Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 1473–1476, February 1992
Immunology

A member of the C/EBP family, NF-IL6β, forms a heterodimer and transcriptionally synergizes with NF-IL6

(transcription factor/leucine zipper/interleukin 6/inflammation)

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Contributed by Tadamitsu Kishimoto, November 1, 1991

ABSTRACT **Using a DNA probe from the DNA-binding portion of the NF-IL6 gene and an antibody against the DNA-binding domain of NF-IL6, we isolated a gene homologous to NF-IL6 in the DNA-binding and leucine zipper domains. This intronless gene, termed NF-IL6β encodes a 269-amino acid protein with a potential leucine zipper structure, and the gene product can bind to the CCAAT homology as well as the viral enhancer core sequence, as in the cases of NF-IL6 and C/EBP. This gene is expressed at an undetectable or a minor level in normal tissues but is induced by lipopolysaccharide or inflammatory cytokines, as in the case of NF-IL6. NF-IL6β easily forms a heterodimer with NF-IL6 *in vitro* and the heterodimeric complex binds to the same DNA sequence as the respective homodimers. When examined by transient luciferase assays, NF-IL6β is consistently a stronger transactivator than NF-IL6. Furthermore, NF-IL6β shows a synergistic transcriptional effect with NF-IL6. These data suggest that NF-IL6β is an important transcriptional activator in addition to NF-IL6 in regulation of the genes involved in the immune and inflammatory responses.**

NF-IL6 was originally identified as a DNA-binding protein responsible for interleukin 1 (IL-1)-stimulated interleukin 6 (IL-6) induction (1). Direct cloning of the human NF-IL6 revealed its homology with C/EBP (2). C/EBP is expressed in liver and adipose tissues and is supposed to regulate several hepatocyte- and adipocyte-specific genes such as the albumin, phospho*enol*pyruvate carboxykinase, 422/aP2 protein, stearoyl-CoA desaturase 1, and insulin-responsive glucose transporter genes (3–8). In contrast, NF-IL6 is expressed at an undetectable or a minor level in normal tissues but is rapidly and drastically induced by lipopolysaccharide (LPS) or inflammatory cytokines such as IL-1, tumor necrosis factor, and IL-6. NF-IL6 can bind to the regulatory region of various acute-phase protein genes and several cytokine genes and can transactivate the promoters of these genes (9–14). These results suggest that NF-IL6 may be involved in regulation of a variety of genes involved in immune and inflammatory responses. NF-IL6 has recently been reported by other groups as AGP/EBP (9), LAP (10), IL-6DBP (11), C/EBPβ (15), and CRP2 (16). C/EBP and NF-IL6 belong to the so-called basic leucine zipper (bZIP) family. bZIP proteins bind to DNA as dimers through a leucine zipper structure that is required for dimerization and an adjacent basic region that makes direct contact with DNA (17). In fact, NF-IL6 and C/EBP can form a heterodimer *in vitro* and functionally interact *in vivo* (11). Accumulating evidence indicates that heterodimer formation between members of the family increases the repertoire of transcription factors and provides a variety of mechanisms of transcriptional regulation (18, 19).

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In the process of the molecular cloning of the NF-IL6 gene, we showed the existence of several other genes related to NF-IL6 by genomic Southern blot analysis (2). We report here the cloning and characterization of an additional member of the C/EBP gene family, termed NF-IL6β.‡ Expression of this clone is also suppressed in normal tissues but is induced by LPS or inflammatory cytokines, as in the case of NF-IL6. NF-IL6β forms a heterodimer with NF-IL6, and this complex synergistically enhances the transcriptional activity. Therefore, this newly discovered member of the C/EBP family is involved in regulation of the genes responsible for immune and inflammatory responses in addition to NF-IL6.

