25). Association of NF-IL6 with Fos or Jun via the leucine zipper region therefore leads to altered DNA-binding specificity of NF-IL6.

Fos and Jun repressed transcription activation by NF-IL6

NF-IL6-1 and NF-IL6-2 likely function as transcription activators and NF-IL6-3 as a repressor, analogous to their rodent counterparts. Reduction of NF-IL6 binding to the NF-IL6 site may lead to reduction or enhancement of transcription mediated by this site, depending on which of the functionally distinct NF-IL6 isoforms is sequestered by Fos and Jun. The functional properties of NF-IL6 proteins and the influence of Fos and Jun were investigated by transient transfections in NT2/D1 cells (Figure 16). NF-IL6-3 is not detected and NF-IL6-2, Fos and Jun are expressed at an extremely low level in these cells. As expected, expression of NF-IL6-2 activates transcription of a reporter CAT gene linked to four tandemly repeated copies of the NF-IL6(IL-6) site (4xNF-IL6-CAT) (Figure 16A). This activation can be down-regulated by the expression of NF-IL6-3 (Figure 16A). Consistent with their inability to bind to this site (Figure 15B), Fos and Jun did not activate the NF-IL6 site directed transcription (Figure 16A). Fos and Jun, however, reduced transcriptional activation by NF-IL6-2 in a concentration dependent manner (Figure 16B).

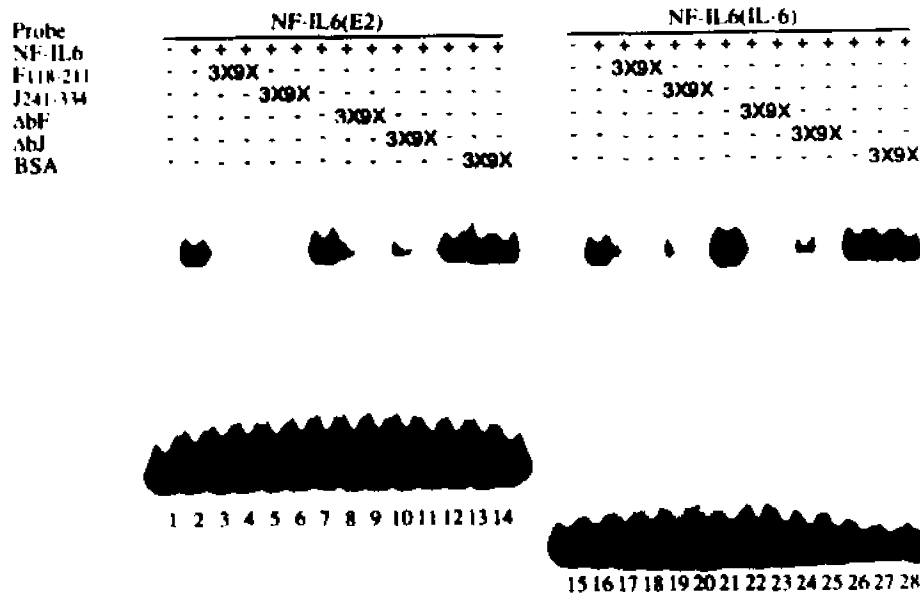


Figure 15. The NF-IL6 DNA-binding activity is reduced by Fos and Jun. Binding of purified recombinant NF-IL6-3 (2.5 pmol) to the NF-IL6(E2) and NF-IL6(IL-6) oligonucleotide probes (70 pmol) was performed in the presence (+) or absence (-) of purified recombinant Fos and Jun proteins. F118-211 and J241-334 are as describe in Figure 12. ΔbF is a derivative of F118-211 which lacks amino acid residues 139 to 144 in the basic region. ΔbJ is a derivative of J241-334 with the deletion of amino acid residues 260 to 266 in the basic region. 3x and 9x represent 3 and 9 times of the molar concentration of NF-IL6-3. BSA is used as a control.

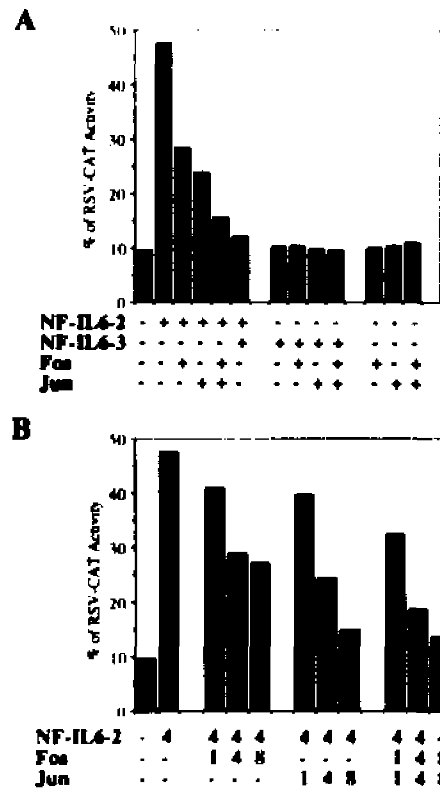


Figure 16. Fos and Jun repress transcription activation by the activator form of NF-IL6. **(A)** The reporter plasmid, 4xNF-IL6-CAT, was cotransfected with pCMV-NF-IL6-2 or pCMV-NF-IL6-3, with or without pCMV-fos and pCMV-jun, into NT2/D1 cells. In each reaction, 10^6 cells were transfected with 20 μg of DNA including 4 μg of 4xNF-IL6-CAT, 4 μg of each of the effector plasmids as indicated (+), and supplemented amounts of carrier DNA, pBluescript. **(B)** The reporter plasmid, 4xNF-IL6-CAT (4 μg), was cotransfected with pCMV-NF-IL6-2 (4 μg) and pCMV-fos or pCMV-jun, or both, in amounts as indicated. In each transfection, a total of 30 μg of DNA and 10^6 cells were used.

These results suggest that NF-IL6 functions as an activator or repressor, depending on the ratio of the isoforms in human cells. Correlated with diminution of the NF-IL6 DNA-binding activity by association with Fos and with Jun, NF-IL6 site dependent transcription activation by the activator forms of NF-IL6 is antagonized by Fos and Jun.

NF-IL6 homodimers and NF-IL6-Fos heterodimers bind to an AP-1 site

Fos and Jun can not bind to most NF-IL6 sites tested so far. Whether NF-IL6 can bind to an AP-1 site was examined by electrophoretic mobility shift assay, using purified NF-IL6-3 and as references recombinant truncated Fos and Jun that contain their bZIP regions (F118-211 and J241-334). NF-IL6 apparently bound to the AP-1 site of the collagenase promoter less efficiently than to the NF-IL6 site of the E2 promoter (Figure 17). Because NF-IL6 also bound to the ATF/NF-IL6* site of the E2 promoter, to which Fos and Jun bound with reduced efficiency (Figure 12), these results suggest that NF-IL6 can bind to the AP-1 binding sites.

Association of NF-IL6 with Fos and Jun reduces the binding of NF-IL6 to the NF-IL6 sites of the IL-6 and E2 promoters (Figure 15). Whether NF-IL6-Fos and NF-IL6-Jun heterodimers can bind to an AP-1 site was examined, using the AP-1 site of the collagenase promoter as a probe. Although NF-IL6 and Jun homodimers compete for binding to the AP-1 site, the formation of DNA-protein complexes containing NF-IL6 and Jun heterodimers was not observed (Figure 18, lane 6 to 8). The NF-IL6-Fos heterodimers (N:F), however, appeared to bind to the AP-1 site as shown by the faster migrating DNA-protein complex (Figure 18A, lane 3 to 5). Binding of NF-IL6-Fos heterodimers to the AP-1 site was dependent on the concentrations of both partners because formation of the NF-IL6-Fos complex (N:F) was enhanced with increasing amounts of either NF-IL6 or Fos (Figure 18B, lane 2 to 9). The sequence specificity for NF-IL6-Fos heterodimers was examined by the use of the ATF/NF-IL6* site. NF-IL6-Fos heterodimers failed to bind to the ATF/NF-IL6* site (Figure 19, lane 6), whereas NF-IL6 and Jun homodimers and Fos-Jun

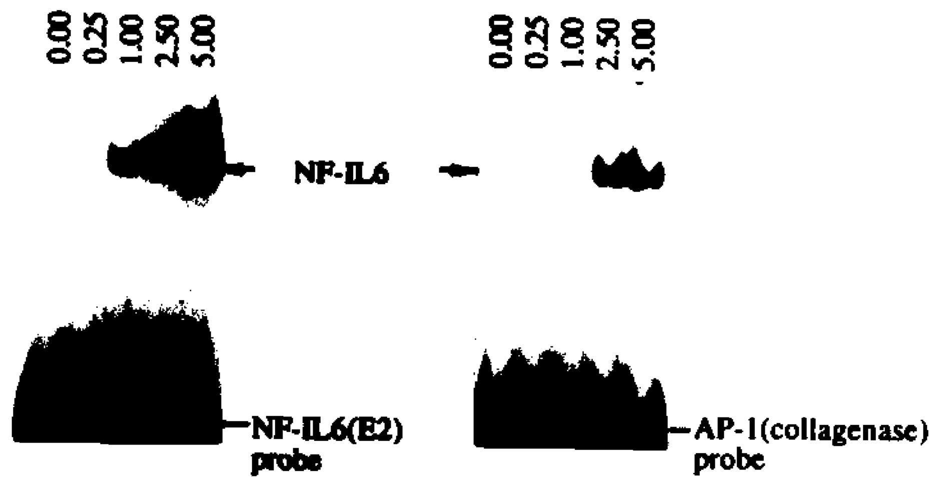
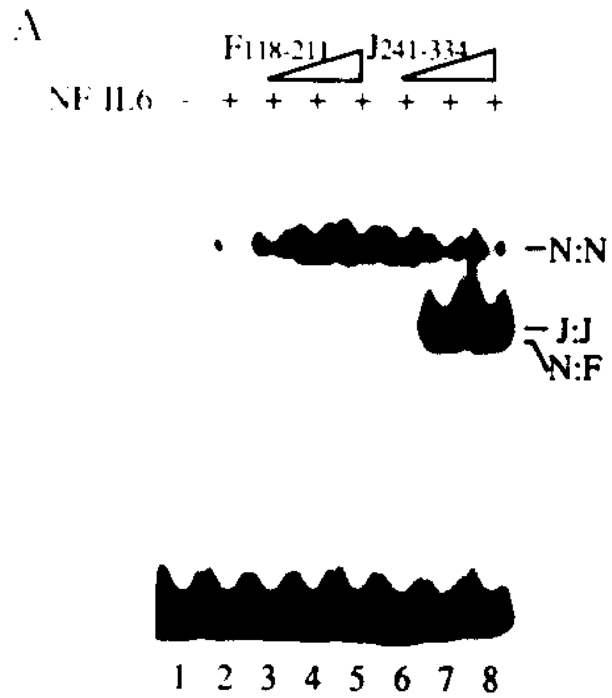
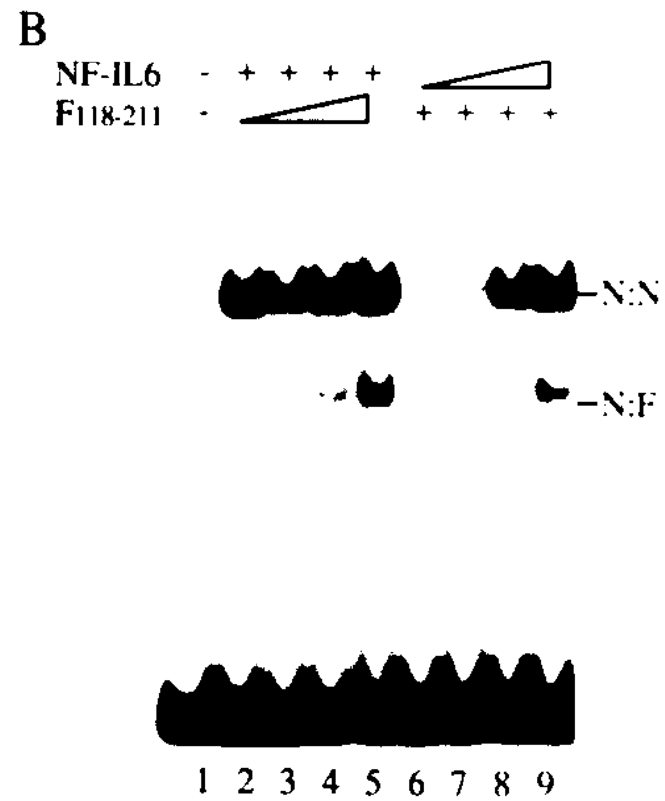


Figure 17. NF-IL6 binds to the AP-1 site with reduced efficiency. Electrophoretic mobility shift assay of binding of NF-IL6-3, in increasing concentrations as indicated, to an oligonucleotide probe (65 pmol) which contains the AP-1 site of the collagenase promoter (AP-1) or the NF-IL6 site of the E2 promoter (NF-IL6). The two probes have comparable specific activity (1.3×10^5 cpm/pmol).

