MOLECULAR AND CELLULAR BIOLOGY, June 1990, p. 2757–2764
0270-7306/90/062757-08$02.00/0

Vol. 10, No. 6

Constitutive and Interleukin-1 (IL-1)-Inducible Factors Interact with the IL-1-Responsive Element in the IL-6 Gene

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Received 29 December 1989/Accepted 13 March 1990

The interleukin-6 (IL-6) promoter is rapidly and transiently activated with other cytokines, including IL-1, tumor necrosis factor, and platelet-derived growth factor, as well as phorbol esters and agents that increase intracellular cyclic AMP. In this study, we have investigated *cis*-acting regulatory elements and *trans*-acting factors responsible for IL-1-induced IL-6 gene expression. Studies on the 5′ deletion mutants of the human IL-6 gene suggested that the IL-1-responsive element was mapped within the IL-6 promoter region (−180 to −123) which was homologous to the c-*fos* serum-responsive enhancer element. Gel retardation assay identified two types of nuclear factors that bound to this region, one constitutive and the other inducible. These two factors recognized a 14-base-pair (bp) palindromic sequence, ACATTGCACAATCT. Furthermore, three copies of this 14-bp palindrome conferred IL-1 responsiveness to the basal enhancerless IL-6 promoter, indicating that a 14-bp-dyad symmetry sequence was an IL-1-responsive element in the IL-6 gene.

Interleukin-6 (IL-6) is a cytokine with a variety of biological activities on a wide range of tissues (19), such as induction of immunoglobulin synthesis in B cells (14, 16), enhancement of myeloma and hybridoma growth (18, 43, 44), regulation of acute-phase protein synthesis by hepatocytes (2, 11), activation of multipotential hematopoietic progenitor cells (17), stimulation of T-lymphocyte growth and differentiation (10, 27, 40), and induction of nerve cell differentiation (32). IL-6 is synthesized by a variety of cells, including macrophages, fibroblasts, T and B lymphocytes, synovial cells, endothelial cells, glia cells, and keratinocytes (31). The expression of the IL-6 gene is induced by a variety of cytokines, including IL-1, tumor necrosis factor, and platelet-derived growth factor, as well as serum, poly (I · C), and cycloheximide (31). Phorbol esters which activate protein kinase C and agents that increase intracellular cyclic AMP (cAMP) enhance the accumulation of IL-6 mRNA (16, 31, 34, 47). Infection with various viruses is also a potent stimulus for IL-6 production (16, 35). The induction of IL-6 mRNA by various stimuli is seen within 1 h, accompanied by an increase in the rate of IL-6 mRNA transcription (45). Recently, the possibility has been suggested that deregulation of the IL-6 gene expression may be involved in the pathogenesis of autoimmune diseases (13, 15, 39) and certain lymphoid malignancies, especially multiple myelomas (18). In order to clarify the mechanisms involved in the abnormal expression of IL-6, it was imperative to investigate the mechanism of the IL-6 gene expression under physiological conditions.

In recent years, considerable information has been accumulated on the role of *cis*-acting elements and *trans*-acting factors that are essential for gene expression. In this study, we showed that IL-1-induced IL-6 gene expression was conferred by a novel 14-base-pair (bp)-dyad symmetry which was present within the region homologous to the c-*fos* serum-responsive enhancer element (SRE). Furthermore, we showed the existence of constitutive and inducible factors which bound to this sequence before and after IL-1 stimulation.

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MATERIALS AND METHODS

Cell lines. A human glioblastoma line, SK-MG-4 was kindly provided by 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/ml). A mouse fibroblast line, L cells, was maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum.

Preparation of nuclear extracts. Cell nuclei were prepared as described by Dignam et al. (6) with some modifications. Glioblastoma cells were rinsed twice in phosphate-buffered saline and then in cold modified reticulocyte standard buffer (RSB; 10 mM NaCl, 10 mM Tris hydrochloride, pH 7.9, 5 mM $MgCl_2$) and kept on ice for 5 min in modified RSB containing 0.1% Triton X-100. They were then broken in a Dounce homogenizer with a tight pestle, pelleted, and rinsed twice in modified RSB containing 100 mM KCl. Proteins were extracted in modified RSB containing 0.4 M KCl from washed nuclei.

DNA transfection. For the stable transformation, L cells were transfected with 20 μg of plasmid and 1 μg of pSV2Neo by calcium phosphate precipitation. Two days later, G418 (GIBCO Laboratories) was added to the medium, and resistant colonies were isolated after 2 to 3 weeks of selection. For the chloramphenicol acetyltransferase (CAT) assay, 10 μg of the CAT reporter plasmid plus 10 μg of high-molecular-weight salmon sperm DNA was transfected into a glioblastoma cell line, SK-MG-4, by using the calcium phosphate procedure. The cells were washed after 20 h and stimulated with IL-1 at a concentration of 100 U and harvested another 24 h later. Cell extracts were prepared by freeze-thawing the cells three times and then were assayed for CAT activity (12).