MATERIALS AND METHODS

Plasmid Construction. The reporter plasmid consists of 5′ flanking region (−179/+12) of the human IL-6 gene linked to the structural sequence of the firefly luciferase gene. NF-IL6β and NF-IL6 expression vectors (CMV–NF-IL6β and CMV–NF-IL6) were constructed by inserting the coding sequences of the respective cDNAs downstream of the cytomegalovirus promoter/enhancer. BS–NF-IL6β plasmid was constructed by inserting the *Sac* I/*Pst* I fragment from the NF-IL6β cDNA clone into the *Sma* I/*Pst* I sites of the Bluescript SK+ vector in-frame with the β-galactosidase gene. BS–NF-IL6 delSpl plasmid was constructed by fusing the first 37 codons of the β-galactosidase gene in-frame with a truncated NF-IL6 cDNA that lacks the internal *Spl* I/*Spl* I fragment. These plasmids were used to transform *Escherichia coli* JM105, and synthesis of the fusion proteins was induced by addition of isopropyl β-D-thiogalactopyranoside.

Isolation of Recombinant Clones Encoding NF-IL6β. An NF-IL6β genomic clone was isolated by screening a human placental genomic library (Clontech) with the NF-IL6 cDNA probe [≈300-base-pair (bp) *Spl* I/*Sac* I fragment including the DNA-binding domain]. Hybridization was carried out in 6× standard saline citrate at 55°C for 16 hr. To isolate NF-IL6β cDNA, a λgt11 expression library prepared from human lung cDNA (Clontech) was screened with an antibody against the common 17 amino acids in the DNA-binding domains of NF-IL6β, NF-IL6, and C/EBP.

Preparation of an Antibody. A synthetic peptide corresponding to the common 17 amino acids (Arg-Arg-Glu-Arg-Asn-Asn-Ile-Ala-Val-Arg-Lys-Ser-Arg-Asp-Lys-Ala-Lys) in the DNA-binding domains of NF-IL6β, NF-IL6, and C/EBP was synthesized by K. Yasukawa (Tohso, Tokyo). The synthetic peptide was coupled to ovalbumin and a rabbit was immunized with the conjugates. Antibodies were affinity purified by using the peptide-conjugated Sepharose 4B (Pharmacia).

Abbreviations: LPS, lipopolysaccharide; IL-1, interleukin 1; IL-6, interleukin 6.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M83667).

Luciferase Assays. P19 embryonic carcinoma cells were cultured in minimal essential medium (α modification) (GIBCO) supplemented with 10% fetal bovine serum. P19 cells were seeded at 1.5×10^5 cells per 60-mm dish. One day later, cells were transfected with 5 μg of the reporter plasmid with the indicated amounts of CMV–NF-IL6β or CMV–NF-IL6 expression vector by the calcium phosphate coprecipitation technique. Cells were harvested 48 hr after transfection and measured for luciferase activity as described (20).

Other Procedures. Gel-retardation assays were carried out as described (1). For analysis of dimerization, a mixture of NF-IL6 and NF-IL6β proteins was preincubated for 15 min at 37°C. Northern blot analysis was performed essentially as described (2), using the antisense nucleotide corresponding to the nucleotide sequence (+187/+225) of the NF-IL6β gene as a probe. DNA sequencing was performed according to the dideoxynucleotide chain-termination method.

RESULTS

Cloning of an NF-IL6 Related Gene. To isolate the NF-IL6-related genes, we screened a human placental genomic DNA library at moderate stringency, using the DNA fragment corresponding to the DNA-binding domain of the human NF-IL6 gene as a probe. Several positive clones were isolated from 2×10^6 recombinant phages. Among them, one clone, termed HPG13, did not hybridize with the NF-IL6-specific probe and seemed to be different from the NF-IL6 gene. The nucleotide sequence corresponding to the region homologous to the DNA-binding domain of NF-IL6 was determined and one of three deduced amino acid sequences included the 17-amino acid stretch that was commonly present in the DNA-binding domains of NF-IL6 and C/EBP (Fig. 1). To obtain cDNA clones corresponding to the genomic clone, we screened a λgt11 cDNA library prepared from human lung with a rabbit polyclonal antibody against the common 17 amino acids and obtained a positive clone, termed HLu5. By using the cDNA insert of the HLu5 clone as a probe, several additional clones were obtained from the human lung cDNA library. The combined cDNA sequence was 1.2 kilobases (kb) long, which was consistent with the length of the band detected by Northern blot analysis in several human cell lines. The large open reading frame encoded a protein of 269 amino acids with a calculated molecular mass of 28.4 kDa. We termed this protein NF-IL6β. The comparison of the genomic sequence from the HPG13 clone with that of the cDNA revealed that the NF-IL6β gene is devoid of introns, as in the cases of the NF-IL6 and C/EBP genes. The sequence of the NF-IL6β gene together with the deduced amino acid sequence is shown in Fig. 2. The transcriptional start site was determined by a primer-extension method and mapped 42 bp upstream of the first methionine (data not shown). A TATA homology was detected 31 bp upstream of the transcriptional start site.