Figure 18. NF-IL6-Fos heterodimers bind to an AP-1 site. **(A)** Binding of purified recombinant NF-IL6-3 (2.5 pmol), as indicated with (+), to an oligonucleotide probe (65 pmol) containing the AP-1 site of the collagenase promoter was performed in the presence of increasing amounts of purified truncated recombinant Fos polypeptide corresponding to amino acid residue 118 to 211 (F118-211) (lanes 3, 0.5 pmol, 4, 1.5 pmol and 5, 2.5 pmol), or Jun polypeptide corresponding to amino acid residue 241 to 334 (J241-334) (lanes 6, 0.5 pmol, 7, 1.5 pmol, and 8, 2.5 pmol). **(B)** Binding of NF-IL6-Fos heterodimers to the AP-1 site was performed by using NF-IL6-3 (2.5 pmol) and increasing amounts of F118-211 (lanes 2 to 5, 0 to 2.5 pmol), or using F118-211 (2.5 pmol) and increasing amounts of NF-IL6-3 (lanes 6 to 9, 0 to 2.5 pmol). The migration of the protein-DNA complexes containing NF-IL6 homodimers, Jun homodimers, and NF-IL6-Fos heterodimers are indicated as N:N, J:J, and N:F, respectively. Electrophoretic mobility shift assay was performed as described in Chapter II Materials and Methods.


A
F118-211
J241-334
NF-IL6 - + + + + + + +
N:N
J:J
N:F
1 2 3 4 5 6 7 8


B
NF-IL6 - + + + +
F118-211 - + + + +
N:N
N:F
1 2 3 4 5 6 7 8 9

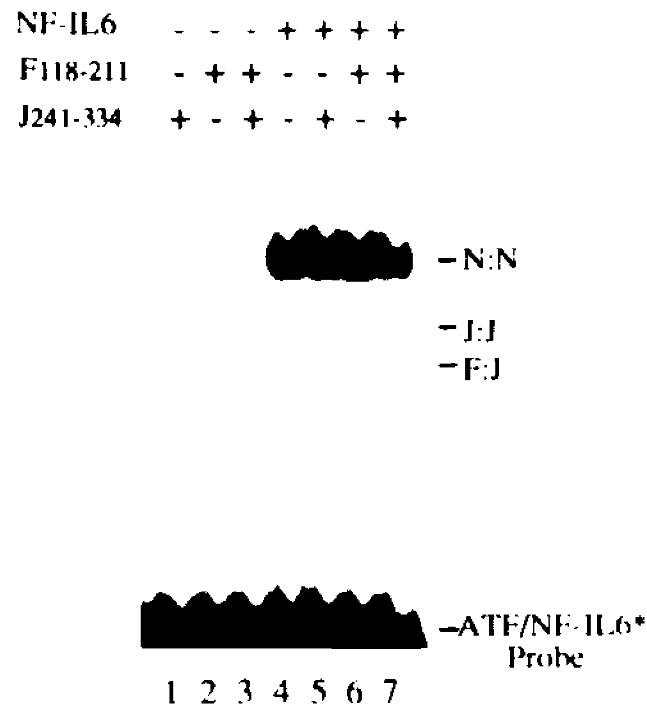


Figure 19. NF-IL6-Fos and NF-IL6-Jun heterodimers do not bind to the ATF/NF-IL6* site of the E2 promoter. Binding of NF-IL6-3 (2.5 pmol) in the presence of F118-211 (2.5 pmol) and/or J241-334 (2.5 pmol) as indicated (+), to an oligonucleotide probe (65 pmol) containing the ATF/NF-IL6* site of the E2 promoter was analyzed by electrophoretic mobility shift assay. N:N, J:J, and N:F represent the NF-IL6 homodimers, Jun homodimers, and NF-IL6-Fos heterodimers, respectively.

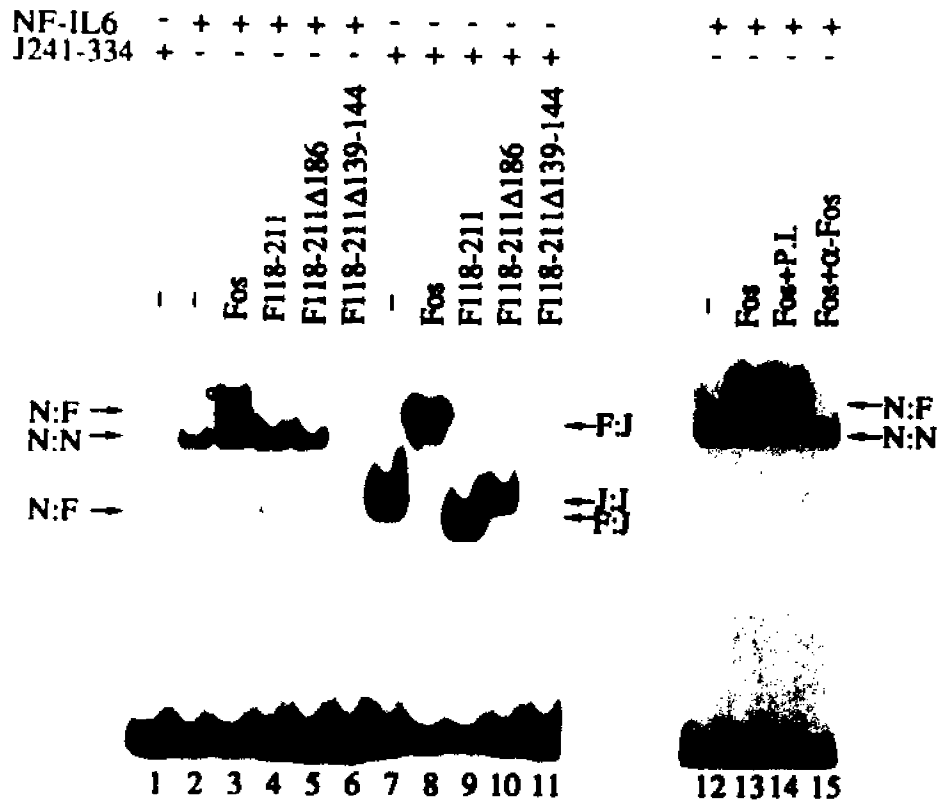
heterodimers can bind to this site (Figure 19, lanes 1,3, and 4). These results suggest that the DNA-binding property of NF-IL6-Fos heterodimers is distinguishable form that of NF-IL6-Jun heterodimers, and that NF-IL6-Fos heterodimers bind to the AP-1 sites selectively.

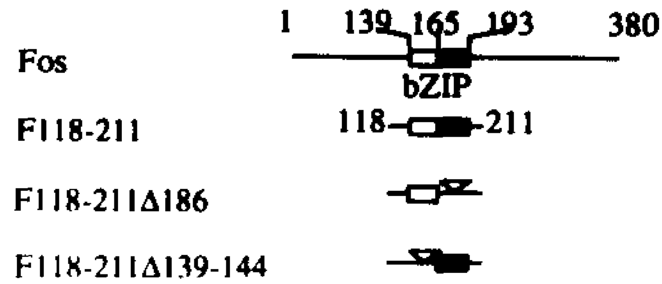
Binding of NF-IL6-Fos heterodimers to an AP-1 site is enhanced by residues outside the bZIP region

The bZIP regions of NF-IL6, Fos and Jun are sufficient for the cross-family association (Figures 9 and 10). To study the contribution of residues within and outside the bZIP region of AP-1 to the formation of stable DNA-protein complexes, I first confirmed that the bZIP region of Fos is sufficient for binding of NF-IL6-Fos dimers, and the control Fos-Jun dimers to the AP-1 site (Figure 20, lanes 4 and 9). A truncated Fos (F139-200) containing the minimal bZIP region is also sufficient for binding of NF-IL6-Fos dimers to the AP-1 site (data not shown). As predicted, deletion of the fourth leucine residue in the bZIP region of Fos (F118-211Δ186) changes the spacing between leucines and inhibits the dimerization between NF-IL6 and Fos, and Fos and Jun (Figure 20A, lanes 5 and 10). Deletion of six amino acids in the basic region of Fos (F118-211Δ139-144) abolished the binding of both NF-IL6-Fos and Fos-Jun heterodimers to the AP-1 site (Figure 20A, lanes 6 and 11). This deletion also reduced the binding of NF-IL6 homodimers to the AP-1 site, presumably due to the formation of NF-IL6-Fos heterodimers that are defective in binding to DNA (Figure 20A, lane 6). These results are in agreement with the deletion analysis of NF-IL6 and confirm that the bZIP region of Fos is necessary and sufficient to mediate the association with NF-IL6 and the binding of NF-IL6-Fos heterodimers to an AP-1 site.

The NF-IL6-Fos heterodimers that contain the full length Fos bound to the AP-1 site more efficiently than those containing a truncated Fos (Figure 20A, lanes 3 and 4). Two complexes in addition to the NF-IL6 homodimers were generated by interactions between the full length Fos and NF-IL6 (Figure 20, lane 3 and 13), as confirmed by the interference

Figure 20. Formation of NF-IL6-Fos heterodimers is dependent on the leucine zipper region. **(A)** Binding of NF-IL6-Fos or Fos-Jun heterodimers to a labeled oligonucleotide probe containing the AP-1 site of the collagenase promoter was analyzed by electrophoretic mobility shift assay using purified recombinant Fos protein and its mutant derivatives (Fos, F118-211, F118-211Δ186, and F118-211Δ139-144), NF-IL6-3 and J241-334. Equal molar amounts (2.5 pmol) of the NF-IL6-3, J241-334, and Fos derivatives were used in each reaction. **(B)** Schematic representation of the Fos derivatives used in panel A. The numbers represent the corresponding positions of amino acid residues present in the Fos protein. The basic (open box) and leucine zipper (hatch box) were as indicated (bZIP). N:N, NF-IL6 homodimers; N:F, NF-IL6-Fos heterodimers; F:J, Fos-Jun heterodimers; o, low mobility complex.

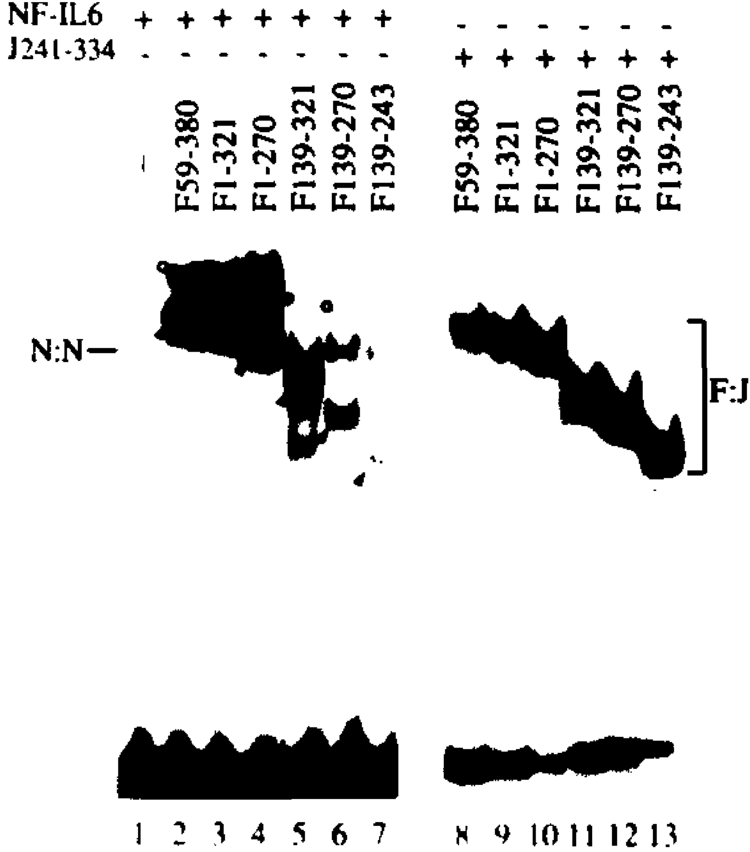

A
NF-IL6 - + + + + + - - - - - + + + +
J241-334 + - - - - - + + + + + - - - -
-
-
Fos
F118-211
F118-211Δ186
F118-211Δ139-144
-
Fos
F118-211
F118-211Δ186
F118-211Δ139-144
-
Fos
Fos+P.I.
Fos+α-Fos
N:F
N:N
N:F
F:J
J:J
F:J
N:F
N:N
1 2 3 4 5 6 7 8 9 10 11
12 13 14 15

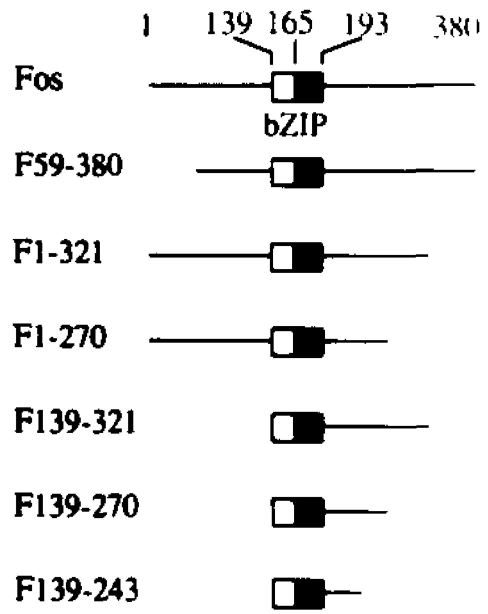

B
1
139
165
193
380
Fos
bZIP
F118-211
118-
-211
F118-211Δ186
F118-211Δ139-144

of complex formation by an anti-Fos antibody (Figure 20A, lane 15). The faster migrating species of the two complexes is likely to be a NF-IL6-Fos heterodimer because there is no indication of degradation of Fos in the control reaction that contains Fos-Jun heterodimers (Figure 20A, lane 8). The low mobility complex (marked with a o) may be due to the formation of a multimeric complex containing NF-IL6 and Fos. This remains to be investigated. These data suggest that although the bZIP region of Fos is sufficient for association with NF-IL6, formation of stable DNA-protein complexes may be enhanced by amino acid residues outside the bZIP region.

