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Interleukin-6 Induction by Tumor Necrosis Factor and Interleukin-1 in Human Fibroblasts Involves Activation of a Nuclear Factor Binding to a κB-Like Sequence

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Using variable-length deletion constructs of the 5′-flanking region of the human interleukin-6 (IL-6) gene linked to the chloramphenicol acetyltransferase gene, we showed that the region from positions −109 to −50 mediated the bulk of the response to tumor necrosis factor (TNF) or interleukin-1 (IL-1), while it was less responsive to forskolin. DNA mobility shift assays and DNase I footprinting analysis identified a nuclear protein from TNF- or IL-1-treated fibroblasts that bound to a region comprising a κB-like element located between positions −72 and −63 on the IL-6 gene. On the basis of these and other experiments, we conclude that TNF and IL-1 apparently activate IL-6 gene expression by closely related mechanisms involving activation of a NF-κB-like factor, whereas the pathway of IL-6 induction by forskolin is, in part, different.

Interleukin-6 (IL-6) is a multifunctional cytokine (19, 21) whose major actions include enhancement of immunoglobulin synthesis (17), activation of T cells (11), and modulation of acute-phase protein synthesis (12). Many different types of cells, including monocytes-macrophages, endothelial cells, and fibroblasts, can produce IL-6 (19, 21). Expression of the IL-6 gene in these cells can be stimulated by a variety of agents. Important among the inducers of IL-6 gene expression are the cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF) (6, 20). Other agents capable of inducing IL-6 include activators of protein kinase C (17, 30), calcium ionophore A23187 (30), and various agents causing elevation of intracellular cyclic AMP (cAMP) levels (37).

In a previous report we showed that, in human fibroblasts, treatment with TNF or IL-1, which stimulated IL-6 production, also induced a rapid and transient accumulation of intracellular cAMP (38). We concluded that the increase in cAMP levels is likely to contribute to the induction of IL-6 by TNF or IL-1. At the same time, our data suggested that other, unidentified signal transduction mechanisms also are important in the induction of IL-6 gene expression by TNF and IL-1. Several recent studies have shown that TNF and IL-1 activate nuclear factors that bind to the immunoglobulin κ light-chain enhancer element κB or to κB-like sequences on some other genes, e.g., the IL-2 receptor α-chain gene and human immunodeficiency virus 1 (8, 25, 27, 32). A sequence (positions −72 to −62) on the human IL-6 gene shares 90% homology with the classical κB sequence (31). The data presented in this report suggest that, in human fibroblasts, the κB-like sequence on the IL-6 gene functions as a major *cis*-acting element for IL-6 induction by TNF or IL-1. We also show that treatment with TNF or IL-1 leads to a rapid activation of the binding of preformed nuclear protein to the κB-like element on the IL-6 gene. Treatment with forskolin caused a less marked activation of the binding of nuclear protein to the κB-like element. We conclude that, in human fibroblasts, the major pathway of IL-6 induction by TNF or IL-1 (mediated by activation of the binding of nuclear protein to the κB-like sequence) is different from the major pathway of IL-6 induction by forskolin.