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NF-IL6 β   SAGKRGPDRGSPEYRQRRERNNIAVRKSRDKAKRRNQEMQQKLVE
NF-IL6     SKAKKTVDKHSDEYKIRRERNNIAVRKSRDKAKMRNLETQHKVLE
C/EBP      GKAKKSVDKNSNEYRVRRERNNIAVRKSRDKAKQRNVETQQKVLE
Ig/EBP     SKKSSPMDRNSDEYRQRRERNNMAVKKSRLKSKQKAQDTLQRVNQ
                .     .     .     .     .
           LSAENEKLHQRVEQLTRDLAGLRQFFKQLPS
           LTAENERLQKKVEQLSRELSTLRNLFKQLPE
           LTSDNDRLRKRVEQLSRELDTLRGIFRQLPE
           LKEENERLEAKIKLLTKELSVLKDLFLEHAH
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FIG. 1. Comparison of C-terminal amino acid sequences of NF-IL6β and other C/EBP family members. The leucine residues of the leucine zipper domain are marked with a solid circle.

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-90                                          CACGTCAGCCGGGGCTAGAAAAGGCGGC
-60  GGGGCTGGGCCCAGCGAGGTGACAGCCTCGCTTGGACGCAGAGCCCGGCCCGACGCCGCC
  1  ATGACGGCCGCGCTCTTCAGCCTGGACGGCCCGGCCGGCGGCGCGCCCTGGCCTGCGGAG
     M  T  A  A  L  F  S  L  D  G  P  A  G  G  A  P  W  P  A  E
 61  CCTGCGCCCTTCTACGAACCGGGCCGGGCGGGCAAGCCGGGCCGCGGGGCCGAGCCAGGG
     P  A  P  F  Y  E  P  G  R  A  G  K  P  G  R  G  A  E  P  G
121  GCCCTAGGCGAGCCAGGCGCCGCCGCCCCCGCCATGTACGACGACGAGAGCGCCATCGAC
     A  L  G  E  P  G  A  A  A  P  A  M  Y  D  D  E  S  A  I  D
181  TTCAGCGCCTACATCGACTCCATGGCCGCCGTGCCCACCCTGGAGCTGTGCCACGACGAG
     F  S  A  Y  I  D  S  M  A  A  V  P  T  L  E  L  C  H  D  E
241  CTCTTCGCCGACCTCTTCAACAGCAATCACAAGGCGGGCGGCGCGGGGCCCCTGGAGCTT
     L  F  A  D  L  F  N  S  N  H  K  A  G  G  A  G  P  L  E  L
301  CTTCCCGGCGGCCCCGCGCGCCCCTTGGGCCCGGGCCCTGCCGCTCCCCGCCTGCTCAAG
     L  P  G  G  P  A  R  P  L  G  P  G  P  A  A  P  R  L  L  K
361  CGCGAGCCCGACTGGGGCGACGGCGACGCGCCCGGCTCGCTGTTGCCCGCGCAGGTGGGC
     R  E  P  D  W  G  D  G  D  A  P  G  S  L  L  P  A  Q  V  G
421  CCGTGCGCACAGACCGTGGTGAGCTTGGCGGCCGCAGGGCAGCCCACCCCGCCCACGTCG
     P  C  A  Q  T  V  V  S  L  A  A  A  G  Q  P  T  P  P  T  S
481  CCGGAGCCGCCGCGCAGCAGCCCCAGGCAGACCCCCGCGCCCGGCCCCGCCCGGGAGAAG
     P  E  P  P  R  S  S  P  R  Q  T  P  A  P  G  P  A  R  E  K
541  AGCGCCGGCAAGAGGGGCCCGGACCGCGGCAGCCCCGAGTACCGGCAGCGGCGCGAGCGC
     S  A  G  K  R  G  P  D  R  G  S  P  E  Y  R  Q  R  R  E  R
601  AACAACATCGCCGTGCGCAAGAGCCGCGACAAGGCCAAGCGGCGCAACCAGGAGATGCAG
     N  N  I  A  V  R  K  S  R  D  K  A  K  R  R  N  Q  E  M  Q
661  CAGAAGTTGGTGGAGCTGTCGGCTGAGAACGAGAAGCTGCACCAGCGCGTGGAGCAGCTC
     Q  K  L  V  E  (L)  S  A  E  N  E  K  (L)  H  Q  R  V  E  Q  (L)
721  ACGCGGGACCTGGCCGGCCTCCGGCAGTTCTTCAAGCAGCTGCCCCAGCCCGCCCTTCCTG
     T  R  D  L  A  G  (L)  R  Q  F  F  K  Q  (L)  P  S  P  P  F  L
781  CCGGCCGCCGGGACAGCAGACTGCCGGTAACGCGCGGCCGGGGCGGGAGAGACTCAGCAA
     P  A  A  G  T  A  D  C  R  *
841  CGACCCATACCTCAGACCCGACGGCCCGGAGCGGACGCCCTGCTGCCGACGCCAGAGCCG