To deduce the amino acid residues outside the bZIP region of Fos that contribute to the cross-family interaction, a panel of amino- and carboxy-terminal deletion mutants of Fos were analyzed. The experimental conditions were modified by the inclusion of poly(dIdC) in the both protein association and DNA-binding reactions, since this modification apparently stablized NF-IL6-Fos heterodimers and reduced nonspecific binding of NF-IL6 homodimers to the AP-1 site. Similar to the results obtained with the full length Fos and NF-IL6-3 (Figure 20A, lane 3), F59-380, F2-321 and F2-270 all formed two stable DNA-protein complexes in molar excess of the NF-IL6 homodimers (N:N) (NF-IL6-Fos heteromers were marked with arrow heads and the low mobility complexes marked with o's) (Figure 21A, lane 2 to 4). F139-321, which lacks the amino-terminal portion of Fos, could form stable NF-IL6-F139-321 heterodimers but could not form the low mobility complex with NF-IL6-3 (Figure 21A, lane 5). These data suggested that the region between amino acid 59 and 139 of Fos contribute to the formation of the low mobility complex. Deletion of 51 amino acid residues between 270 to 321 in the carboxy-extension of Fos markedly reduced the formation of stable NF-IL6-Fos heterodimers (Figure 21A, lane 6; marked with a arrow head). This result was confirmed by an additional deletion of 27 amino acid residues in F139-243 (Figure 21A, lane 7). As controls, all the mutants Fos and J241-334 heterodimers bound to the AP-1 site with comparable efficiency, and did not form any additional complexes (Figure 21A, lane 8 to 13). These data suggest that amino acid residues outside

Figure 21. Regions outside the bZIP domain of Fos enhance the binding of NF-IL6-Fos heterodimers to the AP-1 site. **(A)** Electrophoretic mobility shift assay of binding of NF-IL6-Fos and Fos-Jun heterodimers to a labeled probe containing the AP-1 site. The amino acid residues of the Fos mutants are indicated. Equal molar amounts (2.5 pmol) of the NF-IL6-3, J241-334, and Fos mutant proteins were used in each reaction. The binding conditions were modified to increase the stringency of protein-DNA interaction. One microgram of poly(dIdC) was added prior to the protein association reaction at 37°C for 15 min. Arrow head, NF-IL6-Fos heterodimers; o, low mobility complex of NF-IL6 and Fos; F:J, Fos-Jun heterodimers. **(B)** Schematic representation of the Fos mutant proteins used in panel A. The numbers represent the corresponding positions of amino acid residues present in the Fos protein. The basic (open box) and leucine zipper (hatch box) were as indicated (bZIP).


A
NF-IL6
J241-334
F59-380
F1-321
F1-270
F139-321
F139-270
F139-243
N:N
F:J
1 2 3 4 5 6 7
8 9 10 11 12 13


B
1
139
165
193
380
Fos
bZIP
F59-380
F1-321
F1-270
F139-321
F139-270
F139-243

of the bZIP region either influence the association of Fos with NF-IL6, or the binding of NF-IL6-Fos complexes to the AP-1 site, or both.

Modulation of AP-1 transcriptional activity by NF-IL6

Cross-family dimerization between NF-IL6 and AP-1 family transcription factors resulted in down-regulation of NF-IL6 site mediated transcriptional activation due to reduction of binding of NF-IL6 to the NF-IL6 sites (Figures 15 and 16). The regulation of AP-1 transcriptional activity by NF-IL6 was investigated in NT2/D1 cells by transient transfections. Transcription of a reporter CAT gene linked to two tandemly repeated copies of the AP-1 site (p2XAP-1-CAT) was activated 2-fold by Jun expressed from the cotransfected pCMV-jun, and 4-fold by Fos and Jun expressed from the cotransfected pCMV-jun and pCMV-fos (Figure 22). Activation by Jun, or Fos and Jun, was inhibited by NF-IL6-3, an inhibitory form of NF-IL6 (Figure 22). In contrast, the activator form of NF-IL6, NF-IL6-2, had no effect on the transcriptional activity of Fos and Jun. Therefore, NF-IL6 modulates AP-1 site mediated transcription according to the transcriptional properties of the NF-IL6 isoforms.

NF-IL6 and Jun are coordinately induced by IL-6

M1 myeloid leukemia cells were used as a model to examine the physiological significance of interactions between NF-IL6 and AP-1 proteins. M1 cells undergo macrophage differentiation and growth arrest in response to IL-6 induction (Lord et al., 1993). The steady state mRNAs of NF-IL6 and members of Jun family have been shown to be increased by IL-6 induction (Natsuka et al., 1992, Lord et al., 1993). To investigate the simultaneous regulation of NF-IL6 and AP-1 by IL-6, RNAs isolated from M1 cells following IL-6 induction were analyzed by Northern (RNA) blotting. The levels of NF-IL6 and Jun mRNAs increased concomitantly, to 9-fold (Jun) and 55-fold (NF-IL6) above those of uninduced cells by 48 hours (Figure 23). The elevation of JunB mRNA was similar to

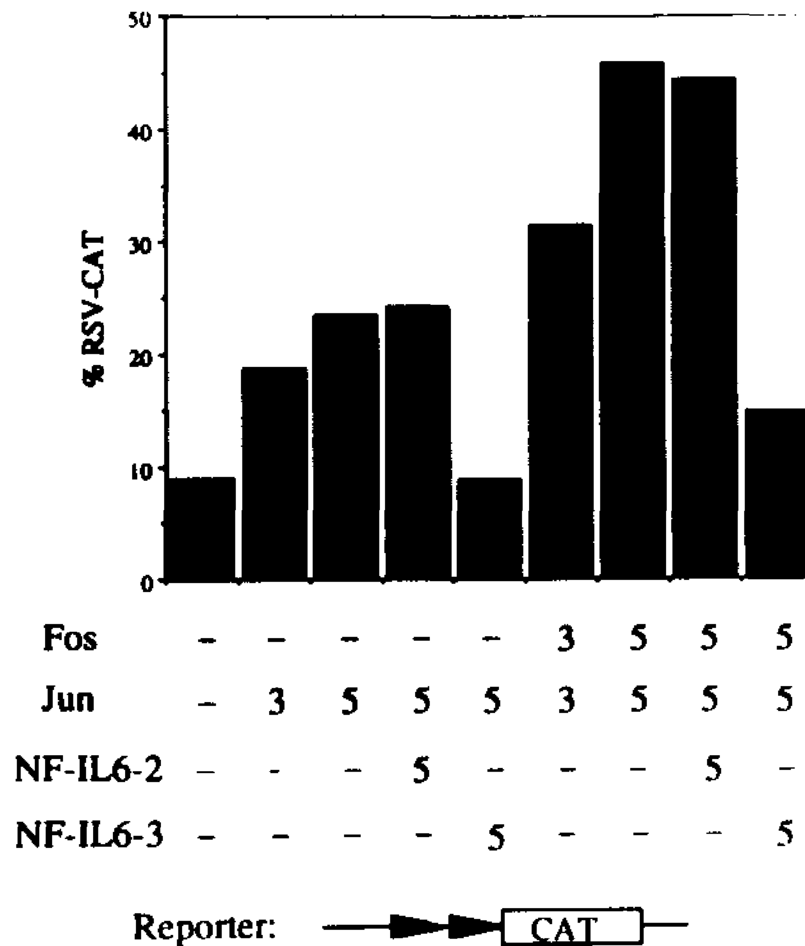


Figure 22. AP-1 site dependent transcriptional activation is modulated by NF-IL6. The reporter plasmid, p2xAP-1-CAT, was cotransfected with pCMV-*jun* (Jun), pCMV-*fos* (Fos), CMV-NF-IL6-2 (NF-IL6-2), and CMV-NF-IL6-3 (NF-IL6-3) into NT2/D1 cells, singly or in combination as indicated. In each reaction, 10^6 cells were transfected with 20 μg of total DNA, which includes 2 μg of the reporter plasmid, 3 or 5 μg of the expression plasmids as indicated, and carrier pBluescript DNA. The cells were harvested at 48 hours after transfection and assayed for CAT activity, expressed as % of RSV-CAT, as described in Chapter II Materials and Methods. The results are representative of three experiments. RSV-CAT is a reference plasmid that contains the long terminal repeat of Rous sarcoma virus linked to the CAT reporter gene.

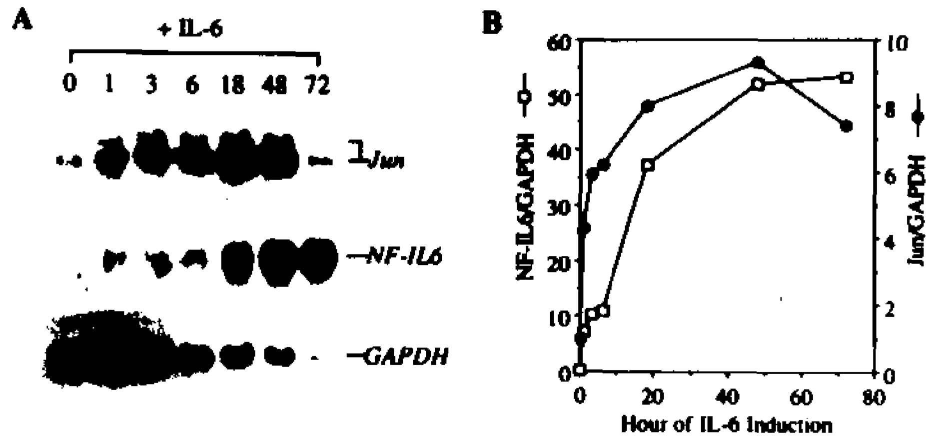


Figure 23. NF-IL6 and Jun mRNAs are coordinately induced by IL-6. (A) Total RNA was isolated from 10^6 M1 cells that were cultured without (0) or with IL-6 for the time (in hours) indicated and analyzed by Northern blotting as described in Chapter II Materials and Methods. The blot was hybridized sequentially with ^{32}P-labeled probes specific to NF-IL6 (the *SmaI-BamHI* fragment of pBS(SK)NF-IL6-1), jun (full length c-jun cDNA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which serves as a control for RNA loading. (B) The hybridization signals were quantified by densitometry scanning of the autoradiographics shown in panel A and expressed as the ratios of NF-IL6 to GAPDH and Jun to GAPDH as a function of time after IL-6 induction.

that of Jun mRNA (data not shown). However, Fos mRNA was not detected nor induced by IL6 (data not shown).