Gel retardation assay. For the binding reaction (1), nuclear extracts (3 to 10 μg) were incubated at room temperature for

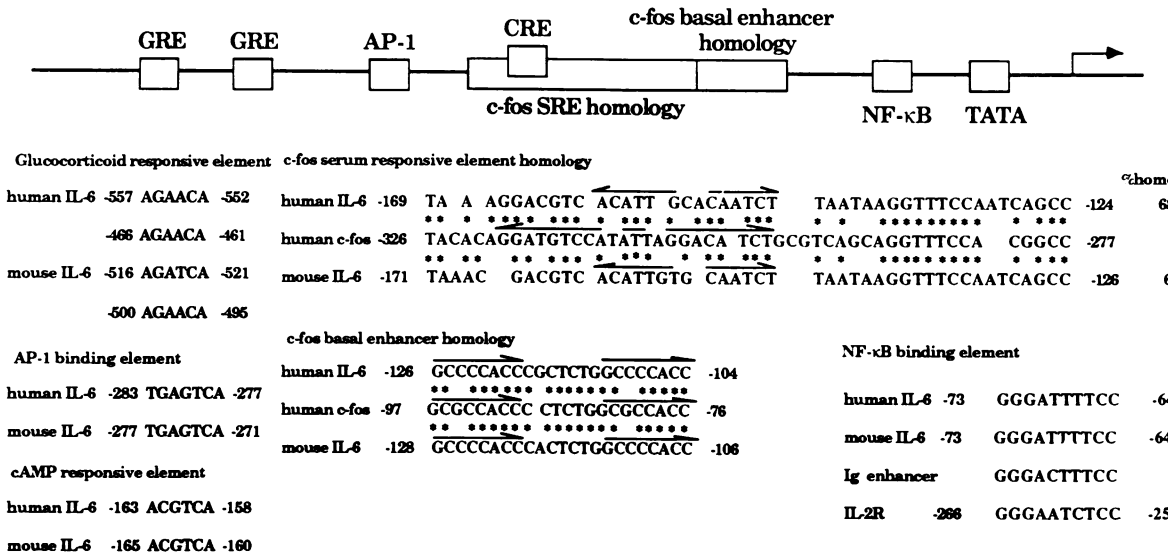


FIG. 1. Transcriptional control motifs identified in the IL-6 promoter.

30 min and the ^{32}P-labeled fragments (0.5 to 2.0 ng) in 25 μl of binding buffer consisting of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*,N'-ethenesulfonic acid) (pH 7.9), 50 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris hydrochloride (pH 7.0), 1 mM dithiothreitol, 15 mM EDTA, 10% glycerol, and various amounts of the carrier polymer, poly(dI-dC). The DNA-protein complexes were separated in a 5% polyacrylamide gel (acrylamide-to-bisacrylamide ratio, 30:1) containing 7 mM Tris hydrochloride (pH 7.5), 3.3 mM sodium acetate, 3.8 mM β-mercaptoethanol, and 1 mM EDTA.

Methylation interference assay. Partial methylation of the end-labeled probe was done by the method of Maxam and Gilbert (24), using 50 ng of probe for methylation and 1.5 M sodium acetate–1 M 2-mercaptoethanol for quenching. This DNA was used for the gel retardation assay as described above. Purified DNA from the band regions was cleaved in 100 μl of 10% piperidine at 90°C for 30 min and then precipitated, rinsed, and loaded onto a 10% sequencing gel.

RESULTS

Transcriptional control element motifs identified in the IL-6 promoter. Comparison of the human (46) and the mouse IL-6 genomic genes (41) revealed the highly homologous regions extending ~350 bp upstream of the transcriptional start. By comparison with known regulatory sequences of various genes, potential transcriptional control elements were identified within the conserved region of the IL-6 promoter. These elements are organized as indicated in Fig. 1.

