The EMBO Journal vol.9 no.6 pp.1897 - 1906, 1990

A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family

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Communicated by G. Köhler

NF-IL6 is a nuclear factor that specifically binds to an IL1-responsive element in the IL-6 gene. In this study the gene encoding NF-IL6 has been cloned by direct screening of a λgt11 library using NF-IL6 binding sequence as a ligand. The full-length cDNA encoded a 345 amino acid protein with a potential leucine zipper structure and revealed a high degree of homology to a liver-specific transcriptional factor, C/EBP, at the C-terminal portion. The bacterial fusion protein bound to the CCAAT homology as well as the viral enhancer core sequences as in the case of C/EBP. Recombinant NF-IL6 activated the human IL-6 promoter in a sequence-specific manner. Southern blot analysis demonstrated the high-degree conservation of the NF-IL6 gene through evolution and the existence of several other related genes sharing the DNA-binding domain. NF-IL6 mRNA was normally not expressed, but induced by the stimulation with either LPS, IL-1 or IL-6. Interestingly, NF-IL6 was shown to bind to the regulatory regions for various acute-phase protein genes and several other cytokine genes such as TNF, IL-8 and G-CSF, implying that NF-IL6 has a role in regulation not only for the IL-6 gene but also for several other genes involved in acute-phase reaction, inflammation and hemopoiesis.

Key words: acute-phase reaction/C/EBP/IL-6/leucine zipper/transcription factor

Introduction

IL-6 is a cytokine with multiple biological activities, a number of which are involved in various aspects of immune and inflammatory responses (Kishimoto and Hirano, 1988). IL-6 stimulates immunoglobulin synthesis (Hirano *et al.*, 1986); stimulates the production of acute-phase proteins by the liver (Andus *et al.*, 1987; Gauldie *et al.*, 1987); enhances myeloma and hybridoma growth (Van Damme *et al.*, 1987; Van Snick *et al.*, 1987; Kawano *et al.*, 1988); and supports the proliferation of hemopoietic progenitor cells (Ikebuchi *et al.*, 1987). IL-6 is produced by a number of different cell types such as fibroblasts, macrophages, T and B lymphocytes, endothelial cells, glia cells and keratinocytes by exposure to a variety of extra-cellular stimuli (Ray *et al.*, 1989). IL-6 is not produced under normal circumstances. However, once some invasions such as bacterial and viral infections, or tissue injuries take place in the host, IL-6 is rapidly and transiently expressed and participates in host defense. IL-1, TNF and PDGF stimulate IL-6 production (Walther *et al.*, 1988; Zhang *et al.*, 1988). In addition it has been shown that IL-6 stimulates its own synthesis (Miyaura *et al.*, 1989; Shabo *et al.*, 1989). Through this complicated cytokine network, host defense mechanisms may be maintained. Constitutive production of the IL-6 gene may have deleterious effects upon the host and therefore its synthesis must be tightly controlled. Accumulating evidence has suggested that deregulation of IL-6 expression may be involved in the pathogenesis of various immunologically mediated diseases and malignancies such as rheumatoid arthritis, cardiac myxoma and myeloma (Hirano *et al.*, 1987, 1988; Kawano *et al.*, 1988). Recent results on transgenic mice harboring the human IL-6 gene showed that IL-6 overproduction could trigger the generation of a massive fatal plasmacytosis and mesangial-proliferative glomerulonephritis (Suematsu *et al.*, 1989). Therefore it is imperative to clarify the regulatory mechanism of the IL-6 gene expression for understanding the deregulation of the IL-6 gene expression and finally the pathogenesis of IL-6-associated disorders.

In this study, we present the isolation of a recombinant clone that encodes NF-IL6, a nuclear factor involved in the IL-6 gene expression. This protein shares high sequence homology with C/EBP, a rat liver nuclear factor (Landschulz *et al.*, 1988a,b) in its DNA-binding domain but differs in its N-terminal domains. In addition it was shown that NF-IL6 bound to the transcriptional regulatory regions found in several acute-phase genes and other cytokine genes, including TNF, IL-8 and G-CSF, implying that NF-IL6 may be involved in the regulation of acute-phase reaction, inflammation and hemopoiesis. Furthermore, NF-IL6 also bound avidly to the viral enhancer core homologies as well as CCAAT homologies as demonstrated in C/EBP. The result may imply the association between virus infection and the constitutive IL-6 production.

Results

Cloning of NF-IL6 that binds specifically to the IL-1-responsive element

We previously showed that transcriptional activation of the IL-6 gene by IL-1 was dependent on the 14 bp palindrome located ~150 bp upstream of the transcriptional initiation site (Isshiki *et al.*, 1990). In a glioblastoma cell line, SK-MG-4, we identified two nuclear factors, one constitutive and the other IL-1 inducible, that specifically bound to the 14 bp palindrome. We refer to these binding factors collectively as NF-IL6 because they revealed identical binding specificity and methylation interference pattern. To clone the gene encoding NF-IL6, we used a screening technique developed by Singh *et al.* (1988). A λgt11 phage library generated from the cDNA of LPS-activated human peripheral monocytes was screened with a ^{32}P-labeled

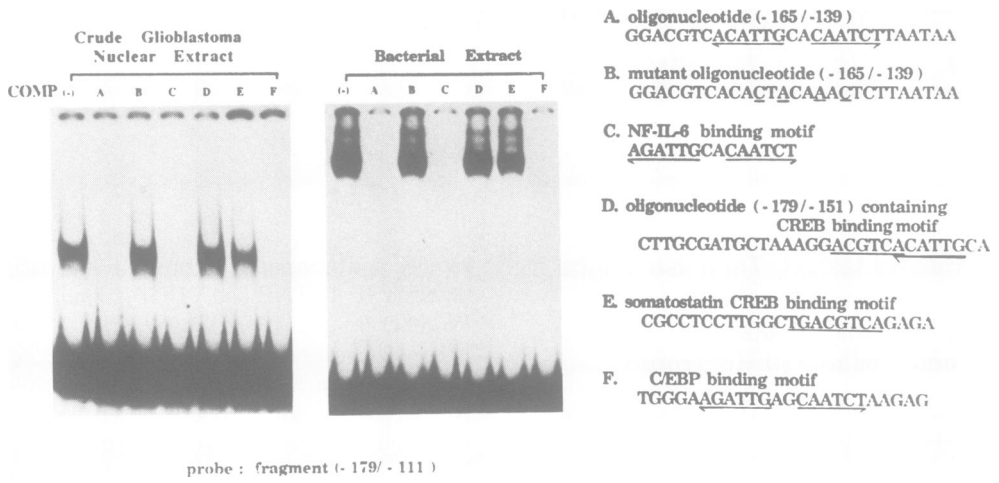


Fig. 1. The λIS3 bacterial fusion protein displays the same binding specificity as crude NF-IL6. A bacterial extract was prepared from λIS3-infected *E.coli* Y1089 cells. An end-labeled fragment (−179/−111 of the human IL-6 gene) was incubated with crude glioblastoma SK-MG-4 nuclear extracts or the bacterial fusion protein in the absence or in the presence of the indicated competitors.

probe consisting of four copies of a 36 bp synthetic nucleotide (−164/−139 bp region) containing the 14 bp palindrome. From 5×10^5 plaques, one positive clone, IS3, was isolated, whose binding to the labeled probe was specifically inhibited by 100-fold molar excess of the cold 14 bp palindromic sequence. To investigate the binding specificity of the protein encoded by the clone IS3, we performed a gel-retardation assay utilizing a bacterial extract from IPTG-induced lysogen (Figure 1). A specific protein–DNA complex was formed when the IPTG-induced cell extract was used, but this complex was not seen in the uninduced extract. The binding specificity of the bacterial extract exactly correlated with the pattern obtained with the crude nuclear extract from SK-MG-4. The complexes formed with the specific probe could be competed away by an excess of unlabeled DNA probe and a 14 bp oligonucleotide corresponding to the NF-IL6 binding site, but not by a mutated version of the same DNA probe or by an unrelated cAMP response element binding motif. As further test for the binding specificity of the fusion protein from IS3, we performed a methylation interference analysis with the same lysogen extract. As shown in Figure 2, three guanosines at positions −152, −150 and −146 in the human IL-6 gene were protected on the non-coding strand, while one guanosine at position −153 was protected on the coding strand. This pattern was identical to the pattern seen with the crude nuclear extract from SK-MG-4, as we previously reported (Isshiki *et al.*, 1990).