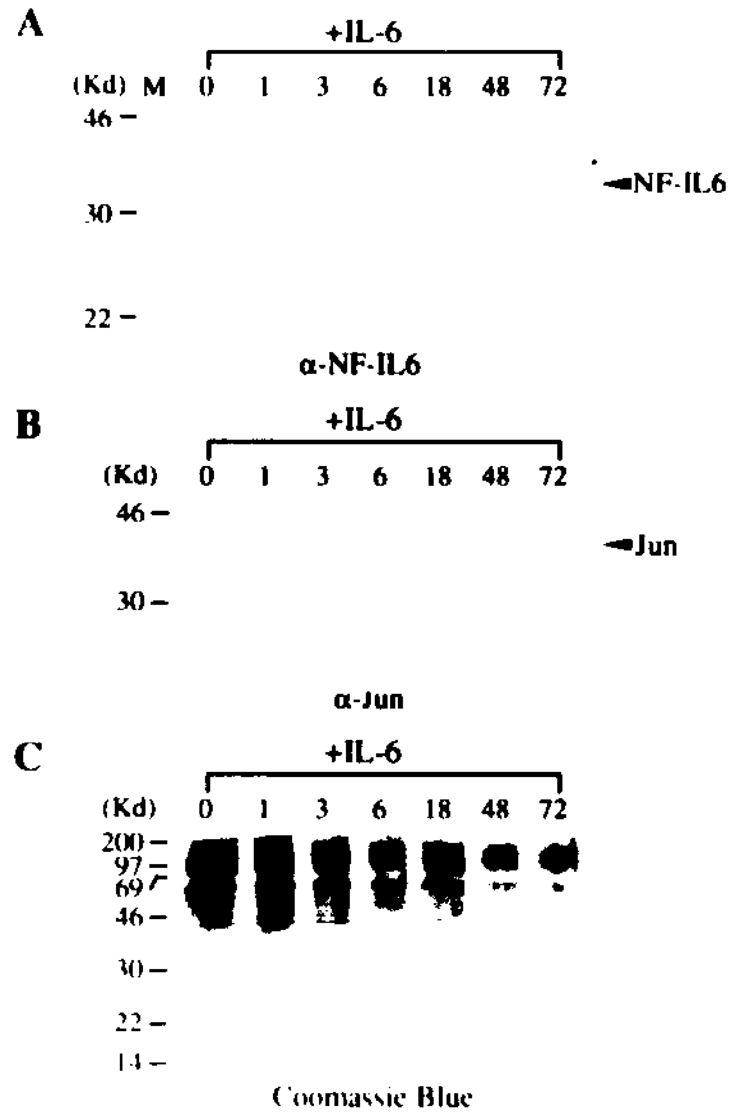
Increases of NF-IL6 mRNA appear to be biphasic. The initial increase occurred within an hour after IL-6 induction, which was followed by a second increase occurring between 6 and 18 hours (Figure 23). Although the significance of this reproducible pattern of regulation remains to be elucidated, the rapid activation of NF-IL6 suggests that it is an immediate-early response gene. Unlike transient activation of most immediate-early response genes by growth factors, the elevated levels of NF-IL6 and Jun mRNA are maintained in the presence of IL-6.

The activations of NF-IL6 and Jun mRNAs in M1 cells by IL-6 induction may also result in elevated levels of proteins. Corresponding to the enhancement at mRNA level, NF-IL6 and Jun proteins were induced in M1 cells by IL-6 induction as examined by immunoblotting analysis (Figure 24A and B). Once induced, the levels of NF-IL6 and Jun proteins were also maintained at elevated levels in the presence of IL-6.

IL-6 regulates association of NF-IL6 and Jun *in vivo*

As discussed earlier, NF-IL6 can associate with Jun and with Fos via their bZIP regions *in vitro*. The coordinated increases of both NF-IL6 and Jun proteins in M1 cells after IL-6 stimulation raise the possibility that they may form stable complexes *in vivo*. I initially attempted to investigate the interaction of NF-IL6 and Jun *in vivo* by coimmunoprecipitation. Antibodies directed against NF-IL6 (α-NF-IL6) and Jun (α-Jun) were used in immunoprecipitation of whole cell lysates prepared from ^{35}S-methione-labeled M1 cells. SDS-PAGE analysis of anti-Jun immunoprecipitates revealed a predominant protein with a relative molecular mass of 39 Kd, which was consistent with the predicted molecular mass of Jun and was not precipitated by the preimmune serum (Figure 25, lanes 3 and 6). In agreement with result obtained by immunoblotting analysis, induction of M1

Figure 24. Coordinate increases of NF-IL6 and Jun proteins by IL-6. Whole cell extracts were prepared from M1 cells cultured in the absence (0) or presence of IL-6 (hours) as indicated. The extracts (25 μg) were then analyzed by immunoblotting with an anti-LAP antibody (α-NF-IL6) which recognizes the rodent counterpart of the NF-IL6 in panel A, or with an anti-Jun antibody (α-Jun) in panel B. "M" represents protein molecular mass standards (Kd). (C) Coomassie blue stained SDS-polyacrylamide gel (12%) that contains the fractionated cell extracts used in panels A and B.


A
+IL-6
(Kd) M 0 1 3 6 18 48 72
46
30
22
NF-IL6
α-NF-IL6
B
+IL-6
(Kd) 0 1 3 6 18 48 72
46
30
Jun
α-Jun
C
+IL-6
(Kd) 0 1 3 6 18 48 72
200
97
69
46
30
22
14
Coomassie Blue

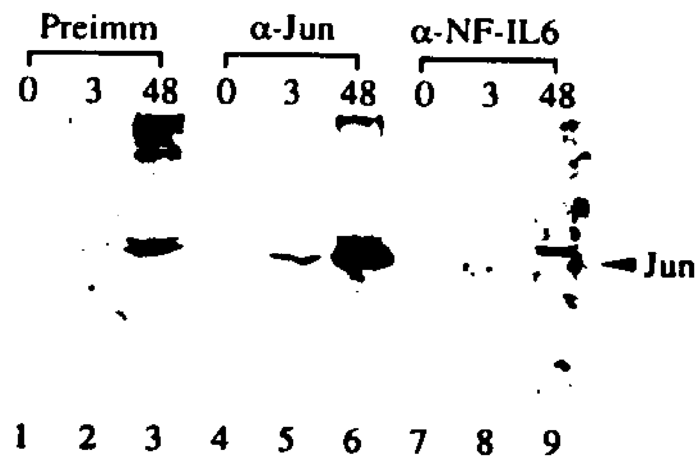


Figure 25. Jun is identified by immunoprecipitation of ^{35}S-methionine-labeled proteins *in vivo*. M1 myeloid leukemia cells (10^6) were uninduced (0) or induced with IL-6 for 3 or 48 hours as indicated and analyzed by metabolic labeling and immunoprecipitation. Jun and NF-IL6 proteins were immunoprecipitated from ^{35}S-methionine-labeled M1 ($2x10^6$ cells) cells using anti-Jun (α-Jun) or anti-NF-IL6 (LAP) (α-NF-IL6) antiserum in the RIPA buffer as described in Chapter II Materials and Methods. A preimmune serum (Preimm) served as a control. The immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography. The Jun protein identified in M1 cells is indicated.

cells with IL-6 resulted in an increase in the amounts of precipitated Jun (Figure 25, lane 4 to 6). Precipitates by anti-NF-IL6(LAP) antibodies, however, did not yield any prominent protein bands, suggesting that the polyclonal antibodies did not recognize NF-IL6 proteins in their native conformation (Figure 25, lane 7 to 9).

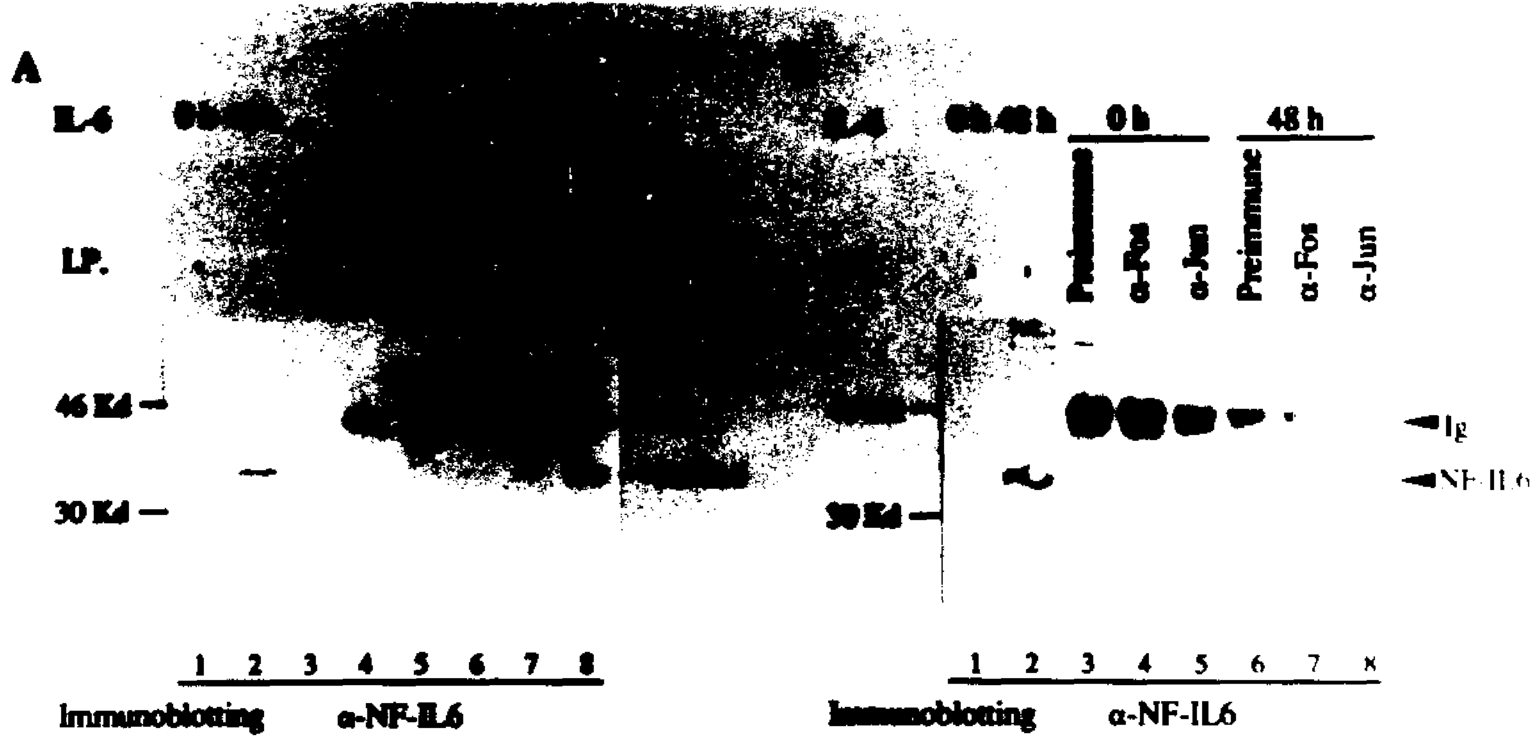
Immunoprecipitation-immunoblotting analysis was used as an alternative approach to analyze the NF-IL6 and Jun association *in vivo*. Whole cell lysates from M1 cells, with or without IL-6 induction, were immunoprecipitated with an anti-Jun antibody (α-Jun), followed by immunoblotting analysis of the immunoprecipitates with an anti-NF-IL6(LAP) antibody (α-NF-IL-6). NF-IL6 was identified in the protein complexes precipitated by anti-Jun antibody from M1 cells treated with IL-6 for 48 hours, but not from control M1 cells (Figure 26A and B, lanes 5 and 8). NF-IL6 was not detected in the protein complexes precipitated by preimmune and anti-Fos antibodies (Figure 26A, lane 7 and Figure 26B, lanes 6 and 7). The association of NF-IL6 and Jun correlates with coordinate elevations of both NF-IL6 and Jun by IL-6 induction. These results are in agreement with direct physical association between NF-IL6 and AP-1 family proteins observed in *in vitro* studies, and demonstrate that NF-IL6 can associate with Jun *in vivo*. Regulated formation of the NF-IL6-Jun complex *in vivo* by IL-6 implies a potential role for heterodimerization between NF-IL6 and AP-1 family proteins in IL-6 nuclear signaling pathway.

DISCUSSION

Cross-family dimerization between NF-IL6 and AP-1 family proteins requires the bZIP region

This study shows that NF-IL6 directly associates with Fos and with Jun *in vitro* in the absence of DNA (Figure 8). Deletion analyses demonstrated that the bZIP region of NF-IL6 was necessary and sufficient for this interaction (Figure 9). Truncated Fos and Jun containing only the bZIP regions were able to associate with NF-IL6 present in nuclear

Figure 26. IL-6 regulates the association of NF-IL6 and Jun *in vivo*. Whole cell extracts (100 μg) prepared from M1 cells without (0h) or with IL-6 treatment for 48 hours (48h) were immunoprecipitated (I.P.). In panel A, an anti-Jun antibody (α-Jun), a preimmune antibody (Preimmune), or no antibody (No Ab) was used in the reaction. In panel B, an anti-Fos (α-Fos), an anti-Jun (α-Jun), or a preimmune antibody (Preimmune) was used. The immunoprecipitates were analyzed by immunoblotting using an anti-NF-IL6 (LAP) antibody (α-NF-IL6) as described in Chapter II Materials and Methods. Lanes 1 and 2 in each panel represent direct immunoblotting of extracts (20 μg) from uninduced (0h) M1 cells or M1 cells induced with IL-6 for 48 hours (48h). The arrows indicate the migration of NF-IL6 protein (NF-IL6) and immunoglobulin heavy chain (Ig), which derives from the antibodies used in immunoprecipitation and cross-reacts with the alkaline phosphatase-conjugated second antibody.