First, two pairs of glucocorticoid responsive elements (5, 33) are located at the positions −557 to −552 and −466 to −461 in the human IL-6 gene. A consensus sequence, TGAGTCA, of the AP-1-binding site (3,21) is found at the position −283 to −277. The AP-1-binding site is present in a number of phorbol-12-myristate-13-acetate (PMA)-inducible promoters and confers inducibility to heterologous promoters. The sequence stretch similar to the human c-*fos* SRE is present at the positions −169 to −124, as reported previously (45). The c-*fos* SRE has been shown to be required for induction of the c-*fos* gene with serum, phorbol esters, and epidermal growth factor. A core sequence, ACGTCA, of the cyclic AMP (cAMP)-responsive element (CRE) (25, 38) is identified within this c-*fos* SRE homology. This sequence is present in several cAMP-regulated promoters and in the upstream promoters of several adenovirus genes. CRE has been shown to mediate cAMP inducibility and to bind a 43-kilodalton (kDa) phosphoprotein (CREB). Downstream of the c-*fos* SRE homology, there was a GC-rich sequence stretch at the positions −128 to −94. This sequence included a direct repeat of the sequence similar to GCCCACC identified in the c-*fos* promoter (8). These sequences have been found to be responsible for basal transcription in the c-*fos* gene. At the positions −73 to −64, the NF-κB-binding site (36) homology is identified.

Regulatory region responsible for the IL-6 mRNA induction by IL-1. To analyze the sequences involved in the IL-1 response, we have constructed a series of 5' end deletion mutants of the human IL-6 gene. Each mutant was transfected in mouse fibroblasts (L cells) whose endogenous IL-6 gene was inducible by IL-1, and stable transformants were obtained. More than 50 colonies from each mutant were mixed, and the expression of the exogenous human IL-6 gene was studied by Northern (RNA) blot analysis before and after IL-1 stimulation. Deletion up to the position −180 did not significantly alter the level of the inducibility (Fig. 2). However, further deletion to −122 resulted in the loss of the inducibility. These results indicated that the −180 to −122 region in the IL-6 promoter was involved in the IL-1 inducibility.

Constitutive and inducible nuclear factors binding to the c-*fos* SRE homology. We next used a gel retardation assay to detect sequence-specific nuclear factors binding to the IL-1-responsive element in the IL-6 promoter region. Nuclear extracts from a human glioblastoma cell line, SK-MG-4, either before or after 6 h of stimulation with IL-1, were incubated with an end-labeled 256-bp fragment corresponding to −242 to +14 of the human IL-6 gene, and the reaction mixtures were subjected to gel electrophoresis. A specific retarded band, B1, was identified upon incubation of the probe with the untreated nuclear extract (Fig. 3A). In addition to this band, a new slower-migrating band, B2, was detected with the stimulated nuclear extract. These complexes were specifically eliminated by competition with the unlabeled probe or fragment A (−179 to −111). No compe-