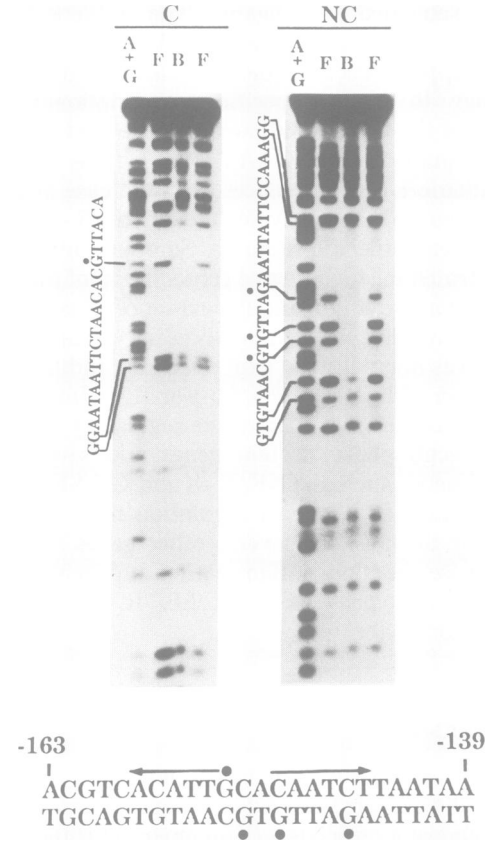
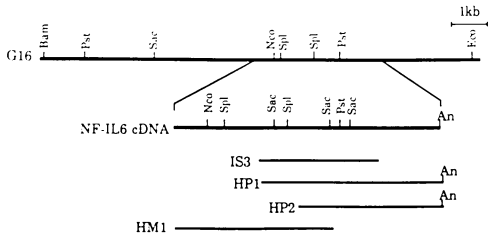


Fig. 2. Methylation interference pattern of the λIS3 bacterial fusion protein. The bacterial extract from λIS3-infected cells was incubated in a binding reaction with an end-labeled, dimethyl sulfate-treated fragment (−179/−111 of the human IL-6 gene). Specific complexes and free probes were separated on preparative band-shift gels. The DNAs were eluted from the gel, chemically cleaved at methylated guanine nucleotides, and fractionated on sequencing gels. Guanine residues that interfere with binding when methylated are indicated by closed circles. C, coding strand; NC, non-coding strand; F, DNA from the free probe; B, DNA from the specific complex.

NF-IL6 is related to C/EBP, a rat liver protein with leucine zipper

Analysis of the cDNA insert of IS3 revealed that it contained an 0.7 kb DNA insert. Using the insert from IS3 as a probe, the cDNA libraries from human peripheral monocytes and a human placenta were rescreened. Several additional clones, one (named HM1) from the human monocyte cDNA library, and two (named HP1 and HP2) from the human placental cDNA library were obtained and sequenced by the chain termination method (Figure 3A and B). No differences of the sequence were observed between the overlapping regions of different cDNA inserts. A single long open reading frame was identified in this sequence. There were two ATG codons in this frame toward the 5′ end of the cDNA. We tentatively assigned the first ATG codon as the initiation codon and deduced the protein sequence from this codon. The predicted