A
IL-6
I.P.
46 Kd
30 Kd
0 h
48 h
Preimmune
α-Fos
α-Jun
Preimmune
α-Fos
α-Jun
Ig
NF-IL6
1 2 3 4 5 6 7 8
Immunoblotting α-NF-IL6
1 2 3 4 5 6 7 8
Immunoblotting α-NF-IL6

extracts prepared from HepG2 cells (Figure 10). In agreement with these data, investigations of the binding of the NF-IL6-Fos dimers to DNA showed that deletion of the bZIP region of Fos abolished its association with NF-IL6 (Figures 20). Mutational analyses of the bZIP region of Fos further suggest that NF-IL6 and AP-1 family proteins associate via the leucine zipper region through coiled-coil interaction.

The bZIP region is highly conserved among members of the C/EBP and AP-1 transcription factor families, raising the question as to the specificity for cross-family association. Formation of homo- and heterodimers of Fos and Jun is determined by the thermostability of the dimers (O'Shea et al., 1992). Fos-Jun dimers are most stable and Fos-Fos dimers are extremely unstable at physiological pH as a result of the presence of acidic residues. In addition, NF-IL6-3 appears to associate with Jun more stably than do the two larger forms of NF-IL6. This result implies that the amino-terminus of NF-IL6, which is lacking in NF-IL6-3 and less conserved between C/EBP members, may influence the association of NF-IL6 with Jun and with Fos.

Regions outside the bZIP domain of Fos appear to contribute to the formation of stable DNA-protein complexes (Figure 21). I have found that two regions outside the bZIP domain of Fos affect the binding of NF-IL6-Fos complexes to DNA. The first region, which is located at the carboxy-terminal portion of Fos, lies between amino acid residues 289 and 321 (Table 6). Deletion of this region greatly reduces the binding of NF-IL6-Fos dimers to the AP-1 site. This region coincides with a previously identified region (F270-321), which is rich in proline and acidic residues and can promote transactivation by Fos-Jun heterodimers (Abate et al., 1991). The second region lies between amino acid residues 59 and 139 at the amino-terminal portion of Fos (Table 6). The presence of this region not only enhances the binding of NF-IL6-Fos dimers to the AP-1 site but also promotes the formation of a low mobility complex in electrophoretic mobility shift assay (Table 6). The conformation and structural features of this complex remain to be determined.

1 139 165 193 380

bZIP

	Formation of NF-IL6-Fos heterodimer complex	Formation of low mobility complex
Fos	+++	++
F1-321	+++	++
F1-270	+++	++
F59-321	+++	++
F139-321	+++	-
F139-270	+	-
F139-255	+	-
F139-243	+	-
F139-200	+	-
F118-211	+	-
F118-211Δ186	-	-
F118-211Δ139-144	-	-

Table 6. A summary of Fos mutants and their abilities to dimerize with NF-IL6 and to form DNA-protein complexes.

Modulation of NF-IL6 and AP-1 transcriptional activities by cross-family association

Although NF-IL6 and AP-1 bind to DNA as dimers through their bZIP regions, their DNA-binding specificities are distinguishable (Figure 12, 13, and 14). NF-IL6 binds to most AP-1 sites, whereas Fos and Jun do not bind to most NF-IL6 sites (Figure 12, 13, and 14). Association of NF-IL6 with Fos or Jun resulted in a reduction of binding of NF-IL6 to two NF-IL6 sites (Figure 15). These data correlated with reduced transcription activation mediated by the activator form of NF-IL6 in transient transfection assays (Figure 16). Sequestration of NF-IL6 by Fos and Jun through dimerization may represent a mechanism for down-regulation of the NF-IL6 site mediated transcription (Figure 27).

Binding of NF-IL6 to the AP-1 site of the collagenase promoter and association of NF-IL6 with Fos and Jun imply that the AP-1 site can be redundantly or cooperatively regulated by NF-IL6 and AP-1 family transcription factors. Regulation of this type is reminiscent of that observed at composite DNA elements by AP-1 and glucocorticoid receptor (Dimond et al., 1990, Minor and Yamamoto, 1992, Kerppola et al., 1993). NF-IL6 comprises transcriptional activator and repressor forms. Activation of the AP-1 site dependent transcription by Fos and Jun is inhibited by NF-IL6-3, but not by the activator form of NF-IL6 (Figure 22). Regulation of the AP-1 site mediated transcription may therefore depend on the ratio of the functionally distinct NF-IL6 isoforms.

The precise mechanism by which NF-IL6-3 down-regulates the AP-1 transcriptional activity is not clear. There are several possibilities that are not mutually exclusive. First, NF-IL6 may compete with Fos and Jun for binding to the AP-1 element (Figure 28). This possibility is supported by the ability of NF-IL6 and Jun homodimers and Fos-Jun heterodimers to bind to the AP-1 site (Figures 17, 18 and 20). Second, NF-IL6, Fos and Jun may compete with one another for the formation of homo- and heterodimers that differ in their DNA-binding affinity to the AP-1 site (Figure 28). This possibility is supported by the

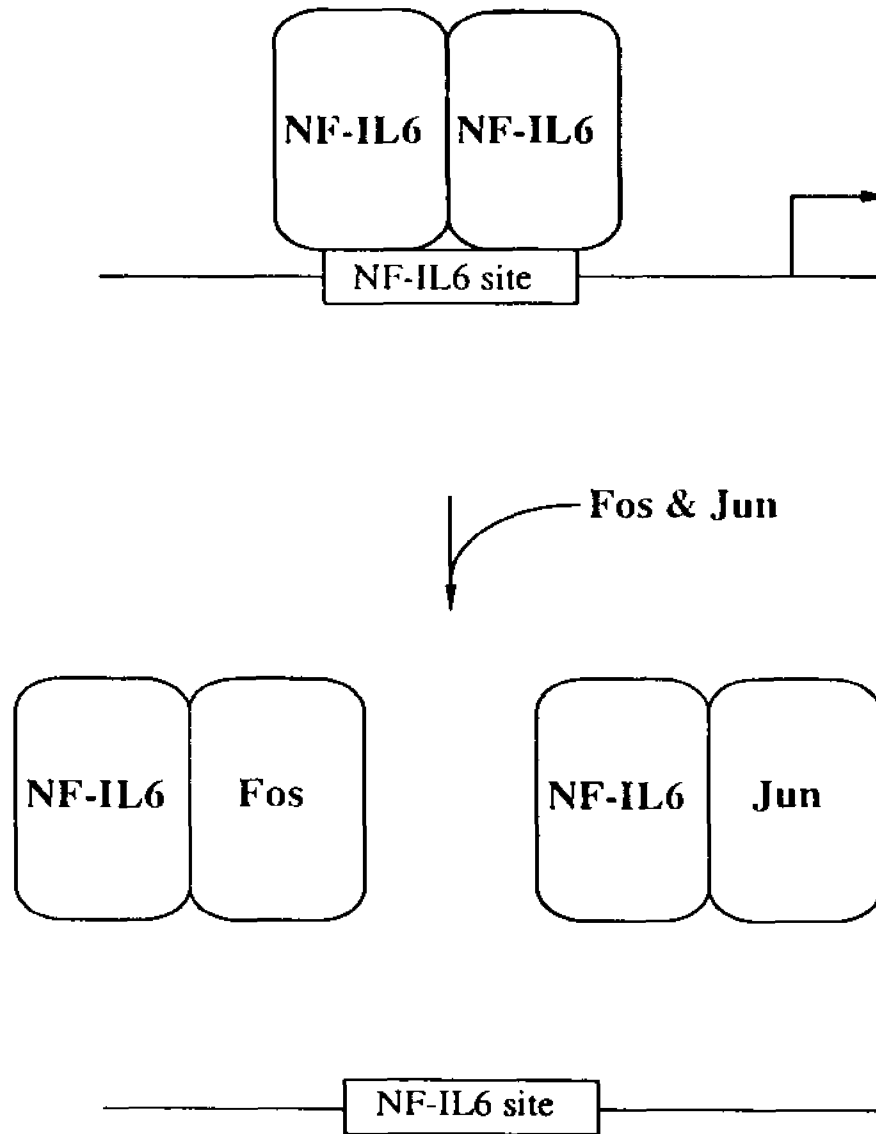


Figure 27. A diagram illustrating the possible mechanism(s) by which Fos and Jun repress transcription activation by NF-IL6.

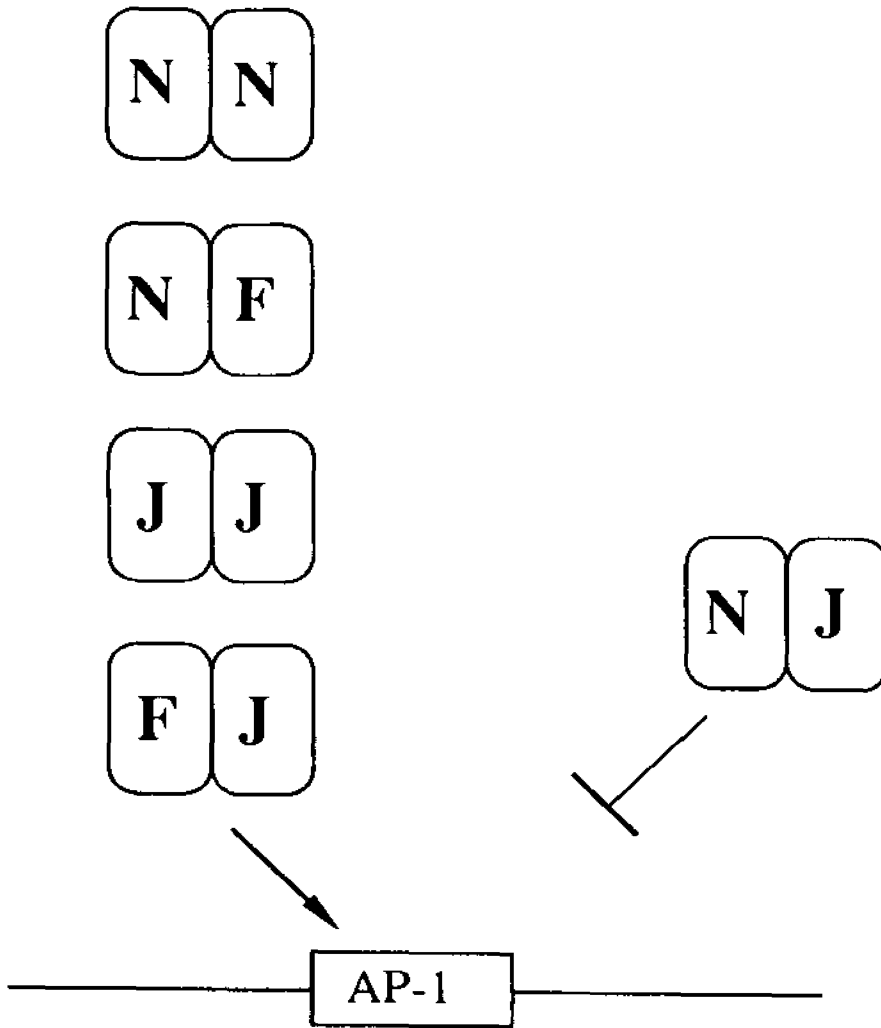


Figure 28. A diagram illustrating regulation of the AP-1 site by NF-IL6 and AP-1 family proteins. N: NF-IL6, F: Fos, J: Jun.

fact that NF-IL6 can dimerize with Fos and with Jun in the absence of DNA (Figure 8 to 10). Third, dimerization and DNA-binding activities of NF-IL6 and AP-1 family proteins may be regulated by posttranslational modification, such as phosphorylation, which remains to be studied.

Implications of cross-family association for IL-6 signaling

Members of the C/EBP family have been implicated in many biological functions. Although NF-IL6 is believed to have a role in IL-6 signaling, the mechanism that underlies the promoter and cell type specificity is not yet understood. As NF-IL6 and AP-1 family transcription factors are regulated by a variety of extracellular stimuli, their cross-family associations may contribute to the differential regulation of promoters by cytokine induction.

Investigation of the coordinate activation of NF-IL6 and Jun in IL-6 induced macrophage differentiation of M1 cells demonstrates that they associate *in vivo* (Figure 26). Regulated formation of the NF-IL6-Jun complex by IL-6 suggests a role of NF-IL6-Jun heterodimerization in determining the promoter and cell type specificity, thereby promoting macrophage differentiation. Fos, but not Jun, is activated by either IL-6 or CNTF in neuronal differentiation (Ip et al., 1992, Bonni et al., 1993). Formation of the NF-IL6-Fos complex may result in activation of genes that are specific for neuronal differentiation since Fos homodimers are not capable of binding to DNA. In this manner, regulated association of C/EBP and AP-1 family proteins may contribute to cell type specific regulation of gene expression and the pleiotropic effect of IL-6.

Chapter IV

CONVERGENT REGULATION OF NF-IL6 AND OCT-1 SYNTHESIS BY INTERLEUKIN-6 AND RETINOIC ACID SIGNALING

Portions of the results contained in Chapter IV has been published (Hsu and Chen-Kiang, 1993, *Molecular and Cellular Biology*, **13**, 2515-2523).