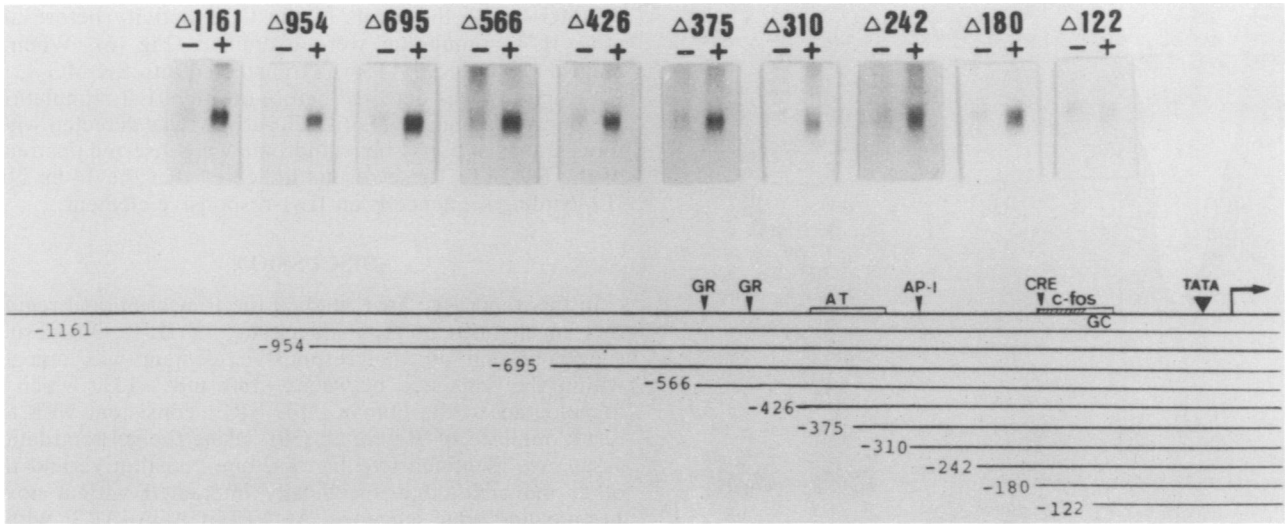


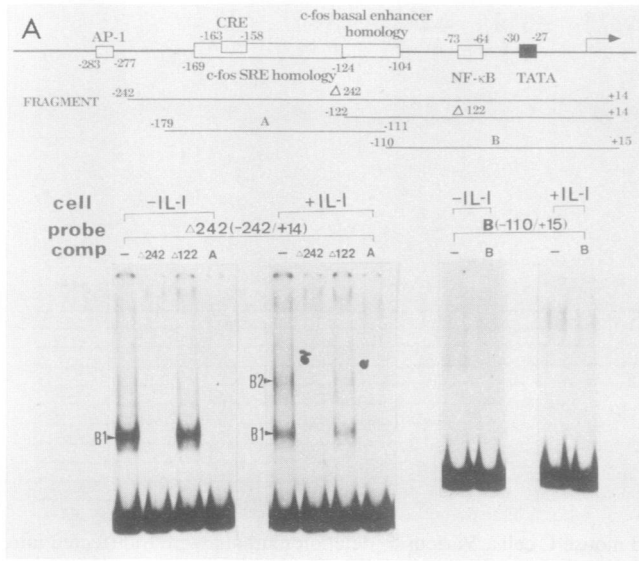
FIG. 2. IL-6 mRNA induction by IL-1 in human IL-6 gene-transfected mouse L cells. Various 5′ deletion mutants were transfected into L cells by the calcium phosphate procedure. Stable transformants were isolated after selection in G418. Twenty micrograms of total RNA were loaded into each lane and analyzed by Northern blot hybridization with a human IL-6 cDNA probe. This experiment was performed three times with different RNA samples from different populations of mixed colonies, and similar results were observed. The amount and integrity of loaded RNA were checked by ethidium bromide staining of 28S rRNA from gels before transfer. Abbreviations: GR, glucocorticoid responsive element; AT, AT-rich sequence; GC, GC-rich c-*fos* basal enhancer-homologous region; c-*fos*, c-*fos* serum-responsive element homologous region.

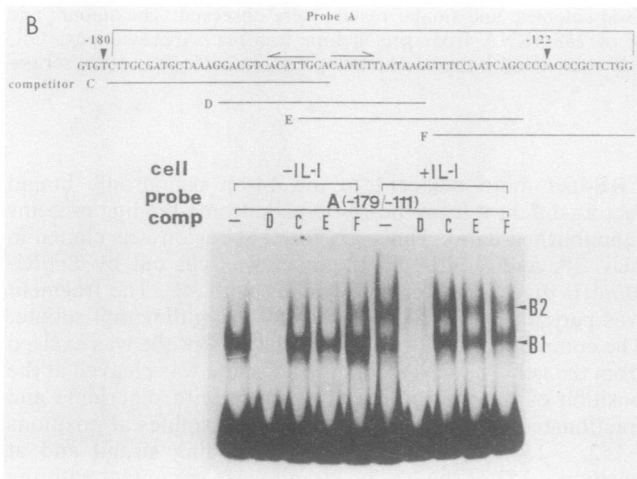
tition was observed with fragment B (−110 to +15). The gel retardation assay using fragment B as a probe did not reveal any retarded bands, although very faint specific retarded bands were detected after long exposure. These results indicated that the binding sites of these complexes were located within the −179 to −111 region of the IL-6 promoter. To define these binding sites more precisely, a series of oligonucleotides (about 30 bp) partially overlapping each other were chemically synthesized and used as unlabeled competitors. Nuclear extracts from SK-MG-4 were incubated with end-labeled fragment A alone or in the presence of each oligonucleotide. The formation of these complexes was inhibited only by oligonucleotide D, suggesting that the protein-binding sites for complexes were within the −164 to −139 region (Fig. 3B). This region was found to correspond exactly to the region homologous to the human c-*fos* SRE. Within this sequence, we could find homology with a cAMP-responsive element, GGACGTCA, and a 14-bp nucleotide containing an inverted repeat of the sequence CAATCT. As oligonucleotide C containing a cAMP-responsive element homology did not compete for these retarded complexes, the most likely binding site was the 14-bp palindromic sequence. Therefore, we synthesized two oligonucleotides with mutations within the 14-bp palindrome as shown in Fig. 3C and used these as competitors. A gel retardation assay was performed using fragment A as a probe. Mutant oligonucleotide D′ did not compete for the formation of these complexes, although mutant oligonucleotide D″ did (Fig. 3C). In addition, the 14-bp palindrome could compete for the formation of these complexes. These results demonstrated that the 14-bp palindrome was involved in the formation of both retarded complexes. To determine the precise binding site for the nuclear factor which formed complex B1, methylation interference experiments were performed. In this experiment, fragment A′ containing the −159 to −111 region was used as a probe instead of fragment A in order to exclude the possibility of the binding of CREB-like proteins, because a CRE-like motif adjacent to the 14-bp palindrome bound factors different from the 14-bp palindrome-binding proteins (unpublished data). The −159 to −111 region was cloned in pUC 18, and a ~100-bp fragment was cut out by *Eco*RI-*Hin*dIII digestion after end labeling with ^{32}P. The fragment was partially methylated in vitro by using dimethyl sulfate. The complex formed with the end-labeled probe was excised from the gel. The DNA from the complex was cleaved at the position of the methylated G residues with piperidine and fractionated on a sequencing gel. The guanines at positions −152, −150, and −146 of the noncoding strand and at position −153 of the coding strand were in contact with the protein (Fig. 4). The other inducible complex, B2, exhibited exactly the same footprinting. Therefore, we referred to the protein(s) binding to the 14-bp palindromic sequence as NF-IL6, collectively.