901  CCGCGTGCCCGCTGCAGTTTCTTGGACATAGACCAAAGAAGCTACAGCCTGGACTTACCA

961  CCACTAAACTGCGAGAGAAGCTAAACGTGTTTATTTTCCCTTAAATTATTTTTGTAATGG

1021 TAGCTTTTTCTACATCTTACTCCTGTTGATGCAGCTAAGGTACATTTGTAAAAAGAAAAA

1081 AAACCAGACTTTTCAGACAAACCCTTTGTATTGTAGATAAGAGGAAAAGACTGAGCATGC

1141 TCACTTTTTTATATTAATTTTTAGGACAGTATTTGTAAGAATAAAGCAGCATTTGAAATG

1201 CCCT
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FIG. 2. Nucleotide sequence of NF-IL6β gene and deduced amino acid sequence. The open reading frame spans 269 amino acids. The transcriptional start site is indicated by an arrow. The TATAAA homology and the polyadenylylation signal are underlined. The leucine residues of the leucine zipper domain are circled.

The C-terminal region of NF-IL6β displayed a very strong homology with that of NF-IL6 or C/EBP. This region includes the leucine zipper region as well as the adjacent basic domain. NF-IL6β also contained a leucine zipper motif, consisting of five leucines in every seventh position. When the amino acid sequence of NF-IL6β was compared with that of NF-IL6 and C/EBP, it shared 75.8% and 81.8% homology in the basic domain and 62.1% and 51.7% homology in the leucine zipper region, respectively. However, the N-terminal region of NF-IL6β was quite different from that of NF-IL6 and C/EBP. Taken together, these results show that NF-IL6β is a newly discovered member of the C/EBP family.

NF-IL6β Recognizes the Same DNA-Binding Sequence as NF-IL6. We examined whether NF-IL6β showed an indistinguishable DNA-binding specificity with NF-IL6 or C/EBP. NF-IL6 and C/EBP are known to bind to the CCAAT homology and the viral enhancer core sequence. NF-IL6 is reported to bind to the regulatory regions for various acute-phase protein genes and several cytokine genes (2, 14). It was shown by competition gel-retardation assays that NF-IL6β bound to the 14-bp palindrome in the IL-6 promoter, several viral enhancer core sequences, and the regulatory regions for the acute-phase protein genes and the cytokine genes, as in the case of NF-IL6 (data not shown). In addition, dimethyl sulfate methylation interference assays demonstrated that NF-IL6β and NF-IL6 made identical contact with the nucleotides on the human IL-6 promoter region (data not shown). These results show that NF-IL6β recognizes the same DNA sequences as NF-IL6.