INTRODUCTION

IL-6 has been shown to promote a cellular activity that functionally substitutes for the adenovirus E1A proteins in transactivating E1A-responsive viral promoters and in complementing E1A-deletion mutant viruses (Spergel and Chen-Kiang, 1991). The transcription factor NF-IL6 can reconstitute this E1A-substituting activity (Spergel et al., 1992). Because E1A-substituting activities have been described in mouse preimplantation embryos and F9 embryonal carcinoma cells (Dooley et al., 1990, Imperiale et al., 1984, La Thangue et al., 1987), NF-IL6 and IL-6 may be functional in cells representing early stages of embryonic development. Additionally, E1A-independent virus replication was stimulated by IL-6 induction (Spergel and Chen-Kiang, 1991). Three cellular factors that are known to enhance viral DNA synthesis *in vitro* may therefore be regulated in IL-6 signaling. The octamer binding protein Oct-1 is one of these three factors. Although Oct-1 is known to be modified posttranslationally by phosphorylation during mitosis and site specific serine phosphorylation negatively regulates its DNA-binding activity, the level of Oct-1 appears not to fluctuate during the cell cycle (Roberts et al., 1991, Segil et al., 1991). In this study, I investigated the regulation of Oct-1 and NF-IL6 by IL-6 in cells representing early stages of mammalian development. The roles of Oct-1 and NF-IL6 in the IL-6 signal transduction pathway and in the regulation of adenovirus by IL-6 will be discussed.

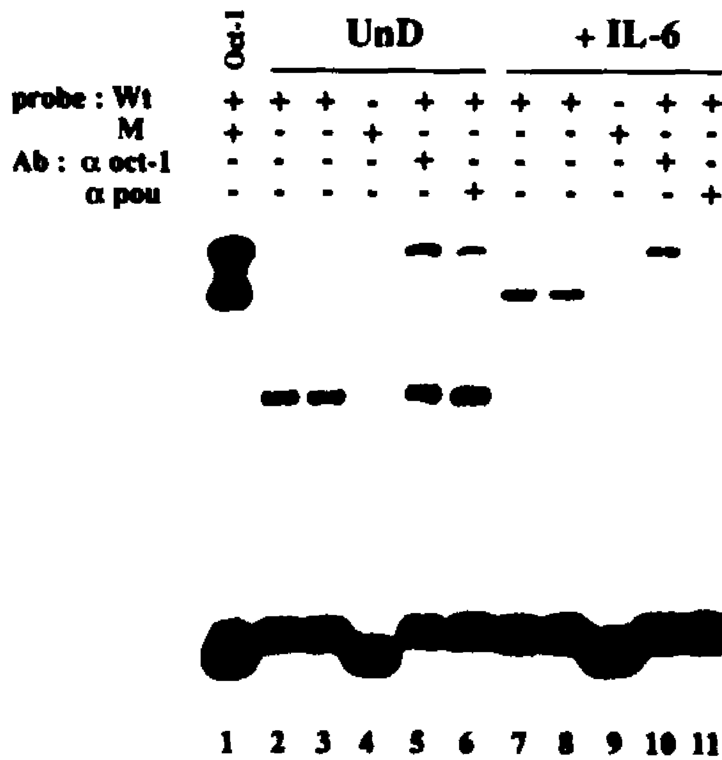
RESULTS

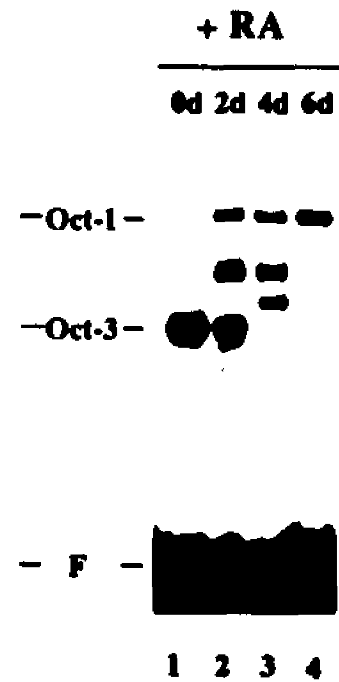
The Oct-1 and Oct-3 DNA-binding activities are regulated by IL-6 and by RA

Human embryonal carcinoma NT2/D1 cells were used as a model to study IL-6 signaling in cells representing early mammalian development. Induction of NT2/D1 cells with IL-6 resulted in an 8- to 9-fold enhancement of the Oct-1 DNA-binding activity, as determined by an electrophoretic mobility shift assay (Figure 29A, lanes 2, 3, 7, and 8). Studies with a mutant DNA probe lacking the octamer element (Figure 29A, lanes 4 and 9) and competition with Oct-1 specific antibodies which inhibit the formation of Oct-1-DNA complexes (α-Oct-1 and α-POU) (Figure 29A, lanes 5, 6, 10, and 11) confirmed that the activity was due to the Oct-1 protein present in the nuclear extracts. Coincident with the enhancement of the DNA-binding activity of Oct-1 was a decrease in the DNA-binding activity of Oct-3 (Figure 29A, lanes 2, 3, 7, and 8), which is expressed in undifferentiated embryonic stem cells (Okamoto et al., 1990, Rosner et al., 1990).

The reciprocal regulation of Oct-1 and Oct-3 DNA-binding activities suggests that the IL-6 signaling pathway is functional in the pluripotent human embryonal carcinoma cells. IL-6 was shown to promote neuronal differentiation of mouse PC12 cells (Satoh et al., 1988). Differentiation of NT2/D1 cells by RA also leads to neuronal differentiation (Andrews 1984) that is accompanied by a loss of Oct-3 mRNA and Oct-3 DNA-binding activity (Okamoto et al., 1990, Rosner et al., 1990). Therefore, regulation of the octamer binding proteins was studied during the course of RA induction in NT2/D1 cells. Oct-1 DNA-binding activity was found to increase by two days after RA induction and reached its maximal level of 10-fold higher than that of uninduced cells by six days after RA induction (Figure 29B). This was parallel with a loss of Oct-3 DNA-binding activity, which was diminished to a level below detection by four days after RA induction (Figure 29B) (Okamoto et al., 1990, Rosner et al., 1990). Two additional octamer sequence specific complexes of intermediate mobility were also regulated temporally in response to RA

Figure 29. IL-6 and RA enhance Oct-1 DNA-binding activity and reduce Oct-3 DNA-binding activity. (A) Nuclear extracts were prepared from NT2/D1 cells that were either uninduced (UnD) or induced with IL-6 for 48 hours (+IL-6) and analyzed by electrophoretic mobility shift assay. The probe used in the assay was an 83 bp *Eco*RI-*Bgl*II fragment of either plasmid pEB-55/106, which contains the terminal repeat of wild-type adenovirus (Wt), or plasmid pEB-44/106, which lacks the octamer binding sequence in the terminal repeat (M). Preincubations of proteins with antibodies, α-oct-1 and α-POU, prior to the binding reactions are indicated (+). Oct-1 is the purified Oct-1 protein from HeLa cells. (B) Nuclear extracts were prepared form RA induced NT2/D1 cells at intervals in days (d) as indicated. The probe used for electrophoretic mobility shift assay was a 51 bp *Dde*I-*Hin*fI fragment of the mouse immunoglobulin heavy chain enhancer. The electrophoretic mobility shift assays were performed as described in Chapter II Materials and Methods.


A
Oct-1
UnD
+ IL-6
probe : Wt
M
Ab : α oct-1
α pou


B
+ RA
0d 2d 4d 6d
—Oct-1—
—Oct-3—

F
1 2 3 4 5 6 7 8 9 10 11
1 2 3 4

although their composition remains to be determined. These data demonstrated that the DNA-binding activities of Oct-1 and Oct-3 can be regulated by two diverse signals, IL-6 and RA.

The synthesis of Oct-1 is stimulated by IL-6 and by RA

The enhancement of Oct-1 DNA-binding activity in nuclear extracts may be attributed to protein modification, compartmentalization, or stabilization of complexes by association with other proteins (Roberts et al., 1991, Segil et al., 1991, Ullman et al., 1991). The latter is less likely because the Oct-1 specific complex derived from all nuclear extracts migrated indistinguishably from that derived from Oct-1 protein purified from HeLa cells (Figure 29A). Whether the regulation of the Oct-1 DNA-binding activity has its molecular basis in the synthesis of Oct-1 was investigated by Northern (RNA) blot analysis using an Oct-1 specific probe (Figure 30). Induction of NT2/D1 cells with IL-6 and with RA resulted in the appearance of a major polyadenylated Oct-1 mRNA species of approximately 10 kb which was not detectable in uninduced cells (Figure 30A, lanes 1 to 3). Additional Oct-1 specific polyadenylated RNAs (6.0, 4.8, 2.4, 1.6 kb), albeit less abundant, were also regulated by IL-6 and by RA.

To investigate the generality of the regulation of Oct-1 synthesis by IL-6 signaling, the level of Oct-1 mRNA was analyzed in NJBC-8 cells as a function of time after IL-6 induction (Figure 30B). NJBC-8 cells are stable transformants of the IL-6 receptor-negative human Jurkat T cells and constitutively express the integrated IL-6Rα gene. As in NT2/D1 cells, the levels of four Oct-1 mRNA species (10, 4.8, 2.4, and 1.6 kb) were increased four-fold by IL-6 within 1 day (Figure 30A, lane 4 to 6). These data provide evidence for the regulation of Oct-1 at mRNA level.

Corresponding to the increases at the mRNA level, the 97 Kd Oct-1 protein, which was indistinguishable from the Oct-1 protein purified from HeLa cells in its electrophoretic mobility, was increased in abundance by three-fold in NT2/D1 cells in response to induction

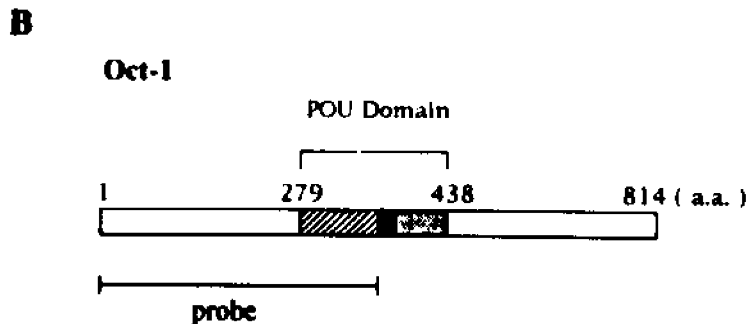


Figure 30. The synthesis of Oct-1 mRNA is regulated by IL-6 and by RA. (A) Polyadenylated RNA was isolated from 10^7 NT2/D1 cells that were uninduced (0), induced with IL-6 for 48 hours (+IL-6), or induced with RA for 8 days (+RA) or from NJBC-8 cells that were uninduced (0) or induced with IL-6 for 1 or 3 days (d) as indicated and analyzed by Northern blotting. (B) The *Xba*I-*Afl*III fragment of a Oct-1 cDNA clone (pBSOct-1) was used as Oct-1 specific probe (Sturm et al., 1988). Northern blotting was performed as described in Chapter II Materials and Methods. The sizes of mRNA species detected by the Oct-1 probe (B) are as indicated. The GAPDH mRNA (1.4 kb) was detected by a probe from pGAPDH (Fort et al., 1985) and used as a reference for RNA loading.

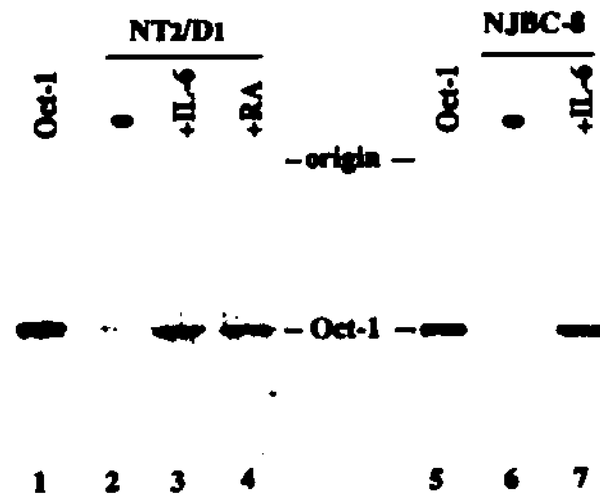


Figure 31. IL-6 and RA enhance the synthesis of Oct-1 protein. Nuclear extracts (20 μg) were prepared from NT2/D1 cells that were uninduced (0), induced with IL-6 for 48 hours (+IL-6), or induced with RA for 8 days (+RA) or from NJBC-8 cells that were uninduced (0), or induced with IL-6 for 24 hours (+IL-6) and analyzed by immunoblotting as described in Chapter II Materials and Methods.

by either IL-6 or RA (Figure 30, lane 1 to 4). The level of Oct-1 protein was also increased 8-fold in NJBC-8 cells after IL-6 induction (Figure 30, lanes 6 and 7). The synthesis of Oct-1 can therefore be regulated in at least two cell types.