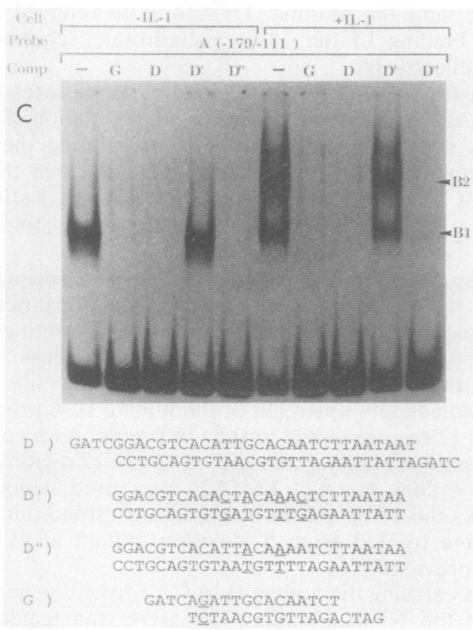
The constitutive and the IL-1-inducible complexes could also be detected in mouse L cells (Fig. 5). Formation of these complexes was completely competed by adding the cold 14-bp oligonucleotide. These results indicated that the appearance of a slower-migrating complex after IL-1 stimulation was not a phenomenon specific to the glioblastoma cell line.

The 14-bp palindrome as an IL-1-responsive element. Furthermore, we examined whether this 14-bp palindromic sequence identified by gel retardation and footprinting analysis was actually involved in the IL-1 responsiveness. Three copies of the 14-bp synthetic NF-IL6-binding site were inserted immediately upstream of the human IL-6 promoter (−122 to +12 region) linked to the CAT gene. As a control, a CAT plasmid containing only the −122 to +12 region of the human IL-6 gene (termed K9CAT) was used, since this region lacks the 14-bp palindrome and has turned out to be unresponsive to IL-1 from 5′ deletion mutant analysis as described previously.

Plasmids carrying the human IL-6-CAT hybrid genes with or without the NF-IL6-binding site were transfected into







SK-MG-4, and the levels of the CAT activity before and after IL-1 stimulation were examined (Fig. 6). When a control vector, K9CAT, was transfected into SK-MG-4, no CAT activity was detected before or after IL-1 stimulation. In contrast, a high level of CAT activity was detected when three copies of the 14-bp palindrome were inserted upstream of the K9CAT. These results indicated that the 14-bp NF-IL6-binding sequence is an IL-1-responsive element.

DISCUSSION

In this report we have studied the transcriptional regulatory mechanism of IL-6 inducibility of IL-1. Our results suggested that an IL-1-responsive element was mapped within the sequence between −180 and −123, which is homologous to the human c-*fos* SRE, consistent with the result obtained by Ray et al. (30). Using the gel retardation assay, we identified two factors, one constitutive and the other inducible, that specifically interacted with a novel 14-bp palindromic sequence, ACATTGCACAATCT, within the c-*fos* SRE homology that was activated by IL-1 treatment. These two DNA-binding proteins exhibited indistinguishable DNA-binding characteristics, as shown by the competition gel retardation assay and the methylation interference assay. Therefore, we referred to these two proteins as NF-IL6, collectively. Furthermore, the 14-bp palindromic sequence within this region has been shown to confer the IL-1 responsiveness to the human IL-6 basal promoter. In the human c-*fos* promoter, a 20-bp region of palindromic sequence is present in the c-*fos* SRE element and has been shown to be both necessary and sufficient for the response of the c-*fos* gene to whole serum, phorbol esters, and epidermal growth factor (42). Interestingly, a closer inspection of the SRE-homologous region within the IL-6 promoter revealed that the 20-bp palindrome of the c-*fos* SRE was replaced by the 14-bp palindrome (Fig. 7). The 14-bp palindrome seen in the c-*fos* SRE homologous region of the IL-6 promoter was quite different from the palindrome in the c-*fos* SRE, and therefore it was suggested that the nuclear factors binding to this 14-bp palindrome might be different from the serum response factor (SRF) that binds to the 20-bp palindrome in the c-*fos* SRE. Recently, direct cloning of NF-IL6 revealed that this protein shared high sequence homology with C/EBP, a rat liver nuclear factor, in its DNA-binding domain but differed entirely in its N-terminal domain (S. Akira, H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T.