B

NF-IL6

```
                                                                      60
GTCCTTCGCGTCCCGGCGGCGCGGCGGAGGGGCCGGCGTGACGCAGCGGTTGCTACGGGC
                                                                     120
CGCCCTTATAAATAACCGGGCTCAGGAGAAACTTTAGCGAGTCAGAGCCGCGCACGGACT
                                                                     180
GGGAAGGGGACCCACCCGAGGGTCCAGCCACCAGCCCCCTCACTAATAGCGGCCACCCCG
                                                                     240
GCAGCGGCGGCAGCAGCAGCAGCGACGCAGCGGCGACAGCTCAGAGCAGGGAGGCCGCGC
                                                                     300
ACCTGCGGGCCGGCCGGAGCGGGCAGCCCCAGGCCCCCTCCCCGGGCACCCGCGTTCATG
                                                                       M
                                                                     360
CAACGCCTGGTGGCCTGGGACCCAGCATGTCTCCCCCTGCCGCCGCCGCCGCCTGCCTTT
Q  R  L  V  A  W  D  P  A  C  L  P  L  P  P  P  P  P  A  F
                                                                     420
AAATCCATGGAAGTGGCCAACTTCTACTACGAGGCGGACTGCTTGGCTGCTGCGTACGGC
K  S  M  E  V  A  N  F  Y  Y  E  A  D  C  L  A  A  A  Y  G
                                                                     480
GGCAAGGCGGCCCCCGCGGCGCCCCCCCGCGGCCAGACCCGGGCCGCGCCCCCCCGCCGGC
G  K  A  A  P  A  A  P  P  A  A  R  P  G  P  R  P  P  A  G
                                                                     540
GAGCTGGGCAGCATCGGCGACCACGAGCGCGCCATCGACTTCAGCCCGTACCTGGAGCCG
E  L  G  S  I  G  D  H  E  R  A  I  D  F  S  P  Y  L  E  P
                                                                     600
CTGGGCGCGCCGCAGGCCCCGGCGCCCGCCACGGCCACGGACACCTTCGAGGCGGCTCCG
L  G  A  P  Q  A  P  A  P  A  T  A  T  D  T  F  E  A  A  P
                                                                     660
CCCGCGCCCGCCCCCGCGCCCGCCTCCTCCGGGCAGCACCACGACTTCCTCTCCGACCTC
P  A  P  A  P  A  P  A  S  S  G  Q  H  H  D  F  L  S  D  L
                                                                     720
TTCTCCGACGACTACGGGGGCAAGAACTGCAAGAAGCCGGCCGAGTACGGCTACGTGAGC
F  S  D  D  Y  G  G  K  N  C  K  K  P  A  E  Y  G  Y  V  S
                                                                     780
CTGGGGCGCCTGGGGGCTGCCAAGGGCGCGCTGCACCCCGGCTGCTTCGCGCCCCTGCAC
L  G  R  L  G  A  A  K  G  A  L  H  P  G  C  F  A  P  L  H
                                                                     840
CCACCGCCCCCGCCGCCGCCGCCCCGCCGAGCTCAAGGCGGAGCCGGGCTTCGAGCCC
P  P  P  P  P  P  P  P  P  A  E  L  K  A  E  P  G  F  E  P
                                                                     900
GCGGACTGCAAGCGGAAGGAGGAGGCCGGGGCGCCCGGGCGGCGGCGCAGGCATGGCGGCG
A  D  C  K  R  K  E  E  A  G  A  P  G  G  G  A  G  M  A  A
                                                                     960
GGCTTCCCGTACGCGCTGCGCGCTTACCTCGGCTACCAGGCGGTGCCGAGCGGCAGCAGC
G  F  P  Y  A  L  R  A  Y  L  G  Y  Q  A  V  P  S  G  S  S
                                                                    1020
GGGAGCCTCTCCACGTCCTCCTCGTCCAGCCCGCCCGGCACGCCGAGCCCCGCTGACGCC
G  S  L  S  T  S  S  S  S  S  P  P  G  T  P  S  P  A  D  A
                                                                    1080
AAGGCCCCCCCGACCGCCTGCTACGCGGGGGCCGGGCCGGCGCCCTCGCAGGTCAAGAGC
K  A  P  P  T  A  C  Y  A  G  A  G  P  A  P  S  Q  V  K  S
                                                                    1140
AAGGCCAAGAAGACCGTGGACAAGCACAGCGACGAGTACAAGATCCGGCGCGAGCGCAAC
K  A  K  K  T  V  D  K  H  S  D  E  Y  K  I  R  R  E  R  N
                                                                    1200
AACATCGCCGTGCGCAAGAGCCGCGACAAGGCCAAGATGCGCAACCTGGAGACGCAGCAC
N  I  A  V  R  K  S  R  D  K  A  K  M  R  N  L  E  T  Q  H
                                                                    1260
AAGGTCCTGGAGCTCACGGCCGAGAACGAGCGGCTGCAGAAGAAGGTGGAGCAGCTGTCG
K  V  L  E  L  T  A  E  N  E  R  L  Q  K  K  V  E  Q  L  S
                                                                    1320
CGCGAGCTCAGCACCCTGCGGAACTTGTTCAAGCAGCTGCCCGAGCCCCTGCTCGCCTCC
R  E  L  S  T  L  R  N  L  F  K  Q  L  P  E  P  L  L  A  S
                                                                    1380
TCCGGCCACTGCTAGCGCGGCCCCCGCGGCGTCCCCCTGGGGCCGGCCGGGGCTGAGACT
S  G  H  C  *
                                                                    1440
CCGGGGAGCGCCCGCGCCCGCGCCCTCGCCCCCNCCCCCNNNNCCGCAAAACTTTGGCAC
                                                                    1500
TGGGGCACTTGGCAGCNGGGGAGCCCGTCGGTAATTTTAATATTTTATTATATATATATA
                                                                    1560
TCTATATTTTGCCAACCAACCGTACATGCAGATGGCTCCCGCCCGTGGTGTATAAAGAAG
                                                                    1620
AAATGTCTATGTGTACAGATGAATGATAAACTCTCTGCTCTCCCTCTGCCCCTCTCCAGG
                                                                    1680
CCCGGCGGGCGGGGCCGGTTTCGAAGTTGATGCAATCGGTTTAAACATGGCTGAACGCGT
                                                                    1740
GTGTACACGGGACTGACGCAACCCACGTGTAACTGTCAGCCGGGCCCTGAGTAATCGCTT
                                                                    1800
AAAGATGTTCTAGGGCTTGTTGCTGTTGATGTTTTGTTTTGTTTTGTTTTTTGGTCTTTT
                                                                    1860
TTTCTATTATAAAAAATAATCTATTTCTATGAGAAAAGAGGCGTCTGTATATTTTGGGAA
                                                                    1920
TCTTTTCCGTTTCAAGCAATTAAGAACACTTTTAATAAACTTTTTTTTGAGAATGGTTAA
AAAAAAAAAA
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C

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NF-IL6          MQRLVAWDPACLPLPPPPPAFKSMEVANFYYEADCLAAAY  40
C/EBP  MESADFYEAEPRPPMSSHLQSPPHAPSNARLWLSPGRGPRAAPSPTCRPGAAGRICEHET  60
       GGKAAPAAPPAARPGPRPPAGELGSIGDHERAIDFSPYLEPLGAPQAPAPATATDTFEAA 100
       SIDISAYIDPAAFNDEFLADLFQHSRQQEKAKAAAGPAGGGGDFDYPGAPAGPGGAVMSA 120
       PPAPAPAPASSGQHHDFLSDLFSDDYGGKNCKKPAEYGYVSLGRLGAAKGALHPGCFAPL 160
       G-AHGPPPGYGCAAAGYLDGRLEPLYERVGAPALRPLVIKQEPREEDEAKQLALAGLFPY 179
       HPPPPPPPPPAELKAEPGFEPADCKRKEEAGAPGGGAGMAAGFPYALRAYLGYQAVPSGS 220
       QPPPPPPPPHPH--ASPAHLAAPHLQFQIAHCGQTTMHLQPGHPTPPPTPV-----PSPH 232
       SGSLSTSSSSSPPGTPSPADAKAPPTACYAGAGPAPSQVKSKAKKTVDKHSDEYKIRRER 280
       PAPAMGAAGLPGPGGSLKGLAGPHPDL-RTGGGGGGGAGAGKAKKSVDKNSNEYRVRRER 291
       NNIAVRKSRDKAKMRNLETQHKVLELTAENERLQKKVEQLSRELSTLRNLFKQLPE-PLL 339
       NNIAVRKSRDKAKQRNVETQQKVLELTSDNDRLRKRVEQLSRELDTLRGIFRQLPESSLV 351
       ASSGHC                                                    345
       KAMGNCA                                                   358
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D

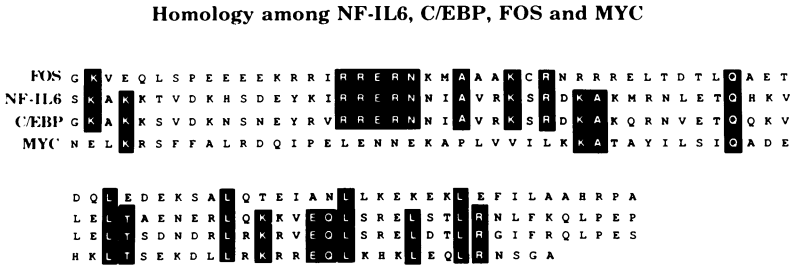