NF-IL6 confers the regulation of Oct-1 DNA-binding activity by IL-6 or RA

Regulation of NF-IL6 mRNA is an early event, occurring within hours after IL-6 induction and in advance of detectable changes in the levels of Oct-1 protein. These data raise the possibility that NF-IL6 mediates the IL-6 or RA induced signals to regulate Oct-1. I found that transient expression of NF-IL6 in NT2/D1 cells with a NF-IL6 expression plasmid in the sense orientation, but not the antisense orientation, led to a 10-fold enhancement of the Oct-1 DNA-binding activity (Figure 32, lanes 2, 5, and 6). Studies with a mutant DNA probe lacking the octamer sequence (Figure 32, lanes 3 and 7) and with an anti-Oct-1 antibody (Figure 32, lanes 4 and 8) confirmed that the enhanced DNA-binding activity was due to the presence of Oct-1. Similar results were obtained for transient expression of NF-IL6 gene in Jurkat cells (Figure 32, lanes 10 and 11). These results suggest that NF-IL6 can confer the IL-6 and RA induced regulation of Oct-1 in at least two cell types.

The abundance and ratio of NF-IL6 proteins are regulated by IL-6 and RA

The NF-IL6 gene is an intronless gene but its mRNA can potentially encode three proteins, which are alternatively translated from different in-frame AUGs (Chapter I Introduction and Figure 33A). To investigate whether there is a correlation between regulation of Oct-1 and NF-IL6 synthesis, the levels of the three NF-IL6 isoforms were analyzed by immunoblotting with anti-P3, which detects all three forms (43, 36, and 18 Kd) but does not cross-react with other C/EBP proteins (Figure 33). Induction with IL-6 led to the appearance of the 36 Kd NF-IL6 protein in both NT2/D1 and NJBC-8 cells (Figure 33B, lanes 6, 7, 11, 12). Induction of NT2/D1 cells with RA resulted not only in a similar increase

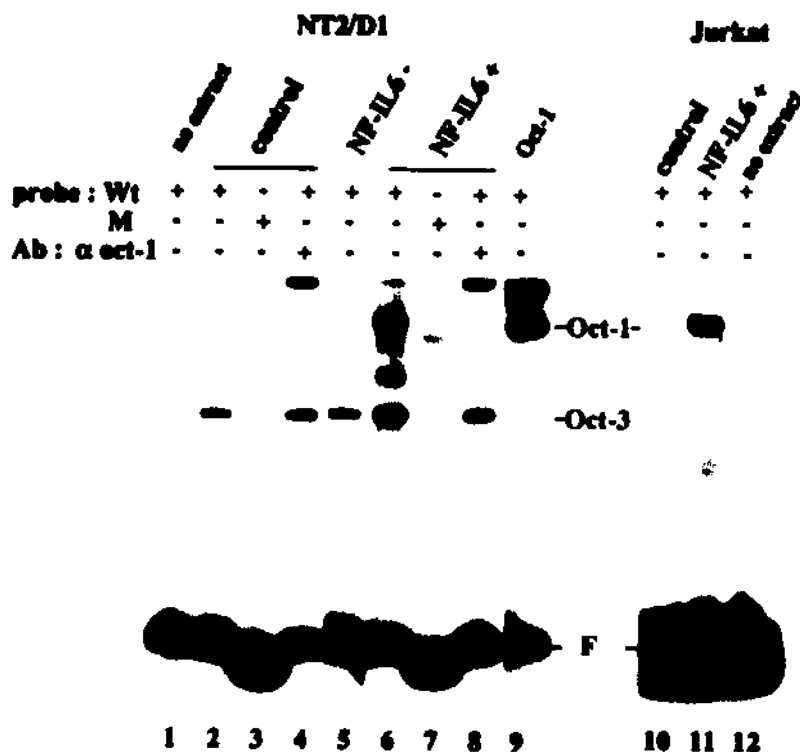


Figure 32. NF-IL6 enhances the Oct-1 DNA-binding activity. Nuclear extracts were prepared from NT2/D1 cells that were not transfected (control) or were transfected with pCMV-NF-IL6(+) (NF-IL6+) or pCMV-NF-IL6(-) (NF-IL6-). Similarly, nuclear extracts were prepared from Jurkat cells that were not transfected (control) or were transfected with pCMV-NF-IL6(+). Binding of Oct-1 protein to the octamer binding sequence was analyzed by an electrophoretic mobility assay as described in Figure 29.

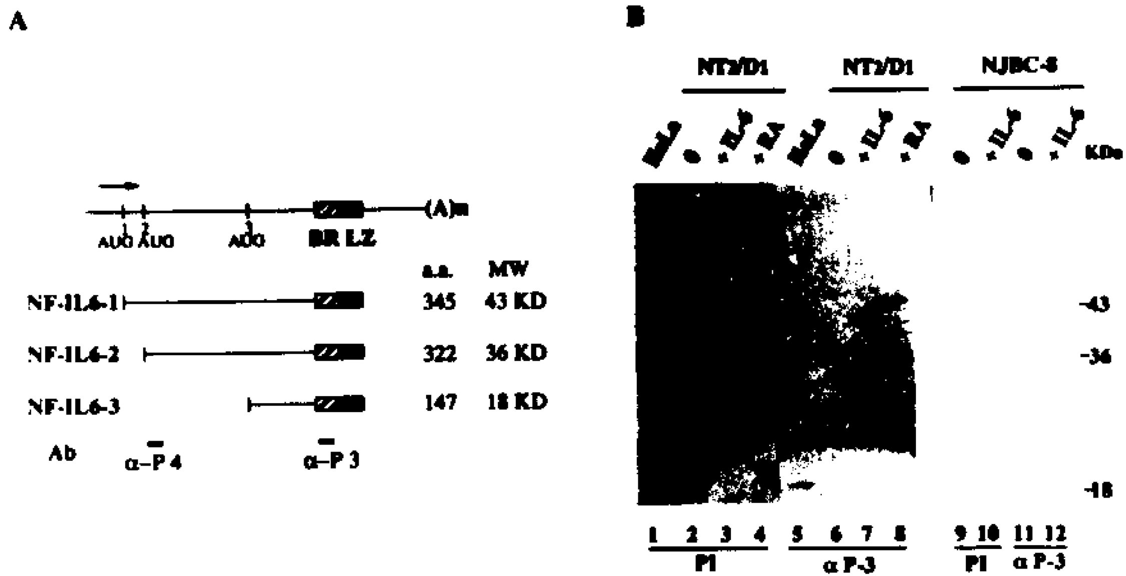


Figure 33. Abundance and ratio of NF-IL6 proteins are regulated by IL-6 and by RA. **(A)** Schematic representation of the NF-IL6 mRNA and proteins. The predicted amino acid residues of the three NF-IL6 proteins initiated in-frame from the first (NF-IL6-1), second (NF-IL6-2), and third (NF-IL6-3) AUGs are indicated. The molecular mass of each protein is estimated by the migration of NF-IL6 proteins relative to protein size markers (not shown). α-P3 is an antiserum that recognizes all three forms of NF-IL6 and α-P4 is an antiserum that recognizes NF-IL6-1 and NF-IL6-2. BR, basic region; LZ, leucine zipper region. **(B)** Nuclear extracts (50 μg) prepared from NT2/D1 cells or NJBC-8 cells as described in Figure 29 and from HeLa cells (20 μg) were analyzed by immunoblotting with preimmune rabbit antisera (PI) or α-P3.

in the 36 Kd protein but also in the synthesis of the 43 Kd protein, which was not detectable in uninduced cells (Figure 33B, lanes 6 and 8). The 18 Kd NF-IL6 protein was not expressed and induced by either IL-6 or RA although it was present in HeLa cells (Figure 33B, lane 5). These data show that the abundance and ratio of the three forms of NF-IL6 can be subject to regulation by cytokine induction. Convergent regulation of Oct-1 by IL-6 and RA signaling thus appears to correlate with the regulation of NF-IL6.

Enhancement of Oct-1 synthesis positively correlates with stimulation of DNA replication

The target genes in this regulatory cascade in NT2/D1 cells are not yet known, as the IL-6 induced phenotype is still being characterized. Our attempts to analyze the regulation of the histone H2B promoter by NF-IL6 and Oct-1 in transient transfection assay have not yielded any conclusive results. In light of the facts that transcriptional activation by Oct-1 may require coactivators (Kristie et al., 1989, Katan et al., 1990, Luo et al., 1992, Ullman et al., 1993) and that knowledge regarding the genes downstream of Oct-1 in NT2/D1 cells is lacking, I examined the stimulatory role that Oct-1 plays in adenovirus DNA replication as an alternative functional assay. Oct-1 has been demonstrated to stimulate the initiation of adenoviral DNA synthesis *in vitro* (Chapter I Introduction) and the octamer sequence is important for viral growth *in vivo* (Hartfield and Hearing, 1991).

Morphological changes in virus-infected NT2/D1 cells (detachment of infected cells) were observed during the course of adenovirus infection. Human cells are permissive hosts for adenovirus, therefore the replication of an E1A-deletion mutant virus, *dl* 312, can be examined. RA was able to stimulate virus DNA replication as well as the detachment of infected cells (Figure 34). NT2/D1 cells supported a low level of viral DNA synthesis in the absence of E1A (Figure 35B). This observation was expected, since E1A-substituting activities have been reported to exist in mouse embryonal carcinoma F9 cells and in cells representing the early stages of embryonic development. Coincidental with the enhancement of Oct-1 synthesis by IL-6 and by RA, replication of adenoviral DNA was accelerated and

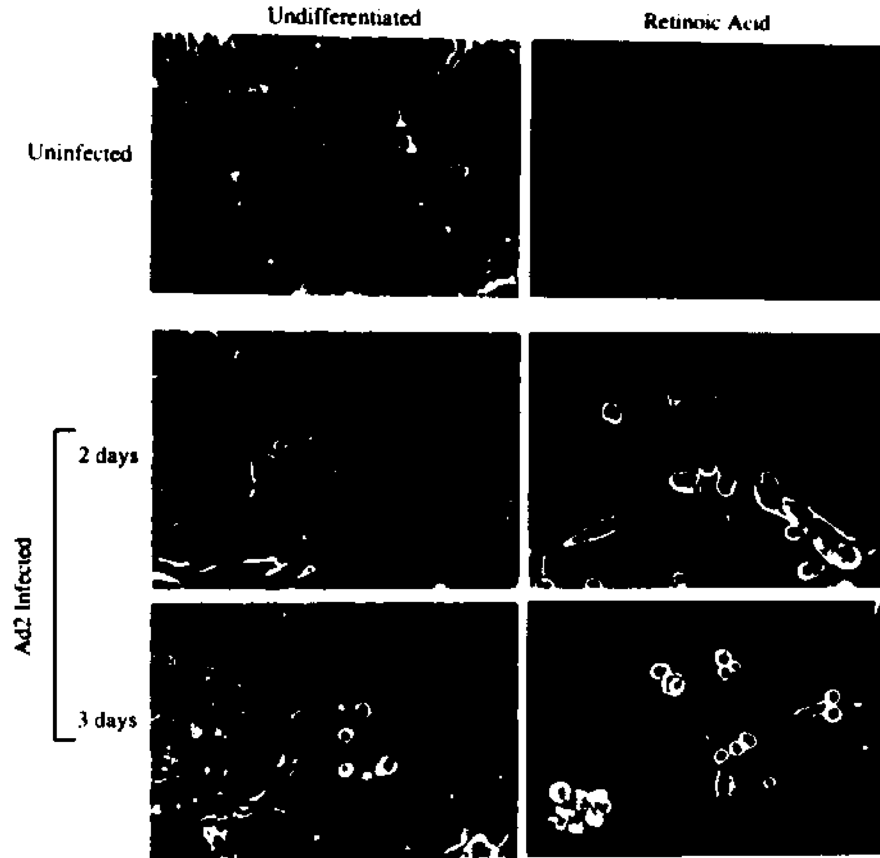
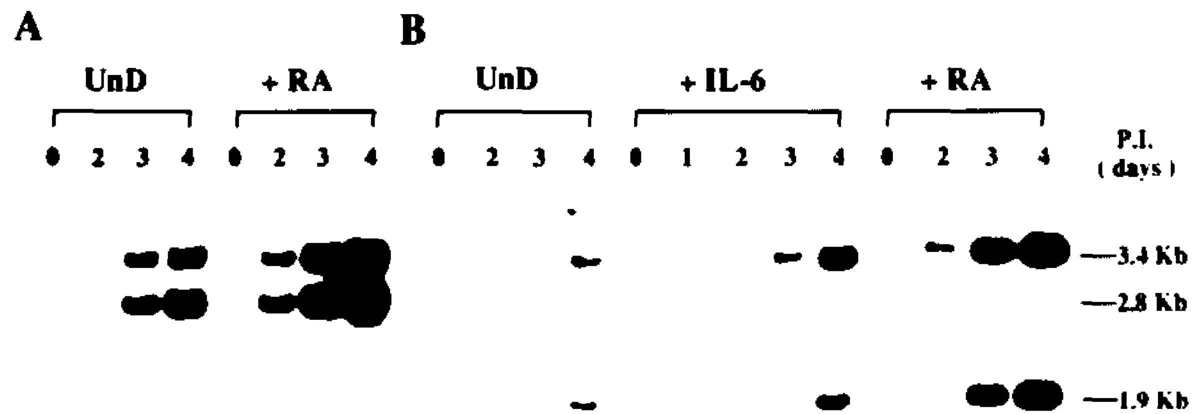
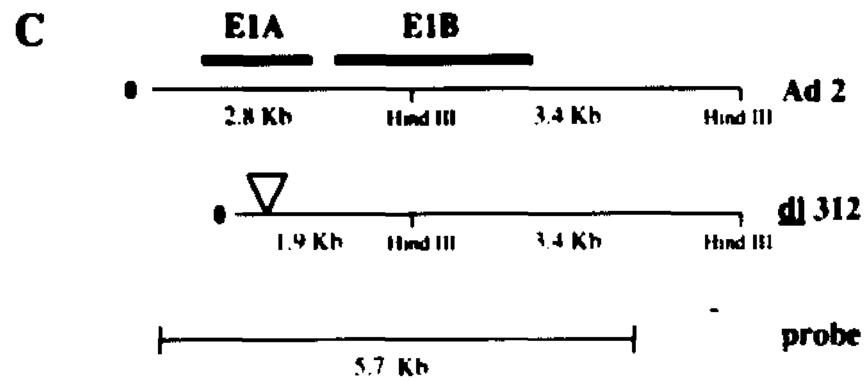


Figure 34. Morphological difference in NT2/D1 cells and adenovirus infected NT2/D1 cells. NT2/D1 cells without induction or with RA induction for 8 days were infected by wild-type adenovirus type 2 (at 10 PFU/cell) (right panel). Mock infection of NT2/D1 and RA induced NT2/D1 cells were used as controls (left panel). Pictures were taken under the microscope with 400 x magnitude.