Identification of *cis*-acting elements on the 5′-flanking region of IL-6 DNA responsive to TNF, IL-1, and forskolin. The 5′-flanking region of the human IL-6 gene contains several sequences resembling known regulatory elements (28, 35, 36), as illustrated in Fig. 1. To identify *cis*-acting elements responsive to TNF, IL-1, and forskolin, a series of 5′-deletion mutants of the IL-6 gene fragment from positions −1158 to +11 were fused to the chloramphenicol acetyltransferase (CAT) reporter gene. Recombinant *Escherichia coli*-derived human TNF (specific activity, 2.0×10^6 U/mg) was kindly supplied by Masafumi Tsujimoto (Suntory Institute for Biomedical Research, Osaka, Japan). Recombinant *E. coli*-derived human IL-1α (specific activity, 2.5×10^9 U/mg) was generously provided by Alvin Stern and Peter Lomedico (Hoffmann-LaRoche, Inc., Nutley, N.J.). Forskolin was purchased from Sigma Chemical Co. (St. Louis, Mo.). Plasmid g26K4, which contains the human IL-6 genomic DNA sequence from positions −2860 to +940 (16), was generously supplied by Walter Fiers and G. Haegeman (University of Ghent, Ghent, Belgium). The sequence −1159 to +11 was released by *Bam*HI and *Xho*I digestion of the plasmid g26K4 and subcloned into pTK · CAT, which was digested with *Bam*HI and *Xho*I to remove the thymidine kinase (TK) promoter. A series of deletion mutants of the 5′-flanking region of the IL-6 gene (positions −742 to +11, −224 to +11, −158 to +11, −109 to +11, and −49 to +11) were then created as follows. The subcloned fragment −1159 to +11 was digested with *Sty*I, *Nhe*I, *Aat*II, *Hae*III, or *Ssp*I; made blunt-ended with Klenow enzyme (Biolabs) or T4 polymerase; and released by *Xho*I digestion. The resulting fragments were subcloned into pTK · CAT (4), (kindly supplied by Herbert Samuels, New York University Medical Center), which was digested with *Bam*HI, filled in with the Klenow enzyme, and then digested with *Xho*I to remove the TK promoter. Activation of the IL-6 promoter region was determined by measuring CAT activities in a transient transfection assay in the GM-637 cell line.

CAT activity was stimulated about 4.5- to 6.5-fold by TNF (30 ng/ml), IL-1 (0.1 ng/ml), or forskolin (10 μM) in cells

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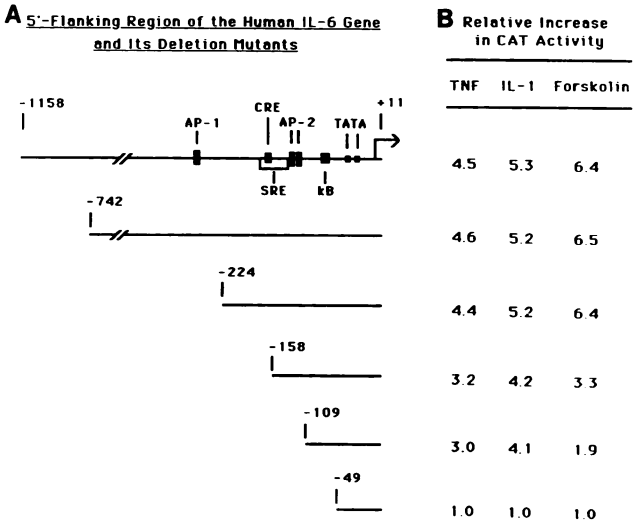

FIG. 1. (A) Summary of deletion analysis of the 5'-flanking region of the human IL-6 gene in human fibroblasts. The GM-637 line of simian virus 40-transformed human skin fibroblasts that were grown in Eagle minimum essential medium with 10% fetal bovine serum was transfected with a series of 5'-deletion constructs derived from the IL-6 genomic DNA fragment −1158 to +11 (the 5'-deletion boundaries are indicated) by the calcium-phosphate coprecipitation procedure (14), as follows. Cells were seeded at a density of 5×10^5 per petri dish (diameter, 100 mm). One-day-old cultures were transfected with 15 μg of $CsCl_2$ gradient-purified plasmids. The cells were exposed to the DNA-calcium phosphate coprecipitate for 24 h. Thereafter, the cells were washed and incubated in medium containing 0.5% fetal bovine serum for 20 h in the absence or presence of various agents, as indicated. The transfected cells were lysed by three cycles of freeze-thawing, and equal amounts of protein from different cell extracts were assayed for CAT activity (13). Protein concentration was determined with reagents from Bio-Rad Laboratories (Richmond, Calif.) by the method of Bradford (2). Abbreviations: SRE, serum response element; CRE, cAMP-responsive element; AP-1 and AP-2, activating protein 1 and 2, respectively. (B) Relative increases (fold induction) in CAT activities were calculated by dividing the percent conversion into the acetylated form of chloramphenicol with extracts from cultures treated with TNF (30 ng/ml), IL-1 (0.1 ng/ml), or forskolin (10 μM) by that in control extracts. Thus, a value of 1.0 represents no increase over the control. The data shown are averages of five independent experiments.