Fig. 3. (**A**) NF-IL6 cDNA clones and a genomic clone. IS3 is an insert of the clone obtained from the human monocyte cDNA library by a direct screening using the NF-IL6-binding motif. HP1, HP2 and HM1 were clones obtained by rescreening the human placental cDNA library and the monocyte cDNA library respectively. Clone G16 was recovered from a human placental genomic library. (**B**) DNA and amino acid sequences of the gene encoding NF-IL6. The sequence was derived from both cDNA and genomic clones. A TATAAA homology is double-underlined. A polyadenylation signal is underlined. The transcription start site is indicated by an arrow. (**C**) Amino acid comparison between NF-IL6 and C/EBP. Identical and conserved amino acids are marked by asterisks and dots respectively. Leucine residues spaced exactly by seven amino acids (leucine zipper motif) are boxed. (**D**) Comparison of the amino acid sequences among NF-IL6, C/EBP, Myc and Fos.

protein contained 345 amino acids with a calculated mol. wt of 36.1 kd. A search of the NBRF protein database and recently published nuclear factor sequences indicated that NF-IL6 contained a region highly homologous to the C-terminal portion of C/EBP, a rat liver nuclear factor with the leucine zipper structure (Landschulz *et al.*, 1988a,b). Figure 3(C) compares the protein sequences of NF-IL6 and C/EBP. The highly conserved region occurs between residues 261 and 345, where there is 73% identity and 95% homology, including conservative changes. The greatest identity is a 44 amino acid segment located immediately N-terminal to the leucine repeat. This region has been shown to be essential for DNA binding in the case of C/EBP. Like C/EBP, NF-IL6 also shares homology with the fos within this highly basic region (Figure 3D). In particular, the 5 amino acid sequence (RRERN) is shared among these three proteins. The homology between NF-IL6 and C/EBP includes the region of leucine zipper structure. NF-IL6 also contains a potential leucine zipper, consisting of five leucines in every seventh position. The amino acid composition of the predicted NF-IL6 protein is enriched for alanine (16%), proline (15%) and serine (8%). The distribution of proline residues is quite atypical. This amino acid is concentrated within two regions located between positions 13 and 19, and between positions 159 and 171 respectively. In particular a cluster of nine prolines is observed between positions 163 and 171. Interestingly, the proline cluster is shared between NF-IL6 and C/EBP at the identical location. In contrast, the putative DNA-binding domain highly homologous to that of C/EBP is almost devoid of proline residues. A serine-rich portion (8 out of 11 residues) is also observed between 218 and 231.

NF-IL6 is an intronless gene

The isolation of two different types of cDNA that share the highly conserved DNA-binding domain cannot exclude the possibility that one gene generates different but related mRNAs by alternative splicing, although the C/EBP gene is known to lack intron. In order to identify whether the NF-IL6 and the C/EBP cDNA are derived from a single gene by alternative splicing or from different genes on different chromosomal locations, we tried to isolate a genomic clone corresponding to the human NF-IL6 cDNA. We screened a human genomic library from placenta using the human NF-IL6 cDNA insert under high-stringency conditions. Several clones were isolated out of 10^6 recombinant phages. One of them, clone G16, was further analyzed (Figure 3A). The sequence of the genomic clone was found to be contiguous with the sequence of the NF-IL6 cDNA, indicating that the NF-IL6 gene is also an intronless gene.

A canonical TATAAA homology was observed in the genomic DNA sequence 231 bp upstream from the first methionine codon of the NF-IL6. To map the NF-IL6 mRNA cap site, an oligonucleotide complementary to the residues +127 to +152 was synthesized and a primer extension assay was carried out on SK-MG-4 mRNA. The result mapped the location of the NF-IL6 mRNA cap site to the site as shown in Figure 3(B). Therefore, overall length of the transcript of NF-IL6 is ~1.8 kb, which is equivalent to the size of mRNA (2.0 kb) identified in several cell lines, taking into account the addition of a polyadenylation tail.

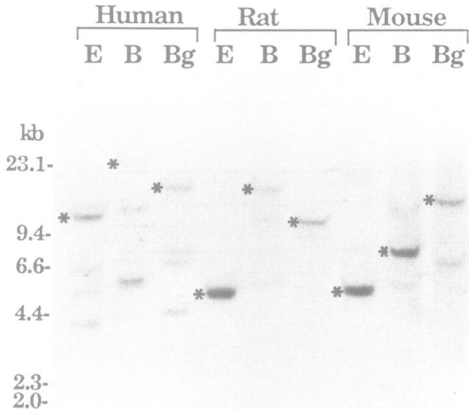

Fig. 4. Southern blot analysis of the NF-IL6 gene. Genomic DNAs from human, rat and mouse were digested with indicated restriction enzymes and hybridized with NF-IL6 cDNA probe (~300 bp *Spl*I–*Sac*I fragment including the putative DNA-binding domain). Washing conditions; 65°C, 0.1 × SSC and 0.1% SDS. In order to identify the NF-IL6-specific band, the same filter was rehybridized with the NF-IL6-specific probe (a ~100 bp *Pst*I–*Eco*RI fragment containing a leucine zipper motif in clone IS3; this *Eco*RI site is derived from the *Eco*RI site of λgt11 vector in the IS3 clone). The band marked corresponds to the NF-IL6-specific one.

Southern blot analysis of the NF-IL6 genomic gene reveals a family of C/EBP

To determine whether the gene encoding for NF-IL6 is conserved at least among other mammals, genomic DNAs of man, mouse and rat were digested with either *Eco*RI, *Bgl*II or *Bam*HI and hybridized with the ~300 bp *Spl*I–*Sac*I fragment containing the putative DNA-binding domain of NF-IL6 cDNA (Figure 4). In all lanes, a single intensely hybridizing band and several less intense bands were identified. This indicated that the NF-IL6 gene was highly conserved through evolution. Furthermore, this result strongly suggested the presence of several other genes with homology to NF-IL6. In fact, during the screening of NF-IL6 genomic clone we could obtain another clone, G13, which also showed high homology to the NF-IL6 and the C/EBP gene in the C-terminal portion (unpublished data).

Anti-peptide antibody inhibits constitutive and IL-1-inducible binding activities

To ascertain that the isolated cDNA actually encodes the native nuclear factor first identified by a gel-retardation assay, we raised polyclonal antisera in rabbits against several synthetic peptides corresponding to the sequences between amino acids 170 and 183 (Pep1), 224 and 242 (Pep2), and 224 and 277 (Pep3). We obtained a high titer of antisera against Pep2 and 3 but not against Pep1. As shown in Figure 5, polyclonal antiserum against Pep3 prevented the formation of both constitutive and IL-1-inducible DNA–protein complexes from SK-MG-4 nuclear extracts. In addition, polyclonal antiserum against Pep2 formed a new, slower migrating DNA–protein complex (data not shown). Taken together, these results strongly suggest that the recombinant NF-IL6 is identical to the NF-IL6 we first identified in the crude nuclear extract from glioblastoma cell line SK-MG-4. This result also suggests that the same protein was involved in both constitutive and IL-1-inducible complex formation,

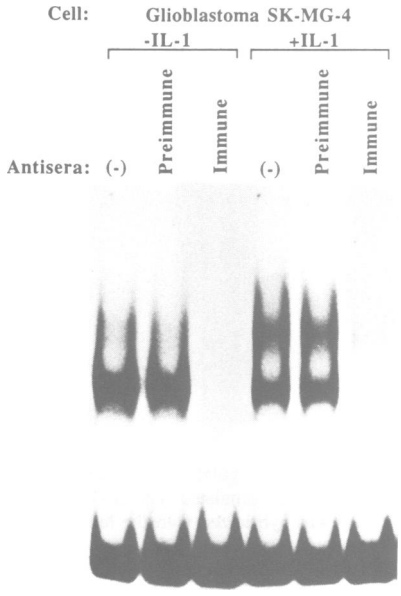