NF-IL6β and NF-IL6 Form a Heterodimer. We examined whether NF-IL6β and NF-IL6 were capable of forming a heterodimer *in vitro*. The NF-IL6β and NF-IL6 proteins were produced in *E. coli* as fusion proteins (BS–NF-IL6β and BS–NF-IL6 delSpl), in which the N-terminal amino acid sequence of β-galactosidase was joined in-frame to an N-terminal truncated NF-IL6β and an internal deletion mutant

NF-IL6, respectively. A gel-retardation assay was performed by using the IL-6 promoter containing the 14-bp palindrome as a probe (Fig. 3). The fusion proteins BS–NF-IL6β and BS–NF-IL6 delSpl generated a slower- and a faster-migrating complex, respectively (lanes 1 and 2). When equimolar amounts of BS–NF-IL6β and BS–NF-IL6 delSpl were preincubated for 15 min at 37°C, a new complex of intermediate migration was observed, demonstrating that NF-IL6β can form a heterodimer with NF-IL6 (lane 3). Interestingly, the homodimers of NF-IL6β and NF-IL6 disappeared completely. This result indicates that the NF-IL6β–NF-IL6 heterodimer is formed more efficiently than the respective homodimers.

Synergistic Transcriptional Activity of NF-IL6β and NF-IL6. To demonstrate that NF-IL6β acts as a transcriptional activator, we performed cotransfection experiments into P19 embryonic carcinoma cells. P19 cells do not express NF-IL6β or NF-IL6 mRNA (unpublished data). The reporter construct contained 5′ flanking region (−179 to +12) of the human IL-6 gene in front of the structural sequence of the firefly luciferase gene. The reporter plasmid was transfected with CMV–NF-IL6β, CMV–NF-IL6, or both into P19 cells, and their transcriptional activities were assayed (Fig. 4*A*). NF-IL6β activated the IL-6 promoter significantly in a dose-dependent manner, while NF-IL6 activated it weakly, even at its high concentration. NF-IL6β was consistently a stronger transactivator than NF-IL6. As shown above, NF-IL6β and NF-IL6 are able to interact *in vitro* through formation of heterodimers. To examine the possibility that the two proteins are also able to interact *in vivo*, we analyzed the transcriptional activity when the cells were cotransfected with both CMV–NF-IL6β and CMV–NF-IL6. The reporter plasmid was transfected into P19 cells together with increasing amounts of CMV–NF-IL6β and a suboptimal constant amount (0.5 μg) of CMV–NF-IL6. We could obtain the synergistic transcriptional activity of NF-IL6β and NF-IL6 on the human IL-6 promoter. However, this synergistic effect was observed only when 0.3–1.0 μg of the NF-IL6β expression vector DNA was added. Furthermore, this effect was modest and, at most, 1.5-fold stronger than the additive sum of their individual activity (Fig. 4*B*).

NF-IL6β Is an Inducible Factor. The tissue distribution of NF-IL6β mRNA was analyzed by Northern blot analysis. To exclude the cross-hybridization of other members of the C/EBP family, we used an antisense NF-IL6β oligonucleotide probe that revealed a single band in genomic Southern blot analysis. We first analyzed the NF-IL6β expression in

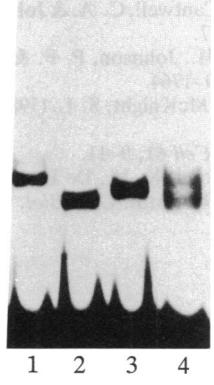

FIG. 3. Heterodimer formation between NF-IL6β and NF-IL6. The BS–NF-IL6β and BS–NF-IL6 fusion proteins reacted with a fragment containing the NF-IL6 binding motif (−179 to −111) of the human IL-6 gene. Lanes: 1, NF-IL6β protein only; 2, NF-IL6 protein only; 3, mixture of NF-IL6β and NF-IL6 proteins incubated for 15 min at 37°C before addition of probe; 4, mixture of the samples after NF-IL6β and NF-IL6 proteins reacted with the probe individually.