FIG. 3. Identification of constitutive and IL-1-inducible complexes in glioblastoma SK-MG-4. (A) Gel retardation analysis. End-labeled, double-stranded fragments Δ242 (−242 to +14) and B (−110 to +15) were incubated with crude glioblastoma SK-MG-4 nuclear extracts. The complexes were separated by electrophoresis on a 5% native polyacrylamide gel. For competition experiments, the labeled probe was incubated with a 100-fold molar excess of competitor. Competitors used in this experiment are shown at the top of the panel. Specific protein-DNA complexes (B1 and B2) are indicated by arrows. comp, competitor. (B) Competition gel retardation analysis with various oligonucleotides. End-labeled fragment A (−179 to −111) was incubated with crude glioblastoma SK-MG-4 nuclear extracts either with no competitor (−) or in the presence of the indicated competitor at a 100-fold molar excess. Competitors used in this experiment are shown at the top of the panel. (C) Competition gel retardation analysis with the 14-bp palindrome and its mutants. The 14-bp palindrome sequence(G) and the mutant sequences (D′ and D″) are shown at the bottom of the panel. Mutated nucleotides are underlined.

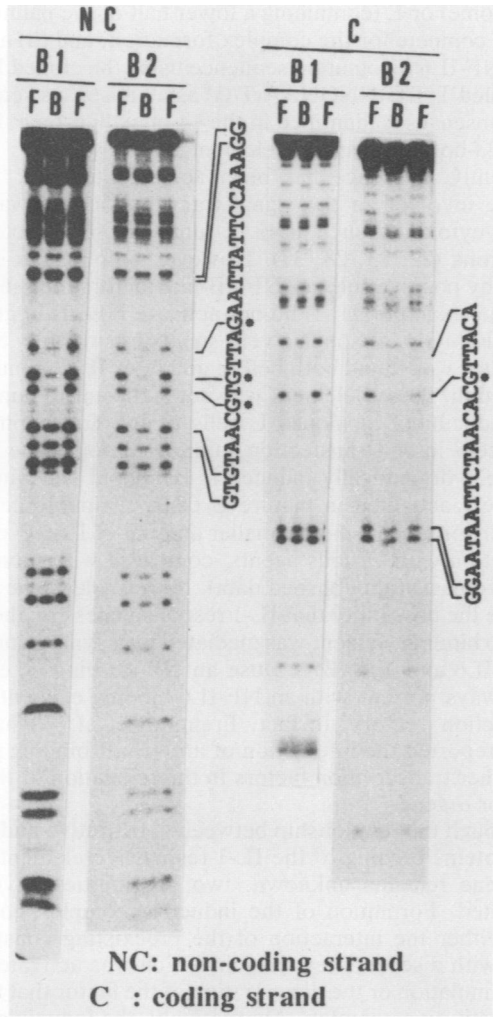


FIG. 4. Methylation interference analysis of complexes B1 and B2. Fragment A′ containing the −159 to −111 region was 5′ end labeled on either the coding (C) and noncoding (NC) strand and partially methylated with dimethyl sulfate. Preparative gel retardation assays were performed with the partially methylated probe and crude extracts from a glioblastoma cell line, SK-MG-4. The bound retarded (B) and free (F) probe bands were eluted, treated with piperidine, and analyzed by electrophoresis on a 10% sequencing gel. A summary of the methylation interference data is shown at the bottom.

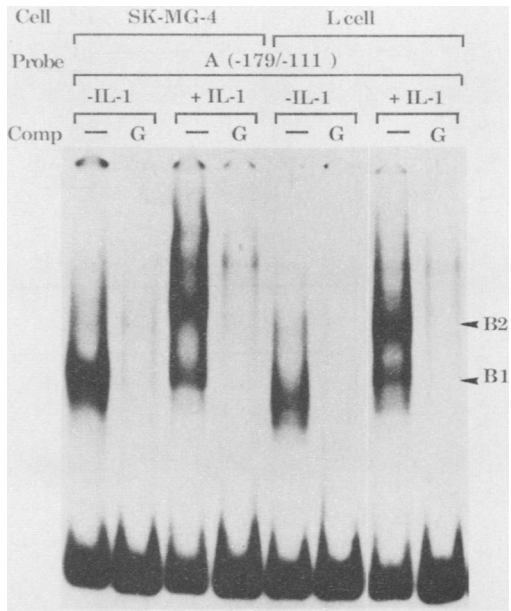


FIG. 5. Identification of an IL-1-inducible complex in mouse L cells. End-labeled fragment A (−179 to −111) was incubated with crude nuclear extracts from mouse L cells either alone (−) or in the presence of the 14-bp palindrome (G). A gel retardation assay performed using the crude glioblastoma nuclear extracts is also shown for comparison.