Fig. 5. Rabbit antisera against λIS3 bacterial fusion protein inhibit constitutive and IL-1-inducible binding activities. A gel-retardation assay was performed using the end-labeled fragment (−179/−111 of the human IL-6 gene). The anti-peptide sera against the putative DNA binding domain of NF-IL6 or the preimmune sera were added to the binding reactions.

considering the identical binding specificity in a gel-retardation assay and the identical methylation interference pattern of these complexes as previously shown (Isshiki *et al.*, 1990). Furthermore, immunoprecipitation of both non-stimulated and IL-1-stimulated SK-MG-4 extracts by these polyclonal antibodies revealed a polypeptide of M_r 38 000 daltons on SDS−PAGE (data not shown). This correlated well with the predicted mol. wt of NF-IL6. This result also indicated that the post-translational modification did not alter the mobility of NF-IL6 itself, supporting the previous speculation (Isshiki *et al.*, 1990) that NF-IL6 activity might be modulated by interaction with other proteins or by dimerization.

NF-IL6 is a positive transcriptional factor

We investigated whether the promoter harboring the NF-IL6-binding sites could be activated by the recombinant NF-IL6. Two types of reporter plasmids, K9CAT and IL-1RE-K9CAT, were used for this experiment. The K9CAT construct contained nucleotides −122 to +12 of the human IL-6 gene, which lacked the 14 bp NF-IL6-binding motif. The IL-1RE-K9CAT construct contained three copies of a synthetic 14 bp NF-IL6-binding site in front of K9CAT. The expression vectors CMV-NFIL6(+) and CMV-NFIL6(−) expressed the NF-IL6 cDNA in a sense and an antisense orientation from the cytomegalovirus enhancer/promoter respectively. Jurkat cells, which did not express the NF-IL6 gene, were co-transfected with various combinations of expression vectors and the reporter plasmids. As a control, the cells were transfected with the reporter plasmids only. As shown in Figure 6, the promoter of IL-1RE-K9CAT could be activated by co-transfection of the correctly expressed NF-IL6 cDNA, but not by co-transfection of the antisense cDNA. As expected,

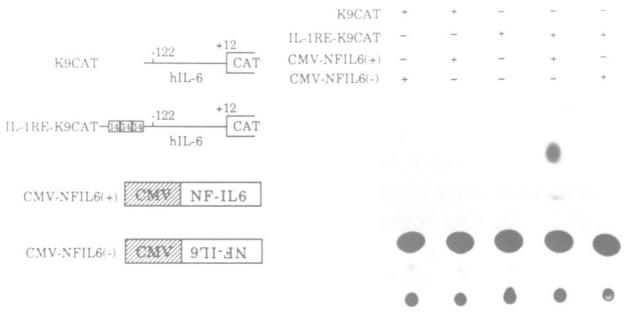

Fig. 6. NF-IL6 expressed in Jurkat cells activates transcription. The IL-1RE-K9CAT construct contains three copies of a 14 bp NF-IL6-binding site immediately upstream of the human IL-6 promoter (−122/+12 region) linked to the chloramphenicol acetyltransferase (CAT). The −122 to +12 region of the human IL-6 promoter lacks the 14 bp NF-IL6-binding site and has turned out to be unresponsive to IL-1 form 5′ deletion mutant analysis. The expression vectors CMV-NFIL6(+) and (−) express the NF-IL6 gene in sense and antisense orientation respectively from the cytomegalovirus enhancer/promoter. A combination of NF-IL6 expression vectors and CAT constructs was transfected into Jurkat cells, which do not express NF-IL6 mRNA, and 40 h later CAT activity was analyzed. This experiment was repeated three times and similar results were obtained. The result of one representative experiment is shown. The conversion in co-transfection of CMV-NFIL6(+) and IL-1RE-K9CAT was 9.2% while the conversion in other combinations was <0.2%.

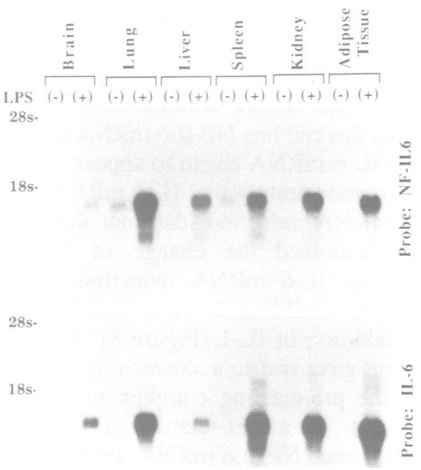

Fig. 7. Tissue distribution of NF-IL6 expression in mice. mRNA was obtained before and after LPS stimulation from various tissues of mice and poly(A) RNA (2 μg per lane) was hybridized with the NF-IL6-specific probe (a ~100 bp *Pst*I−*Eco*RI fragment containing a leucine zipper motif in clone IS3). The same filter was rehybridized with the mouse IL-6 probe. For preparation of mRNA from LPS-stimulated tissues, 20 μg of LPS was injected i.v. into each mouse and 4 h later the mouse was killed. The mouse NF-IL6 mRNA is ~200 bp smaller in length than the human counterpart (~2 kb).

the promoter of K9CAT could not be activated by NF-IL6. Taken together, these results demonstrate that NF-IL6 is a positively acting transcriptional factor. The effect of transfected NF-IL6 on the gene activation was not so drastic. This may be explained by the importance of the post-translational modification of NF-IL6 for transcriptional activation as described below.

Transcriptional regulation and post-translational modification of NF-IL6

To investigate the profile of the expression of NF-IL6 mRNA, we performed Northern blot analysis of RNAs from various sources. To exclude the possibility that a band observed in Northern blot was due to cross-hybridization of another member of the gene family, we used a NF-IL6-specific probe that revealed a single band in Southern blot analysis. We first analyzed the NF-IL6 expression in various mouse tissues. As shown in Figure 7, normal tissues did not express NF-IL6, although a small amount of NF-IL6 mRNA was expressed in lung, spleen and kidney. However, all tissues expressed NF-IL6 after LPS stimulation. This result showed that NF-IL6 was an inducible transcriptional factor. Rehybridization of the same filter with the mouse IL-6 probe showed that the pattern of NF-IL6 induction correlated well with that of IL-6 induction quantitatively. When induction of NF-IL6 protein was studied on mouse kidney and spleen cells, NF-IL6 protein was detected as a faster-migrating complex similar to that seen in SK-MG-4 in a gel-retardation assay as early as 1 h after LPS stimulation, although there was no detectable NF-IL6-binding motif-specific complexes in the absence of stimulation (data not shown). In contrast, NF-IL6 mRNA and protein were expressed constitutively in many cell lines examined, including a glioblastoma cell line SK-MG-4, hepatoma cell lines HepG2 and Hep3B, mouse macrophage cell lines J774 and WEHI3 and mouse L cells (data not shown). In mouse myeloid cell line M1, which differentiates into a macrophage by IL-6 stimulation, NF-IL6 mRNA was not expressed before stimulation but induced after stimulation. In this cell line NF-IL6 mRNA could be induced by 3 h while IL-6 mRNA began to appear around 6 h after stimulation, demonstrating that IL-6 mRNA was preceded by NF-IL6 mRNA induction (data not shown).

We next examined the change of NF-IL6 mRNA accompanied by IL-6 mRNA induction in glioblastoma SK-MG-4. In this cell line IL-6 was rapidly and drastically induced by addition of IL-1 (Figure 8). At the same time IL-1 treatment gave rise to a slower-migrating complex in addition to the pre-existing complex in the glioblastoma nuclear extract in a gel-retardation assay. However, SK-MG-4 expressed NF-IL6 mRNA constitutively and there was little increase of NF-IL6 mRNA after IL-1 stimulation. These results indicate that IL-1-mediated IL-6 induction is most likely to occur due to modification of pre-existing NF-IL6 in this cell line. This may also explain the fact that induction of IL-6 mRNA by IL-1 is not blocked by cycloheximide in SK-MG-4 and does not require *de novo* protein synthesis (unpublished data). As the post-translational modification of NF-IL6 did not involve significant alterations in its electrophoretic mobility on SDS−PAGE, and the amount of the faster-migrating complex diminished upon induction, it is possible that formation of the inducible complex could involve the interaction of the pre-existing constitutive complex with a second new protein which was activated after IL-1 stimulation or dimerization of the constitutive complex.