transfected with constructs with 5' boundaries at positions −1158, −742, or −224 and a 3' boundary at position +11 (Fig. 1). Deletion to position −158 led to a partial reduction in the stimulation of CAT activity in response to all three stimuli. Such deletion to position −158 would lead to the destruction of the serum response element-like domain, which was previously proposed to mediate IL-6 induction in response to a variety of stimuli (28). Further deletion to position −109 did not lead to a significant additional change in CAT activity after induction with TNF or IL-1, but the response to forskolin was further reduced. Further deletion to position −49 led to a complete loss of responsiveness to all three agents that we used; the deleted region comprises the sequence GGGATTTTCC (positions −72 to −63), which is highly homologous to the immunoglobulin κ light-chain enhancer sequence GGGACTTTCC, which acts as the NF-κB-binding site (31).

Activation of nuclear protein(s) binding to the IL-6 DNA fragment −109 to −50 after the treatment of cells with TNF, IL-1, or forskolin. The deletion analysis described above suggested that the region between positions −109 and −50 is important for the activation of the IL-6 gene, especially in response to TNF or IL-1. To determine whether this sequence interacts specifically with nuclear protein(s), FS-4 cultures were treated with TNF for various periods of time, the cells were homogenized, and nuclear extracts were prepared as follows. Cultures (approximately 5×10^8 cells in total) were incubated with or without the inducing agents for various periods of time, as indicated. The cells were then broken by homogenization in buffer A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF]) and centrifuged at $1,000 \times g$ for 10 min at 4°C to separate the nuclei from the cytosolic fraction. The nuclei were washed once with buffer A. Nuclear proteins were extracted in buffer C (20 mM HEPES [pH 7.9], 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) on ice for 30 min as described by Dignam et al. (7). The extracted nuclear proteins were dialyzed against buffer D (20 mM HEPES [pH 7.9], 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) for 5 h, divided into portions, frozen in liquid nitrogen, and stored at −70°C. The protein concentration was quantitated by the method of Bradford (2). The isolated nuclear proteins were incubated with the ^{32}P-end-labeled IL-6 DNA fragment −109 to −50. To resolve the DNA-protein complexes that were formed, a DNA mobility shift assay was then carried out essentially as described previously (31).

Incubation with TNF led to the appearance of a protein(s) that formed two distinct complexes with the labeled IL-6 DNA fragment, marked I and II in Fig. 2A. Complex formation was apparent with extracts prepared 30 min after TNF addition, peaked at about 5 h, and remained detectable for at least 9 h. UV cross-linking analysis showed that the proteins that bound to DNA in complexes I and II displayed identical molecular weights under denaturing conditions (data not shown), suggesting that a common protein that comes into direct contact with DNA is shared by the two complexes and that the difference in gel mobility is due to some protein-protein interaction rather than the presence of two different DNA-binding proteins. The formation of the complexes demonstrated by DNA mobility shift was specific, because the appearance of both complexes I and II was inhibited in the presence of an excess of the unlabeled IL-6 DNA fragment −109 to −50 (Fig. 2B). Incubation of FS-4 cells with IL-1 also led to the appearance of the nuclear protein(s) that formed complexes I and II (Fig. 2C). To determine whether protein synthesis was necessary for the appearance of the nuclear protein(s) that interacted with IL-6 DNA, FS-4 cells were incubated with TNF in the presence or absence of cycloheximide. Cycloheximide not only failed to block the appearance of the protein(s) that interacted with IL-6 DNA but, in fact, increased the density of both bands (Fig. 2D). In addition, treatment with cycloheximide alone also led to the formation of the same two complexes, albeit less efficiently than did treatment with TNF. These results suggest that formation of complexes I and II is due to the activation of preformed protein(s), in good agreement with many earlier reports showing that activation of NF-κB in different cell lines does not require protein synthesis (1, 22, 31, 32). Finally, treatment of FS-4 cells with forskolin for 2 h also weakly activated the protein(s) that formed complexes I and II (far right lane in Fig. 2D).