Nakajima, T. Hairano, and T. Kishimoto, EMBO J., in press). The cloned NF-IL6 revealed a binding specificity in a gel retardation assay and a methylation interference pattern identical to those of the NF-IL6 identified in SK-MG-4 nuclear extracts. Analysis of the sequence that NF-IL6 bound revealed that NF-IL6 and C/EBP recognized identical nucleotide sequences. NF-IL6 could bind to sets of considerably divergent sequences, as presented in the case of C/EBP. Therefore, a palindromic structure is not always required for the binding of NF-IL6. This is also reflected in the result that even mutant oligonucleotide D′, which disrupted a palindromic structure, could inhibit the formation of the constitutive and the inducible complexes (Fig. 3C).

To date, two signal transduction pathways, that is, the adenylate cyclase pathway and the protein kinase C pathway, have been implicated in modulating IL-6 gene expression. In this regard it is noteworthy that the IL-6 gene carries a consensus binding site for the PMA-inducible transcription factor, AP-1. We have identified a DNA-protein complex specifically interacting with the AP-1-binding site in the promoter region of the IL-6 gene, which was specifically competed for by anti-c-*fos* antibody. These results demonstrated that a Jun-Fos heterodimer interacted with this sequence (unpublished data). However, this site does not seem to be important for the IL-6 induction by PMA, considering the results of the CAT assay done by Ray et al. (30). CRE is known to be responsible for the response to the cAMP pathway. In the IL-6 gene, a CRE motif is located adjacent to the 14-bp palindrome. A cross-competition gel retardation assay using oligonucleotides containing the IL-6 CRE motif and the somatostatin CRE strongly indicated that the CRE motif in the IL-6 promoter could bind CREB (unpublished data). At present we cannot exclude the possibility that the CRE in the SRE-homologous region, but not the 14-bp palindrome, is responsible for the induction of IL-6 mRNA through the cAMP pathway. Ray et al. recently showed that a 23-bp IL-6 multiresponse element (−173 to −151) including the CRE motif is responsible for induction by IL-1, tumor necrosis factor, and serum as well as by the activators of protein kinase A (forskolin) and protein kinase C (phorbol ester). They also identified several sequence-specific complexes that were increased in intensity in HeLa cell nuclear extracts after stimulation. Although the 23-bp multiresponse element contains the upper half of the 14-bp

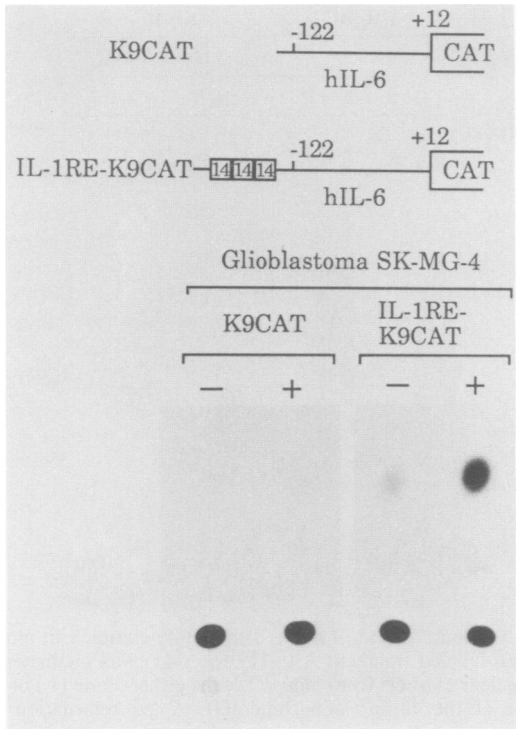


FIG. 6. The 14-bp palindromic NF-IL-6-binding motifs confer IL-1 responsiveness to the human IL-6 promoter CAT chimeric construct. K9 CAT contained the −122 to +12 region of the human IL-6 gene in front of the CAT gene. Three copies of the 14-bp palindrome (5′-AGATTGCACAATCTGATCAGATTGCACAAT CTGATCAGATTGCACAATCT-3′) were inserted immediately upstream of the human IL-6 gene (−122 to +12) in K9CAT. The binding of NF-IL6 to this multimerized palindrome was ascertained by a gel retardation assay. K9CAT and IL-1RE-K9CAT constructs were transfected into a glioblastoma cell line SK-MG-4 by the calcium phosphate procedure. Twenty hours after transfection, SK-MG-4 cells were stimulated with IL-1, and the CAT activity was assayed another 20 h later. This experiment was performed three times with similar results. The result of one representative experiment is shown. The percent conversion in this experiment was as follows. K9CAT IL-1(−), <2.0%; K9CAT IL-1(+), <2.0%; IL-1 RE-K9CAT IL-1(−), 12%; IL-1 RE-K9CAT IL-1(+), 47%.