Taken together, these data show that activity of NF-IL6 is regulated both at the transcriptional and the post-translational levels.

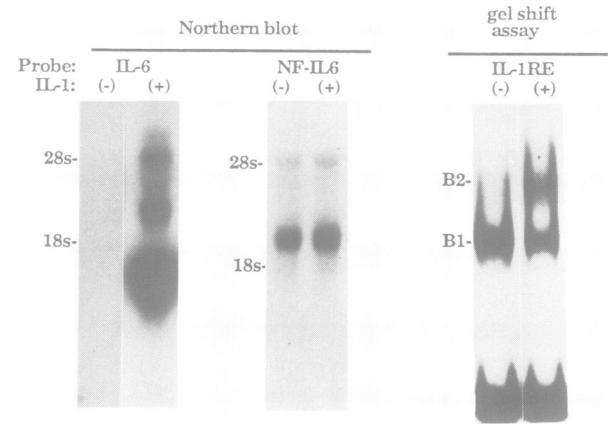

Fig. 8. Activity of NF-IL6 is regulated at the post-translational level. Glioblastoma SK-MG-4 was stimulated with IL-1 (100 U/ml) for 6 h. Poly(A) RNA (2 μg) was hybridized with the human IL-6 probe or the NF-IL6-specific probe. The probe used for a gel-shift assay was a fragment containing the NF-IL6-binding motif (−179/−111 of the human IL-6 gene).

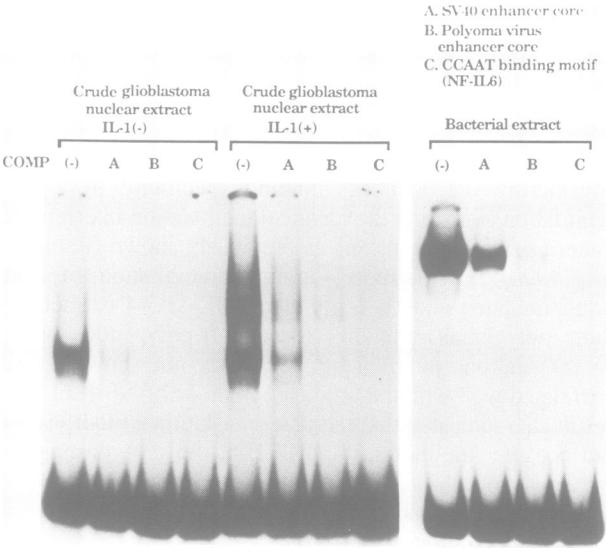

Fig. 9. NF-IL6 binds to viral enhancer core sequences. End-labeled fragment (−179/−111 of the human IL-6 gene) was incubated with crude nuclear extracts from glioblastoma SK-MG-4 or recombinant NF-IL6 in the absence or in the presence of the indicated competitors and electrophoresed.

NF-IL6 binds to the viral enhancer core sequence and the CCAAT homology identified in several acute-phase reaction associated genes

Since C/EBP binds avidly to both CCAAT homologies and viral enhancer core homologies, NF-IL6 was also expected to bind to the viral enhancer core sequences. As shown in Figure 9, the complexes formed in the presence of the crude nuclear extract from SK-MG-4 or the bacterial fusion protein could be competed by viral enhancer core sequences such as the SV40 enhancer core and the polyoma virus enhancer core.

We further analyzed the sequence that NF-IL6 recognized. Competition analysis of the binding of NF-IL6 with the

Table I. Summary of NF-IL6 recognition sequences

A	G	A	T	T	G	T	G	C	A	A	T	G	T	IL-6 (human)	−145/−158	Yasukawa *et al.* (1987)
A	C	A	T	T	G	T	G	C	A	A	T	C	T	IL-6 (mouse)	−160/−147	Tanabe *et al.* (1988)
T	G	A	T	T	T	T	G	T	A	A	T	G	G	Albumin (DE1)	−105/−92	Lichtsteiner *et al.* (1987)
A	G	A	T	T	G	A	G	C	A	A	T	C	T	Albumin, −3.5 kb HS	−3.5 kb	Liu *et al.* (1988)
G	T	C	T	T	A	A	G	C	A	A	A	G	C	α1-Antitrypsin site C	−200/−213	Costa *et al.* (1988)
G	T	A	T	T	A	G	G	A	C	A	T	G	T	Transthyretin site 2	−1.8 ~ −1.9 kb	Costa *et al.* (1988)
A	T	G	T	T	G	A	G	T	A	A	G	A	T	Transthyretin site 3	−1.8 ~ −1.9 kb	Costa *et al.* (1988)
G	T	G	T	G	A	A	G	C	A	A	G	A	G	Haptoglobin site A	−169/−156	Oliviero and Cortese (1989)
G	A	A	T	T	A	C	G	A	A	A	T	G	G	Haptoglobin site C	−71/−58	Oliviero and Cortese (1989)
A	A	G	T	T	G	T	G	C	A	A	T	G	G	α1-Acid glycoprotein	−5 kb	Prowse and Baumann (1988)
T	A	G	T	G	G	C	G	C	A	A	A	C	T	CRP	−59/−46	Arcone *et al.* (1988)
C	A	G	T	G	A	T	G	T	A	A	T	C	A	Hemopexin site A	−118/−105	Poli and Cortese (1989)
A	A	G	T	T	G	A	G	A	A	A	T	T	T	422 (aP2)	−148/−135	Christy *et al.* (1989)
G	G	C	T	G	A	G	G	A	A	A	T	A	C	Stearolyl-CoA desaturase	−76/−63	Christy *et al.* (1989)
A	C	A	A	G	T	T	G	C	A	A	C	A	T	CPS1	−120/−107	Howell *et al.* (1989)
G	G	A	T	T	T	G	G	A	A	A	G	T	T	TNFα (human)	−183/−196	Hensel *et al.* (1989)
G	T	T	T	T	G	T	G	A	A	A	T	C	G	G-CSF (mouse)	−170/−183	Nishizawa *et al.* (1990)
A	C	A	T	A	A	T	G	A	A	A	A	G	A	IL-8	−67/−54	Mukaida *et al.* (1989)
G	G	G	T	G	T	G	G	A	A	A	G	T	C	SV40 enhancer	251/238	Johnson *et al.* (1987)
T	G	G	T	T	T	T	G	C	A	A	G	A	G	Polyoma enhancer	5216/5229	Johnson *et al.* (1987)
A	T	C	T	G	T	G	G	T	A	A	G	C	A	MSV enhancer	−231/−218	Johnson *et al.* (1987)
			T	T	N	N	G	N	A	A	T			(consensus)		
				(G)							(G)					

Abbreviations: DE1, distal element 1; HS, hypersensitive site; CRP, C-reactive protein; CPS1, carbamyl phosphate synthetase 1; TNF, tumor necrosis factor; G-CSF, granulocyte-colony stimulating factor; MSV, murine sarcoma virus.

published binding sequences to which C/EBP-like proteins bound revealed that NF-IL6 and C/EBP recognized the same nucleotide sequences and that the best fit was the consensus T(T/G)NNGNAA(T/G) for NF-IL6 (Table I).

Of particular interest were the sequences seen in several acute-phase protein genes such as the C-reactive protein, hemopexin, haptoglobin and α1-acid glycoprotein genes (Table I). These sequences have been identified as the IL6-responsive elements (Prowse and Baumann, 1988; Oliviero and Cortese, 1989; Poli and Cortese, 1989). Complex formation by these IL6-responsive elements in the presence of hepatoma cell nuclear extracts was completely inhibited by the 14 bp palindromic NF-IL6-binding sequence (data not shown). This result suggested that NF-IL6 involved in the IL-6 gene expression also participated in the expression of the genes regulated by IL-6. Therefore, we examined if NF-IL6 mRNA could be induced in hepatocytes by acute-phase inducers, IL-6 or IL-1. As shown in Figure 10, the administration of either IL-6 or IL-1 induced NF-IL6 mRNA drastically in mouse liver cells. Even in the hepatoma cell lines HepG2 and Hep3B that express NF-IL6 mRNA and protein constitutively, a several-fold increase of NF-IL6 mRNA was demonstrated (data not shown). However, the modification of NF-IL6 in hepatoma cells seemed to differ from that seen in SK-MG-4. Several DNA−protein complexes were formed using the IL6-responsive element of the hemopexin gene in the presence of the non-stimulated HepG2 extracts, and the intensity of these complexes increased after IL-6 stimulation without formation of any new complexes. These results strongly suggest the involvement of NF-IL6 in the gene regulation for acute-phase proteins.

Furthermore, NF-IL6 could bind to the GPE1 element of the G-CSF gene. The GPE1 element was shown to be

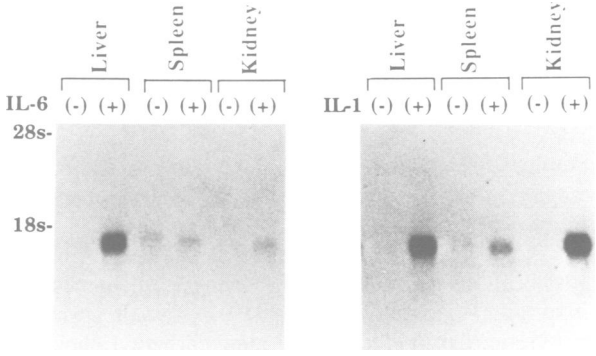

Fig. 10. Induction of NF-IL6 mRNA by IL-6 and IL-1 in mouse liver. For preparations of mRNA from IL-6- and IL-1-stimulated tissues, 10 μg (5 × 10^4 units) of IL-6 and 1 μg (2 × 10^4 units) of IL-1 were injected i.v. into each mouse and 4 h later the mouse was killed. Poly(A) RNA (2 μg per lane) was hybridized with the NF-IL6-specific probe.

required for the constitutive expression of G-CSF in human carcinoma cells (CHU-2) and for its LPS-inducible expression in macrophages (Nishizawa *et al.*, 1990). NF-IL6 has also been shown to bind to the sequences within the regulatory region of IL-8 and TNF (Table I). These results suggest that NF-IL6 may take part in the regulation of G-CSF, IL-8 and TNF genes.

Discussion

Existence of a C/EBP family

The high degree of sequence homology within the DNA-binding domains of NF-IL6 and C/EBP suggests that the