κB-like sequence is responsible for both the binding of

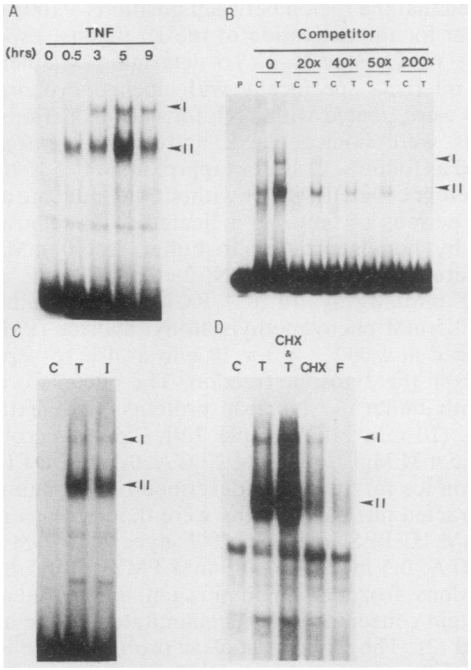

FIG. 2. Activation of nuclear factor(s) binding to IL-6 DNA by TNF, IL-1, and forskolin revealed in the DNA mobility shift assay. The FS-4 line of human diploid foreskin fibroblasts was grown in Eagle minimum essential medium supplemented with 5% fetal bovine serum as described previously (24). FS-4 cells were treated with TNF (30 ng/ml) and/or other agents, as indicated. Nuclear extracts (5 μg) were mixed with ^{32}P-end-labeled IL-6 DNA fragment −109 to −50. The reaction mixtures contained 0.1 ng of the DNA probe (labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase), 10 mM Tris hydrochloride (pH 7.5), 50 mM NaCl, 1 mM EDTA, 0.1 mM DTT, 2 μg of poly(dI-dC), and 10% glycerol in the presence of 5 μg of nuclear extracts in a total volume of 20 μl. The mixtures were incubated at room temperature for 15 min and electrophoresed on a 4% native polyacrylamide gel. The gels were analyzed by autoradiography. (A) DNA mobility shift assay with nuclear extracts from cultures treated with TNF for 0, 0.5, 3, 5, or 9 h. (B) Nuclear extracts prepared from control FS-4 cells or FS-4 cultures treated for 30 min with TNF were incubated with the ^{32}P-labeled IL-6 DNA fragment −109 to −50 in the absence or presence of a 20-, 40-, 50-, or 200-fold excess of the unlabeled IL-6 probe used as a competitor. The DNA mobility shift assay was then carried out. Abbreviations: P, probe alone with no nuclear extract; C, nuclear extract from control cells; T, nuclear extract from TNF-treated cells. (C) Nuclear extracts prepared from control FS-4 cultures or FS-4 cultures treated for 30 min with TNF or IL-1 (0.1 ng/ml) were incubated with the ^{32}P-labeled IL-6 DNA fragment −109 to −50 and analyzed by the DNA mobility shift assay. Abbreviations: C, control; T, TNF-treated cultures; I, IL-1-treated cultures. (D) Nuclear extracts were prepared from control FS-4 cells or cultures treated for 30 min with TNF alone or TNF with 10 μg of cycloheximide per ml or for 2 h with 10 μM forskolin. The nuclear extracts were incubated with ^{32}P-labeled IL-6 DNA fragment −109 to −50, and a DNA mobility shift assay was carried out. Abbreviations: C, control; T, TNF; CHX, cycloheximide; F, forskolin.