Figure 35. IL-6 and RA enhance the E1A-independent adenovirus DNA replication. **(A)** Replication of wild-type adenovirus type 2 DNA in NT2/D1 cells without induction (UnD) or with RA induction for 8 days prior to and during infection (at 10 PFU/cell) was analyzed by Southern blotting at 0 through 4 days postinfection (PI) as described in Chapter II Materials and Methods. The ^{32}P-labeled 5.7 kb probe detects the 3.4 and 2.8 kb adenovirus *Hin*dIII genomic fragments. **(B)** Replication of E1A-deletion mutant virus *dl*312 DNA in NT2/D1 cells without induction (UnD), with RA induction (+RA) (as in panel A), or with IL-6 induction for 2 days prior to and during infection (+IL-6) was analyzed postinfection (at 10 PFU/cell) as indicated. Deletion of the majority of the E1A coding sequence in *dl*312 virus resulted in a reduction of the 2.8 kb *Hin*dIII fragment to 1.9 kb. **(C)** Schematic representation of the left end 15% of the adenovirus genome. The DNA probe is the insert of p3Wt18 as described in Chapter II Materials and Methods.


A
B
UnD
+ RA
UnD
+ IL-6
+ RA
0 2 3 4 0 2 3 4 0 2 3 4 0 1 2 3 4 0 2 3 4
P.I.
(days)
3.4 Kb
2.8 Kb
1.9 Kb


C
E1A
E1B
0
Ad 2
2.8 Kb
Hind III
3.4 Kb
Hind III
0
dl 312
1.9 Kb
Hind III
3.4 Kb
Hind III
probe
5.7 Kb

stimulated (Figure 35A). The replication of an E1A-deletion mutant virus, *dl* 312, was enhanced 10-fold by IL-6 and 50-fold by RA. This stimulation was independent of E1A (Figure 35B). The regulation of NF-IL6 and Oct-1 synthesis by two signals positively correlated with one of the functions of Oct-1; the stimulation of adenovirus DNA replication.

DISCUSSION

Regulation of Oct-1 synthesis by two signals: a converged regulatory cascade

The induction of Oct-1 at the levels of mRNA and protein synthesis provides the first evidence that the ubiquitously expressed *oct-1* gene can be regulated by external stimuli (Figure 30 and 31). Regulation of Oct-1 by two diverse signals, IL-6 and RA, suggests that Oct-1 may be an intermediate in both signaling pathways (Figures 29, 30, and 31). NF-IL6 may mediate the regulation of Oct-1 induced by IL-6 and RA (Figure 32). Regulation of NF-IL6 is rapid and precedes that of Oct-1. Oct-1 may be downstream of NF-IL6 in the regulatory cascade shared by the two signals. In addition, Oct-3 DNA-binding activity is also regulated in NT2/D1 cells by IL-6 and RA (Figure 29). Together with the regulation of Oct-2 in B cells, the octamer binding proteins may represent a subset of DNA-binding proteins which respond to IL-6 signals in a cell type specific fashion.

Initial thought to be an accessory factor for transcription of housekeeping genes by RNA polymerase II and III (Sive et al., 1986, Carbon et al., 1987, LaBella et al., 1988, Tanaka et al., 1992), Oct-1 may play a biological role in inducible regulation of the IL-2 promoter in T lymphocytes and in the transcription of immunoglobulin genes in B cells (Ullman et al., 1991, Luo et al., 1992). Identification of the regulatory genes that are downstream of and regulated by Oct-1 in IL-6 signaling remains to be studied.

Potential translational regulation of NF-IL6 by extra cellular stimuli

The results presented in this study have provided the first example of differential regulation of NF-IL6 isoforms by signal transduction. The sequences encompassing the first and second translational initiation sites of NF-IL6 mRNA imperfectly match to the consensus sequences for efficient initiation of translation. A leaky mechanism proposed in the ribosomal scanning model has been suggested for this alternative initiation of translation (Descombes and Schibler, 1991). Regulation of the abundance and the ratio of the three NF-IL6 isoforms by two signaling pathways lends biological significance to this possibility and suggests novel mechanism(s) by which the two signals are transduced. These results may have functional implications in cellular regulation by extracellular stimuli. Whether and how NF-IL6 may be regulated by translational control that is initiated by IL-6 and RA remain to be explored.

Regulation of adenovirus by IL-6 is bipartite and E1A-independent

The enhancement of Oct-1 synthesis by IL-6 and by RA correlates with the stimulation of adenovirus DNA replication in NT2/D1 cells. This result supports a physiological role of Oct-1 in DNA replication. Differentiation of embryonal carcinoma cells does not necessarily lead to down-regulation of the cellular E1A-substituting activity. The cellular E1A-substituting activity was initially found to activate the E1A-responsive viral early genes in the absence of E1A and to be diminished in differentiated F9 cells by RA induction. Contrary to that, differentiation of NT2/D1 cells by IL-6 or RA led to stimulation of E1A-independent viral replication (Figure 35B). The DNA replication assay used in this study is different from the assay used in previous experiments. Stimulation of adenovirus replication does require the participation of viral early proteins which are essential components for DNA synthesis. The possible explanation for these differences is that a clonal variation in the level of endogenous E1A-substituting activities in embryonal cacinoma cells and in their regulation during differentiation. The regulation of adenovirus

by IL-6 could be bipartite and intricately orchestrated by means of regulation of NF-IL6 and Oct-1. First, NF-IL6 leads to transactivate the viral early genes at the early stage of viral infection. Second, Oct-1 is activated and stimulates DNA replication during the late-phase of viral life cycle. In view of the fact that many cellular promoters contain binding sites for both NF-IL6 and Oct-1, the interplay of NF-IL6 and Oct-1 may represent one consequence of signaling by IL-6 and by RA.

Chapter V

CONCLUSION

Physical and functional interactions between NF-IL6 and AP-1 family proteins have been demonstrated in this study. The results provide new insights into the interplay of transcription factors in the IL-6 signaling pathway. The transcription factor NF-IL6 was shown to associate with Fos and with Jun. Other members of NF-IL6 and AP-1 families may also interact with one another. The generality of this cross-family interaction remains to be determined. The specificity for homo- and heterodimerization may be influenced by the primary amino acid sequences within and outside the bZIP domain. Two regions outside the bZIP domain of Fos were found to enhance the DNA-binding efficiency of NF-IL6-Fos complexes (Chapter III). These two regions, which are rich in proline residues, have also been described to promote the transactivating activity of Fos-Jun dimers. One of these two regions, which is amino-terminal to the bZIP domain of Fos, seems to promote the formation of a multimeric protein-DNA complex (low mobility complex) as shown by electrophoretic mobility shift assay (Chapter III). Although the formation of this low mobility complex may be due to an intrinsic structural change, the ability of the bZIP proteins to form two-, three-, and four-stranded coiled-coils has been reported (Harbury et al., 1993). Determination of the conformation and composition of this low mobility complex may give new insights into the interplay of bZIP proteins. Because Fos-Jun and Jun-Jun dimers can induce DNA bends of different orientations and magnitudes (Kerppola and Curran, 1991a), NF-IL6-Fos and NF-IL6-Jun dimers may also contact DNA differently. DNA bends of different orientations and magnitudes may play a role in mediating distinct functions.

Even though NF-IL6-Fos heterodimers bound efficiently to the AP-1 site, the DNA sequences bound by NF-IL6-Jun heterodimers have not been identified. NF-IL6 has been shown to dimerize with C/ATF, a recently identified member of the ATF family, and direct its binding to a subclass of asymmetric cAMP responsive elements with higher efficiency *in vitro* (Vallejo et al., 1993). Therefore, it is possible that NF-IL6-Jun and NF-IL6-Fos

heterodimers recognize a subclass of high affinity binding sites. Identification of these binding sites will reveal the potential target genes and downstream effects that are regulated by this cross-family association.

Studies of the functional interactions between NF-IL6 and AP-1 family proteins have been carried out by the use of synthetic promoters. Investigations with natural promoters will be necessary to confirm these functional studies. Studies of transcription of the *TSG-6*, a gene encoding a hyaluronan-binding protein, in IL-1 and TNF-α signaling support cooperative regulation of this native promoter by NF-IL6 and AP-1 (Klampfer, personal communication). Activation of the *TSG-6* was activated by NF-IL6 according to the ratio of activator to repressor in transiently transfected cells. This repression is likely to be due to the binding of NF-IL6-3 to both NF-IL6 and AP-1 sites or the dimerization of NF-IL6 with Fos and Jun. Interactions between NF-IL6 and AP-1 family proteins may also play a role in the regulation of their own synthesis. Fos protein has been shown to auto-repress its own promoter (Lucibello et al., 1989). The repression is independent of the AP-1 and ATF sites where Fos complexes usually bind. The major target for Fos mediated auto-repression is thought to reside in the serum response element. NF-IL6 has been shown to regulate the *c-fos* promoter by binding to a site in the serum response element (Metz and Ziff, 1991). Formation of the NF-IL6-Fos complex may participate in auto-regulation of *c-fos* promoter by Fos protein.

Studies on the mechanism(s) of IL-6 signal transduction have revealed that alternative to the NF-IL6 pathway, the IL-6 signals can be rapidly and transiently mediated by APRF (Lütticken et al., 1994). Activation of APRF by tyrosine phosphorylation upon IL-6 induction may be one of the earliest events in IL-6 signaling. However, the functional relationship between these two pathways is not known. Studies presented in this thesis have focused on the NF-IL6 pathway. In addition to the finding that NF-IL6 associates with AP-1, my results also suggest that Oct-1 is downstream of NF-IL6 in the IL-6 signaling pathway (Chapter IV). Regulation of the octamer binding proteins by IL-6 induction suggests that

they represent a subset of DNA-binding proteins that respond to IL-6 signals in a cell type specific manner (Chapter IV). The results may have implications in B cell differentiation induced by IL-6. Transcription of the immunoglobulin genes has been shown to be activated by IL-6 during terminal B cell differentiation, suggesting that regulated transcription factors play crucial roles in mediating the IL-6 responses. One of the prominent B cell specific transcription factors is Oct-2 which may also be downstream of NF-IL6 and regulated by NF-IL6. Oct-2 may cooperate with NF-IL6 to regulate transcription of the immunoglobulin genes. In preliminary studies, I have found that a protein complex which is B cell specific interacts with the immunoglobulin heavy chain enhancer (data not shown). Binding of this complex to the immunoglobulin heavy chain enhancer appears to depend on the NF-IL6 binding site. The participation of NF-IL6 and Oct-2 in this complex remains to be studied in the future.

Coordinate elevations of NF-IL6 and Jun trigger their association in IL-6 stimulated macrophage differentiation. If other members of NF-IL6 and AP-1 families also form complexes *in vivo*, cell type specific formation of these complexes will be important for cellular differentiation. The combinatorial formation of dimers may also be imposed by the intracellular concentrations of proteins that are subject to regulation by external stimuli. Studies of the regulation and interaction of IL-6 regulated nuclear proteins will aid in our understanding of the IL-6 signal transduction pathway, the cell type specific transcription, and the pleiotropic effect of IL-6.

Chapter VI

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