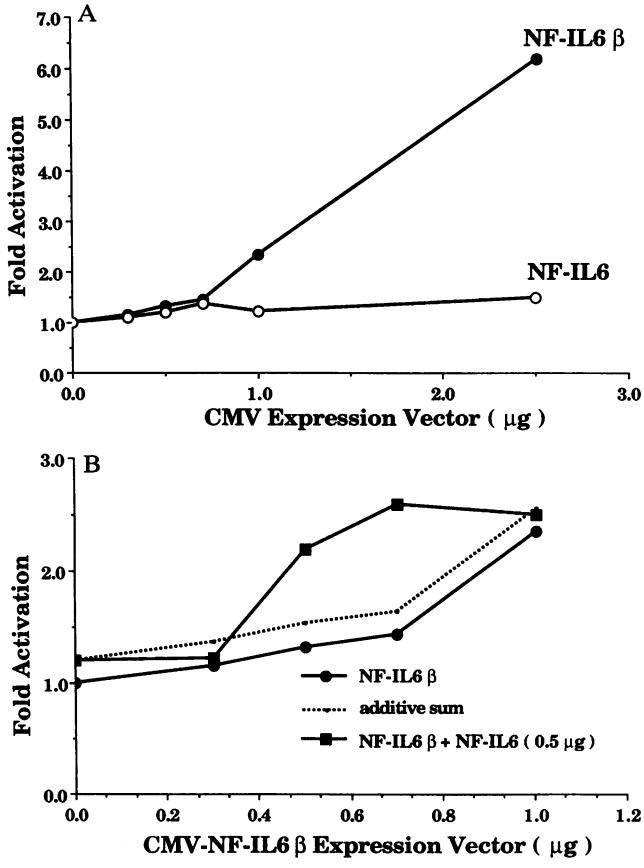

FIG. 4. Synergistic transcriptional activation of NF-IL6β and NF-IL6 *in vivo*. The reporter construct consists of 5′ flanking region (−179 to +12) of the human IL-6 gene linked to the structural sequence of the firefly luciferase gene. The reporter plasmid was transfected into P19 cells together with increasing amounts of CMV–NF-IL6β or CMV–NF-IL6 expression vectors (*A*), or together with increasing amounts of CMV–NF-IL6β and a suboptimal constant amount (0.5 μg) of CMV–NF-IL6 (*B*). Additive sum, calculated sum of the responses of the reporter plasmid transfected with CMV–NF-IL6β and CMV-NF-IL6 individually.

various mouse tissues. The molecular size of the murine NF-IL6β mRNA is ≈1.2 kb, which is similar in length to the human counterpart. As shown in Fig. 5, normal tissues did not express NF-IL6β, although a small amount of NF-IL6β mRNA was expressed in lung, kidney, and spleen. However, all tissues expressed NF-IL6β after LPS stimulation. We next examined the NF-IL6β expression in many cell lines (data not shown). A mouse macrophage cell line, WEHI3, and a mouse stromal cell line, ST2, expressed NF-IL6β mRNA constitutively. However, NF-IL6β mRNA was undetectable in many cell lines and could be detected after stimulation—for example, in mouse myeloid cell line M1 by IL-6, in osteosarcoma cell line MG-63 by IL-1, in glioblastoma cell line SK-MG-4 by IL-1, and in hepatoma cell lines HepG2 and Hep3B by IL-6. These results show that NF-IL6β is induced by LPS, IL-6, or IL-1 in various tissues and cell lines.