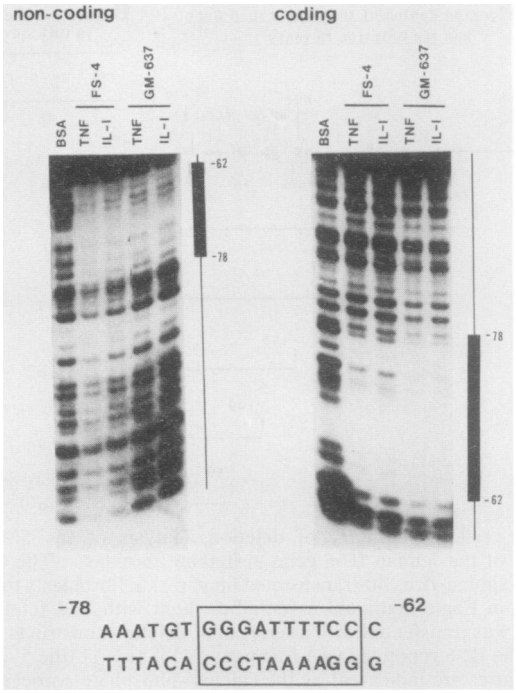

FIG. 3. DNase I footprinting analysis of the IL-6 sequence protected by the nuclear extracts. FS-4 or GM-637 cells were treated with TNF (30 ng/ml) or IL-1 (0.1 ng/ml) for 30 min. Nuclear extracts prepared from FS-4 or GM-637 cells (60 μg) were incubated with 0.1 ng of DNA probe (fragment −109 to −50 of the IL-6 genomic DNA sequence) that was ^{32}P-labeled at the 5′ end of either strand by using the same binding conditions as described for the DNA mobility shift assay in the legend to Fig. 2, except that the incubation volume was 50 μl. The samples were then adjusted to 2 mM $CaCl_2$ and 5 mM $MgCl_2$ and treated with DNase I (2 U/ml; Boehringer Mannheim) for 30 s at room temperature. The reactions were stopped and analyzed on a 8% sequencing gel (26). BSA, Bovine serum albumin. The DNA sequence shown is that of the protected region, and the boxed sequence is the IL-6 κB-like motif.

nuclear protein and transcriptional activation. To localize the protein-binding site, we carried out DNase I footprinting analysis, using the IL-6 DNA fragment −109 to −50, which were labeled at the 5′ end of either strand, and nuclear extracts derived from FS-4 or GM-637 cells stimulated with either TNF or IL-1 (Fig. 3). This analysis revealed that a 17-base-pair region with boundaries at positions −78 and −62 was completely protected by the nuclear proteins from both FS-4 and GM-637 cells stimulated with either TNF or IL-1. This protected region comprises the κB-like sequence (positions −72 to −63).

To gain further evidence for the role of the κB-like sequence in the 5′ flanking region of the IL-6 gene, we examined the interaction of a synthetic 28-mer oligonucleotide containing the IL-6 DNA sequence that includes the κB-like element (Fig. 4A), with nuclear proteins extracted from control or TNF-treated FS-4 cells. The DNA mobility shift assay revealed no retarded bands with the extract from control cells (Fig. 4B, lane 1), whereas formation of complexes I and II was evident with the extract from TNF-treated cells (Fig. 4B, lane 2). In addition, a 200-fold excess of the unlabeled synthetic IL-6 DNA fragment completely abolished the appearance of the two complexes (Fig. 4B, lane 3). In contrast, as shown in lane 4 of Fig. 4B, little competition was seen with a 200-fold excess of a mutant 28-mer oligonucleotide in which the GGG sequence at the 5′ end of the κB-like motif was replaced with TAC (Fig. 4A).