NF-IL6 and C/EBP genes may have arisen by duplication of a common ancestral gene. Southern blot analysis of genomic DNAs from mouse, rat and human showed that the DNA-binding domain of the NF-IL6 gene was highly conserved, at least during evolution of higher vertebrates. In addition, we have identified the existence of the several related genes with a region highly homologous to the DNA-binding domain of NF-IL6 on the basis of Southern blot analyses. We have obtained another genomic clone, G13, from a human placental phage library, which showed a high of homology with the DNA-binding domain of C/EBP or NF-IL6 but very little sequence conservation at other portions. Although we do not know whether this gene is functional or not, this fact indicates the existence of the selective pressure that worked on the maintenance of this DNA-binding domain.

Although C/EBP was originally considered to be a liver-specific DNA-binding protein (Johnson *et al.*, 1987), C/EBP does not appear to be strictly cell-type specific, at least as judged by foot-printing and gel-retardation assays (Lichtsteiner *et al.*, 1987; Costa *et al.*, 1988; Liu *et al.*, 1988). The binding activity of C/EBP was more abundant in liver than other tissues, but the relatively high level of binding was observed in cells not expressing the albumin. Furthermore, the recent result of *in vitro* transcription of the albumin gene showed that a synthetic promoter composed of multimerized C/EBP motifs and a TATA box was well transcribed even in spleen, suggesting that C/EBP may not be involved in liver-specific expression of the albumin gene (Maire *et al.*, 1989). However, the expression of the C/EBP gene, which was studied by a combination of Northern blot and antibody-staining assays, revealed that C/EBP mRNA was detected in liver and fat but not found in spleen or brain (Birkenmeier *et al.*, 1989; Xanthopoulos *et al.*, 1989). This discrepancy may be explained by the present result that there exist several C/EBP-related proteins, each of which is expressed in a different tissue. So far, a C/EBP-binding site has been identified only in the liver-specific genes such as albumin (Friedman *et al.*, 1989), α1-antitrypsin and transthyretin (Costa *et al.*, 1988), and carbamyl phosphate synthetase I (Howell *et al.*, 1989). However, our results strongly suggest that C/EBP-like factors participate in regulation of some other genes, including IL-6, in other tissues. Cloning by cross-hybridization with the sequence from the C/EBP DNA-binding domain will uncover more members of this family.

NF-IL6 may be involved in the expression for acute-phase proteins and other cytokines

Acute-phase proteins play important protective roles in the host defense against tissue damage and infection. IL-6 is a major hepatocyte-stimulating factor that regulates the production of a wide spectrum of acute-phase proteins. IL-6 has been shown to activate transcription of the human haptoglobin, hemopexin and C-reactive protein genes in human hepatoma cell lines (Morrone *et al.*, 1988). Recently, IL6-responsive elements have been identified in the promoters of these liver-specific genes (Prowse and Baumann, 1988; Oliviero and Cortese, 1989; Poli and Cortese, 1989). More interestingly, we have noticed that these IL6-responsive elements are very similar to the NF-IL6- or the C/EBP-binding motif. In fact, the complexes formed by these IL6-responsive elements were completely competed away with the 14 bp palindromic NF-IL6-binding motif. Furthermore, the antibody against the NF-IL6 DNA-binding domain blocked the formation of these complexes (unpublished data). Taken together, these results strongly indicated that NF-IL6 may be involved in the transcriptional activation of these genes by IL-6. In this regard, the difference of NF-IL6 and C/EBP expression between normal hepatocytes and hepatoma cells is noteworthy. C/EBP is constitutively expressed in adult hepatocytes but is more than one order of magnitude less in hepatoma cells (Friedman *et al.*, 1989). It has also been shown that hepatoma cell lines transcribe the albumin gene at a much lower rate than normal hepatocyte (Clayton *et al.*, 1985). In contrast, NF-IL6 is not expressed in adult hepatocytes but is induced after LPS, IL-1 or IL-6 stimulation. In hepatoma cell lines HepG2 and Hep3B, NF-IL6 is expressed constitutively. Furthermore, it was demonstrated that NF-IL6 expression is augmented in hepatoma cells by addition of IL-6 (unpublished data). Thus, NF-IL6 expression is reciprocally related to the expression of C/EBP. Therefore, it is interesting to speculate as follows. C/EBP regulates a broad spectrum of liver-specific genes such as albumin and transthyretin in normal physiologic condition. NF-IL6 expression is suppressed under this condition. However, once acute-phase reaction takes place, NF-IL6 is rapidly induced and may be involved in the induction of acute-phase proteins, although the possibility cannot be excluded that C/EBP is also involved in induction of the acute-phase protein genes. Further study will be required to confirm this hypothesis.

NF-IL6 is drastically and abundantly induced in all tissues after stimulation with LPS or other agents. Many cytokines, including IL-1, TNF, IL-6, IL-8 and G-CSF, are released from macrophages and several other cell types by LPS. This result implies that NF-IL6 may be involved in the genes activated by LPS. Recently, it has been shown that the −169 to −182 bp region of the promoter of the murine G-CSF gene functions as an LPS-inducible enhancer element in macrophages (Nishizawa *et al.*, 1990; S.Nagata and M. Nishizawa, personal communication). This region is also very similar to the NF-IL6-binding motif, and it was in fact shown that a recombinant NF-IL6 bound to this region. In addition, we have identified the existence of NF-IL6 mRNA and NF-IL6 protein in human carcinoma cells (CHU-2) that secrete G-CSF in large amounts. Furthermore, we have observed binding of NF-IL6 to the regulatory regions of the TNF (Hensel *et al.*, 1989) and the IL-8 genes (Mukaida *et al.*, 1989; K.Matushima and N.Mukaida, personal communication), that are both produced by LPS-stimulated monocytes. In summary, these results strongly indicate that NF-IL6 may be responsible for the expression of acute-phase proteins and various LPS-inducible cytokines.

Hence, the interaction of a common nuclear factor, NF-IL6, with different target genes that participate in acute-phase reactions may lead to the amplification via IL-6-mediated NF-IL6 induction as shown in hepatocytes, resulting in a more rapid and stronger biological effects.

Virus and IL-6-associated disorders

IL-6 may be one of the principal cytokines for generalized autoimmune disease. Several tumor cells such as cardiac myxoma, cervical cancer cells and bladder carcinomas aberrantly produce a large amount of IL-6, and patients with such tumors often show autoantibody production and