DISCUSSION

In the present study, we described the cloning and characterization of an additional member of the C/EBP gene family, which we termed NF-IL6β. This gene revealed high sequence homology with NF-IL6 in the basic and leucine zipper domains but differs from it in the N-terminal domain. Both NF-IL6β and NF-IL6 exhibited the same sequence specific-

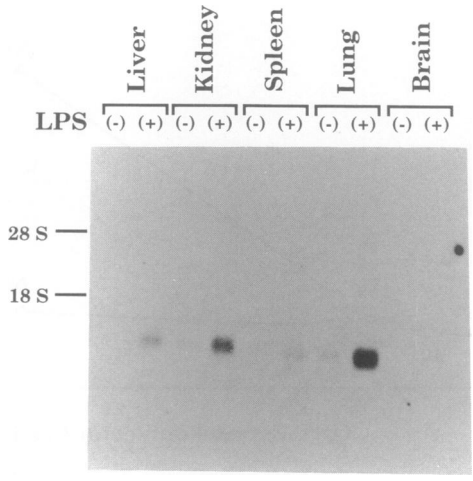

FIG. 5. Tissue distribution of NF-IL6β expression in mice. mRNA was obtained before and after LPS stimulation from various tissues of mice, and poly(A) RNA (2 μg per lane) was hybridized with the 40-mer NF-IL6β-specific antisense oligonucleotide probe. For preparation of mRNA from LPS-stimulated tissues, 20 μg of LPS was injected intravenously into each mouse and 4 hr later the mouse was killed.

ity as evidenced by competition gel-retardation assays and methylation interference assays. NF-IL6β is expressed at a low level in normal tissues but is induced by LPS, IL-1, or IL-6, as in the case of NF-IL6. Despite the similarity in the pattern of expression between NF-IL6β and NF-IL6 in normal tissues, NF-IL6 mRNA is expressed constitutively in many cell lines, whereas NF-IL6β mRNA is not expressed or induced by several stimuli. This may indicate that NF-IL6 is more easily induced than NF-IL6β. Our present study also showed that NF-IL6β is a strong transcriptional activator compared with NF-IL6. Furthermore, NF-IL6β and NF-IL6 are capable of forming a heterodimer *in vitro* and show a transcriptional synergistic effect *in vivo*. The heterodimer is formed more preferentially than its respective homodimers. This observation indicates that the presence of small amounts of NF-IL6β would give rise to only the heterodimeric complexes without formation of NF-IL6β homodimers. Although we have not quantitated the molar amounts of NF-IL6β and NF-IL6, the previous data demonstrated that NF-IL6 is a major contributor to the DNA–protein complexes detected by gel-retardation assays in hepatoma cell lines as well as in a glioblastoma cell line (2, 10). From these observations, we speculate as follows. During the early phase of inflammation, the homodimers of NF-IL6 are predominant and activate several promoters of the genes associated with inflammation. However, the NF-IL6 homodimer may not be enough for activation of some other genes. During the late phase of inflammation, NF-IL6β is induced and replaces one component of the NF-IL6 homodimer to give rise to a more potent activator of the heterodimeric complex, which is in turn involved in regulation of the genes newly activated or the genes more active in this phase. Further experiments will be required to clarify functional differences among the homo- and heterodimers of NF-IL6β and NF-IL6.

While this manuscript was being prepared for publication, papers describing the cloning and characterization of C/EBPδ (15) and CRP3 (16) were published. These sequences were very similar (84.8% identical) to NF-IL6β. Therefore, NF-IL6β could be the human counterpart of the mouse C/EBPδ and CRP3.

We thank Dr. N. Miura for providing us with P19 cells, Dr. K. Yasukawa (Tohso Inc.) for synthesizing the peptide, and Toyo Ink MFG. Co., Ltd. for supplying the luciferase assay kit (Pica Gene). We also thank Ms. K. Kubota and Ms. K. Ono for their excellent secretarial assistance. This study was supported by a Grant-in-Aid for Specially Promoted Research from the Ministry of Education, Science and Culture.

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