To test further whether the κB-like element on the IL-6 gene mediates induction by TNF, IL-1, or forskolin, plasmids containing the synthetic wild-type κB-like sequence or the mutant κB-like sequence placed in front of the TK promoter and CAT structural gene were used to transfect GM-637, MG-63, or HeLa S3 cells. With the wild-type IL-6

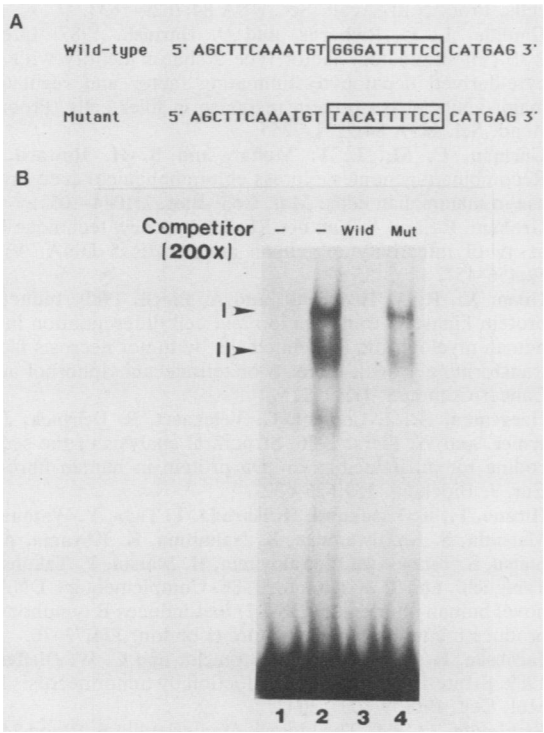

FIG. 4. Competition studies with wild-type and mutant synthetic IL-6 κB-like oligonucleotides. (A) Sequences of the wild-type and mutant 28-mer oligonucleotides used in the experiments shown in panel B and Table 1. These synthetic oligonucleotides were cloned into pTK · CAT, which was digested with *Hind*III and *Bam*HI at the polylinker site. (B) Nuclear extracts were prepared from control FS-4 cells (lane 1) or cells treated with TNF (30 ng/ml) for 30 min (lanes 2 to 4). The extracts (5 μg) were incubated with ^{32}P-end-labeled IL-6 κB-like synthetic oligonucleotide in the absence (lanes 1 and 2) or presence of a 200-fold excess of the unlabeled wild-type (lane 3) or mutant (lane 4) IL-6 κB-like oligonucleotides. (The sequences of the two oligonucleotides used are shown in panel A). The DNA mobility shift assay was done as described in the legend to Fig. 2.

κB-like sequence, CAT activity was significantly increased after stimulation with TNF or IL-1 in all of the cells that we used, with the largest increase being seen after IL-1 treatment in MG-63 cells (Table 1). After stimulation with forskolin, CAT activity was clearly increased in HeLa S3 cells and slightly increased in GM-637 cells, but it did not increase at all in MG-63 cells. This variation in the response to different stimuli (and especially to forskolin) in different cell types suggests that the role of different pathways in the stimulation of IL-6 gene expression via NF-κB activation is tissue type specific. Upon transfection of the mutant IL-6 κB-like sequence into the same cells, transcription of the CAT gene was not stimulated by any of the treatments. In other experiments, the synthetic κB-like sequence has also been shown to drive transcription from the truncated IL-6 promoter (positions −49 to +11) in response to TNF or IL-1 in GM-637 or MG-63 cells (data not shown).

Discussion and conclusions. Data from this study suggest that the increase in cAMP levels and the resulting activation of protein kinase A, which we previously demonstrated occurs in human fibroblasts after treatment with TNF or IL-1 (38), is not the major pathway of IL-6 gene activation by TNF and IL-1. The latter conclusion is based on the following observations. (i) In the CAT assay, the fragment −109 to −50 of the IL-6 gene was highly responsive to TNF or IL-1, while it was barely responsive to forskolin (Fig. 1). (ii) TNF and IL-1 were more effective than forskolin in activating nuclear protein binding to the IL-6 κB-like element (Fig. 2D), and (iii) TNF and IL-1 were effective in driving CAT expression in GM-637 and MG-63 cells transfected with constructs containing the κB-like sequence, while forskolin was not (Table 1). Thus, other pathways, possibly activation of protein kinase C (3, 15) or some other unidentified mechanisms, are likely to contribute to the activation of the IL-6 gene and of the NF-κB-like factor by TNF and IL-1. However, it is likely that the relative importance of different pathways leading to IL-6 gene expression varies depending on the type of tissue involved. For example, in human FS-4 cells IL-6 gene activation by TNF or IL-1 occurred in the absence of protein kinase C activation (37).