autoimmune symptoms (Kishimoto and Hirano, 1988). One of the striking examples of abnormal production of IL-6 and autoimmune diseases is rheumatoid arthritis (Hirano *et al.*, 1988). Unregulated expression of the IL-6 gene is also involved in the pathogenesis of Castleman's disease, which is characterized by lymphadenopathy with massive infiltration of plasma cells and systemic symptoms such as hypergammaglobulinemia, increase in acute-phase proteins and anemia (Yoshizaki *et al.*, 1989).

It has been assumed that viral persistence and reactivation have a considerable potential to cause or at least trigger autoimmune phenomena (Notokines *et al.*, 1984). Rheumatoid arthritis has been ascribed to a number of viruses (Bennett, 1985). In this regard, it is noteworthy that NF-IL6, which regulates IL-6 production, also binds to viral enhancer core homologies. An enhancer core sequence is known to be critical for efficient transcriptional activity of viral enhancers and replication (Weiher *et al.*, 1983). Induction of the viral productive cycle in latently infected cells probably occurs in response to changes in differentiation state of the host cells or in response to activation of these cells by external stimuli, including antigens, cytokines and heterologous viral *trans*-activators (Cullen and Greene, 1989). In such cases, the provirus may be activated by the action of the host transcription factors. Recently, both IL-1 and TNF have been shown to induce NF-$\varkappa$B and stimulate the HIV enhancer (Lowenthal *et al.*, 1989; Osborn *et al.*, 1989). Like NF$\varkappa$B, NF-IL6 may activate certain proviruses as well as the IL-6 gene. Preliminary study shows that NF-IL6 binds to the enhancer of HIV-1. Further study will disclose the role that NF-IL6 plays in viral gene transcription and replication.

Materials and methods

Cell lines

A human glioblastoma cell line, SK-MG-4, was kindly provided by Dr R.Ueda, Aichi Cancer Institute. SK-MG-4 cells were grown in RPMI 1640 containing 10% fetal calf serum, 1 mM glutamine, penicillin (100 U/ml) and streptomycin (100 μg).

Plasmid construction

K9CAT contains the human IL-6 promoter (position -122 to $+12$) linked to the CAT gene. For construction of expression vectors CMV-NFIL6(+) and (−), a fragment extending from 102 bp upstream of the first ATG of the NF-IL6 gene to 30 bp 3′ of the polyadenylation signal sequence was isolated from the NF-IL6 genomic clone G16 and inserted downstream of the cytomegalovirus promoter/enhancer in a sense and an antisense orientation respectively.

Isolation of recombinant clone encoding NF-IL6

A λgt11 library made from LPS-stimulated human peripheral monocyte cDNA (Clontech, Palo Alto, CA) was screened according to the original method developed by Singh *et al.* (1988) and the method, with some modifications, of Miyamoto *et al.* (1988). *Escherichia coli* strain Y1090 was infected with 5×10^4 recombinant phages and plated on a 150 mm plate. After 4 h of incubation at 42°C, nylon membranes (Nytran, Schleicher and Schuell) saturated in 10 mM IPTG were overlaid on the plates and incubated at 37°C overnight. Subsequently, the filters were chilled at 4°C for 20 min. The filters were immersed in BLOTTO (5% non-fat milk powder, 50 mM Tris, pH 7.5, 1 mM EDTA and 1 mM DDT) for 1 h at 4°C. For screening, filters were incubated in TNE-50 containing ^{32}P-labeled probe (four times repeated oligonucleotides D) and 10 μg/ml of salmon sperm DNA. After 1 h incubation, the filters were washed with TNE-50, dried and exposed to X-ray film at −70°C. Approximately 5×10^5 plaques were screened, and only one was positive. Libraries from LPS-stimulated human peripheral monocyte cDNA and human placenta cDNA (Clontech) were screened using an insert and several positive clones were obtained. For genomic clones, the human placental genomic library (Clontech) was screened using the same insert.

Preparation of anti-NF-IL6 serum

Three peptides with the sequences AELKAEPGFEPAD (Pep1), STSSSSSPPGTPSPAD (Pep2) and SKAKKTVDKHSDEYKIRR (Pep3) were synthesized from the predicted amino acid sequence of NF-IL6 by Dr Yasukawa, Tohso Inc., Tokyo. The synthetic peptides were coupled to the carrier protein OVA as described previously (Hirano *et al.*, 1987). Rabbits were immunized with the conjugates (0.2 mg/rabbit, five times at 2 week intervals) and sera were collected. Antibodies were purified by affinity chromatography using peptide-conjugated Sepharose 4B (Pharmacia).

Gel-retardation assay

Nuclear extracts were prepared according to Dignam *et al.* (1983). A fragment (−179/−111 bp region) containing the NF-IL6 binding site was end-labeled using [^{32}P]deoxynucleotides and Klenow enzyme. Binding reactions were essentially as described previously (Aguilera *et al.*, 1987). Nuclear extracts (3 μg) were incubated at 4°C for 30 min with the ^{32}P-labeled fragment in 30 μl of binding buffer consisting of 10 mM HEPES (pH 7.9), 50 mM NaCl, 5 mM Tris−HCl (pH 7.0), 1 mM dithiothreitol, 15 mM EDTA, 10% glycerol and varying amounts of the carrier polymer poly(dI-dC) (P-L Biochemicals). The DNA−protein complexes were then separated in 5% polyacrylamide gel (acrylamide to bisacrylamide ratio of 30:1) containing 7 mM Tris−HCl (pH 7.5), 3.3 mM sodium acetate, 3.8 mM β-mercaptoethanol and 1 mM EDTA. For band shifts with antisera added, binding reactions were performed as described above except that the antisera were added to a final dilution of 1:100 for 2 h prior to addition of the probe.

Methylation interference assay

The probe containing the −179/−111 bp region, ^{32}P-labeled at one 5′ end, was partially methylated with dimethyl sulfate (DMS) as described by Maxam and Gilbert (1977). The methylated DNA probe was incubated with the lysogen bacterial extract. After electrophoretic separation as above and brief autoradiography, the protein-bound and free oligonucleotide probes were cut out and recovered from the gel. After cleavage with piperidine, approximately equal amounts of radioactivity for each sample were electrophoresed on 10% acrylamide/6 M urea gels and autoradiographed.

CAT assay

For transfection of Jurkat cells, the DEAE−dextran procedure was used. Co-transfections contained 5 μg of expression vector and 5 μg of reporter plasmid. At 40 h after transfection, cells were harvested and lysed by four cycles of freeze and thaw. Cell extracts from 2×10^6 cells were analyzed for CAT activity by the method of Gorman *et al.* (1982).

Acknowledgements

We would like to thank Ms M.Kawata and Ms K.Kubota for their secretarial assistance. This work was supported in part by grants from the Ministry of Education, Science and Culture.

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Received on January 26, 1990; revised on February 26, 1990

Note added in proof

The accession number in the EMBL database for the sequence shown in Figure 3 is X52560.