palindrome, it is evident that NF-IL6 is quite different from the nuclear factors they identified and, furthermore, that NF-IL6 cannot bind to the upper half of the palindrome because (i) the results of the competition gel retardation assays (Fig. 3B and C) demonstrated that only the 14-bp palindromic sequence inhibited the complex formation, but either oligonucleotide C (containing an upper half of the palindrome) or E (containing a lower half of the palindrome) did not compete for the complex formation, and (ii) analysis of the NF-IL6 recognition sequence using the cloned NF-IL-6 revealed T(T/G)NNGNAA(T/G) as a consensus sequence. This consensus is identified in the 14-bp palindrome, but not in the 23-bp multiresponse element.

Recently, evidence has been accumulated that NF-κB may be involved in the signal transduction pathway with several cytokines, such as IL-1, tumor necrosis factor, and interferons (22, 23, 28, 37). However, our results did not show any positive roles of NF-κB-like factors in IL-6 induction. For example, IL-1 did not activate NF-κB in glioblastoma SK-MG-4, although a very small amount of an NF-κB-like factor was found both before and after IL-1 stimulation. In addition, the κB element was not sufficient to confer the IL-1 inducibility in mouse L cells or in glioblastoma SK-MG-4 cells in our transfection studies. Although the NF-κB binding is dramatically induced in lymphoid cells and constitutively activated in mature B cells, lymphoid cell lines generally produce a much smaller amount of IL-6 even after stimulation with various agents, compared with monocytes and fibroblasts (unpublished data). Nevertheless, we cannot exclude the possibility that IL-1 responsiveness by the 14-bp NF-IL6 binding element was mediated by a synergistic effect of NF-IL6 and NF-κB because an NF-κB-binding element was always present with an NF-IL6-binding element in our transfection vectors. In fact, Freimuth et al. (9) have recently reported the interaction of an NF-κB-binding protein with other transcription factors in the regulation of the IL-2 receptor α gene.

Although the relationship between constitutive and inducible proteins binding to the IL-1-responsive element in the IL-6 gene remains unknown, two possibilities have been postulated. Formation of the inducible complex could involve either the interaction of the preexisting constitutive factor with a second new protein which was activated after IL-1 stimulation or the dimerization of the factor that formed the constitutive complex. Alternatively, the inducible factor could be quite different from the constitutive factor, as for several well-documented examples of different transcriptional factors with identical DNA-binding specificities, as seen in the cases of the octamer-binding protein (20) and CCAAT box-binding protein (7) and NF-κB (4). Our data (Akira et al., in press) may support the former speculation, because the anti-peptide antibody against the putative DNA-binding domain of the cloned NF-IL6 competed for the formation of both complexes. However, further experiments will be required to determine the nature of these two complexes, and this will provide a more complete picture of the mechanisms for the signal pathway leading to the IL-6 induction as well as for the abnormal IL-6 expression involved in several immunological disorders.

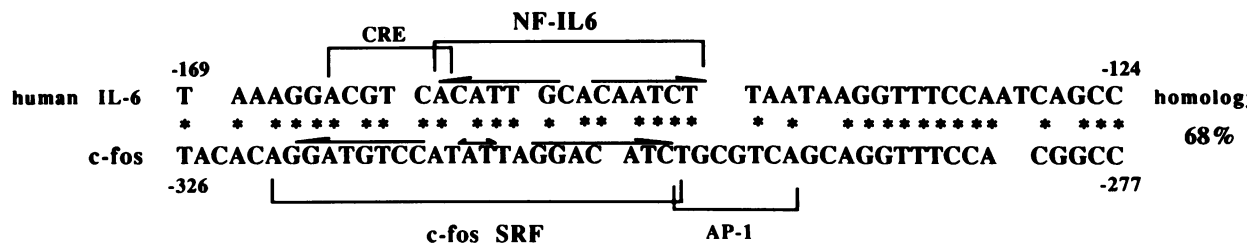


FIG. 7. Comparison of transcriptional control element motifs in the human IL-6 and c-*fos* genes. SRF, Serum response factor-binding-dyad symmetry element.

ACKNOWLEDGMENTS

We thank Moriyuki Sato and Seiji Sato (Kyowa Hakko Kogyo Co., Tokyo, Japan) for oligonucleotides and cell culture, respectively. We thank Edward Barsumian for the critical review of the manuscript and M. Kawata and K. Kubota for their secretarial assistance.

This work was supported in part by grants from the Ministry of Education, Science, and Culture, Japan, and from the Sankyo Foundation of Life Science.

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