TABLE 1. Induction of CAT activity after transient transfection of different cells with constructs containing wild-type or mutant IL-6 κB-like sequence fused with TK promoter

Construct containing[a]:	Transfected cell line[b]	Increase in CAT activity after treatment with[c]:		
		TNF	IL-1	Forskolin
Wild-type κB	GM-637	2.9 ± 0.5	2.4 ± 0.3	1.4 ± 0.2
	MG-63	3.0 ± 0.3	7.3 ± 0.4	1.0
	HeLa S3	2.2 ± 0.2	2.9 ± 0.2	3.7 ± 0.3
Mutant κB	GM-637	1.0	1.0	1.0
	MG-63	1.0	1.1	ND[d]
	HeLa S3	1.0	1.0	1.0

[a] Synthetic wild-type or mutant IL-6 κB-like oligonucleotides (see Fig. 4A) were cloned into the polylinker *Hind*III and *Bam*HI sites in a sense orientation on pTK · CAT and the resulting constructs were used for transient transfection of cell lines.

[b] GM-637 cells and the MG-63 line of human osteosarcoma cells (American Type Culture Collection, Rockville, Md.) were grown in minimum essential medium with 10% fetal bovine serum. HeLa S3 cells (American Type Culture Collection) were grown in Dulbecco minimum essential medium with 10% fetal bovine serum. Transfections were performed as described in the legend to Fig. 1.

[c] Values designate the relative increase in CAT activity (fold induction) ± standard deviations, calculated as described in the legend to Fig. 1. A value of 1.0 represents no increase in CAT activity over the level found in untreated cells. Results given here are the means of three independent experiments.

[d] ND, Not done.

Our results are somewhat different from those reported by Ray et al. (28), who used deletion analysis to localize IL-6 enhancer sequences responsive to TNF, IL-1, activators of protein kinase C, cAMP agonists, viruses, and epidermal growth factor. Based on CAT assays in transiently transfected HeLa S3 cells, Ray et al. (28) concluded that the region between positions −225 and −113 in the 5'-flanking region of the IL-6 DNA is largely responsible for inducibility by all of the agents tested, with the exception of one of the viruses (pseudorabies). Our data confirm that the region identified by Ray et al. (28) contains some responsive element(s), but we also provide evidence for an important positive regulatory region located further downstream, i.e., the κB-like element located between positions −72 and −63.

It is not yet clear whether the nuclear protein from activated fibroblasts that binds to the κB-like element on IL-6 DNA is the same as classical NF-κB from B or T lymphocytes. As with classical NF-κB (1), we have found that FS-4 cells contain, in the cytoplasm, a latent form of NF-κB that is activatable by treatment with deoxycholate (data not shown). On the other hand, preliminary data suggest that some biochemical properties of the nuclear

factor from FS-4 cells are different from those of classical NF-κB. Irrespective of whether there is one NF-κB or a family of related NF-κB-like proteins, it is apparent that it plays an important role in the regulation of cytokine expression, including beta interferon (IFN-β) (9, 23, 34) and, as shown in this study, IL-6. It is interesting that many agents leading to the induction of IFN-β [e.g., various viruses and the double-stranded RNA poly(I) · poly(C)] also induce IL-6 (5, 39). This similarity in the inducibility of IL-6 and IFN-β may be due, in part, to the role of NF-κB in driving the expression of both cytokines, together with the demonstrated capacity of viruses and poly(I) · poly(C) to activate NF-κB (34). Recently, both IL-1 (33) and TNF (18, 29) were shown to induce IFN-β, and this action, too, is likely related to the activation of NF-κB by IL-1 and TNF, as well as their ability to stimulate the synthesis of interferon regulatory factor type 1, another transcription factor that is thought to play a role in the regulation of IFN-β expression (10).

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