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Multiple Regulatory Elements in the Interleukin-6 Gene Mediate Induction by Prostaglandins, Cyclic AMP, and Lipopolysaccharide

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Induction of interleukin-6 (IL-6) gene expression is mediated by numerous agents involving all major signal transduction pathways. We have compared the effects of prostaglandins and their second messenger cyclic AMP (cAMP) with the effect of lipopolysaccharide (LPS) on IL-6 gene expression. We demonstrate that secretion of IL-6 is induced by cAMP in murine monocytic PU5-1.8 cells, even though to a lesser extent than by LPS. Nevertheless, cAMP and prostaglandins of the E series in the presence of theophylline induce transcription of the IL-6 promoter more strongly than LPS, suggesting distinctive effects of cAMP and LPS on posttranscriptional events. Mutations within four regulatory elements, namely, the multiple response element (MRE), AP-1, NF-IL6, and NF-κB sites, significantly reduce, but do not completely abrogate, inducibility by cAMP and prostaglandin E1, whereas alterations of four additional sites have no effects. LPS-induced promoter activity, however, is almost completely abolished by mutations in the NF-κB site, suggesting that a single regulatory element is crucial for inducibility by LPS. Stimulation by cAMP is correlated with the binding of inducible factors to the AP-1, NF-IL6, and NF-κB elements, whereas factors binding to the MRE are constitutively expressed. Recombinant cAMP response element-binding protein binds to the MRE, indicating a potential role for this factor in the cAMP response. Our results suggest that cAMP and prostaglandins act through multiple, partially redundant regulatory elements to induce IL-6 expression in monocytic cells. Nuclear events that overlap partially with the LPS response but also exhibit distinctive features are involved.

Interleukin-6 (IL-6) was originally described as a factor which induces immunoglobulin synthesis in B cells, promotes the growth of B-cell hybridomas and plasmacytomas, stimulates acute-phase protein synthesis in hepatocytes, and plays a role in hematopoiesis and cytotoxic T-cell differentiation (86). In the meantime, this list has expanded into an impressive spectrum of biological activities which have been compiled in several recent reviews (33, 40, 72). In addition to the classical functions mentioned above, IL-6 has been implicated in inflammation (34), viral infection (3), autoimmunity (39), development (43), and malignant cell growth. The significance of upregulated IL-6 production as well as the potential clinical usefulness of the cytokine in a large number of human diseases is currently under investigation (17).

The wide range of responses mediated by IL-6 in a variety of cell types and organs is paralleled by a multitude of agents and conditions that regulate expression of the IL-6 gene (73). Physiologic activators include traumatic and thermal tissue injury, many other cytokines and growth factors, bacterial products like lipopolysaccharide (LPS) and mycobacterial heat shock protein, viruses, serum, cycloheximide, and T-cell mitogens; IL-6 expression is inhibited by IL-4, IL-10 (20), IL-13 (52), glucocorticoids (64), the products of the tumor suppressor genes retinoblastoma and p53 (69), and the adenoviral E1A proteins (37).

IL-6 expression is mediated by all major signal transduction pathways, involving a variety of different protein kinases and second messengers among which intracellular Ca^{2+} levels, protein kinase C, double-stranded RNA-dependent kinase, and cyclic AMP (cAMP) are the most prominent (66, 74, 89). cAMP and prostaglandins, its physiologic agonists, are felt to be of particular significance in immunologic systems because they have certain immunosuppressive properties (38) and also differentially regulate cytokine production by T-cell subpopulations (11, 58, 79). They thus contribute to the important decision about which type of immune response is elicited under different circumstances (62). The ability of prostaglandins and cAMP to induce expression of the IL-6 gene in fibroblasts has previously been reported (89, 91). However, activation of the IL-6 gene in monocytic cells by cAMP has not been uniformly observed; some reports have shown no response (73) or partial responses (47), whereas others have described induction of IL-6 expression in certain experimental systems (7, 32, 61, 70, 87).

The 5′ flanking region of the IL-6 gene contains a number of putative *cis*-acting elements which might be modulated by cAMP or prostaglandins. Some of these regulatory elements have been demonstrated to be required for inducible and tissue-specific transcriptional regulation (73) (Fig. 1). They include a region of homology to the c-*fos* serum response element encompassing a multiple response element (MRE) (65), an NF-IL6 binding site (2), and a potential recognition sequence for members of the *ets* family of transcription factors (75). Two glucocorticoid response element (GRE) homologies and an AP-1 consensus site are located further upstream from the c-*fos* homology region (84), whereas the downstream portion contains a potential GATA–helix-loop-helix (HLH) (54, 59) and an NF-κB site (45, 76, 90). The classical example for a cAMP-regulated transcription factor is cAMP response element-binding protein (CREB) (15, 88), which becomes activated upon phosphorylation by cAMP-dependent protein kinase A (27) and could potentially bind to a sequence contained within the MRE. However, transcription factors

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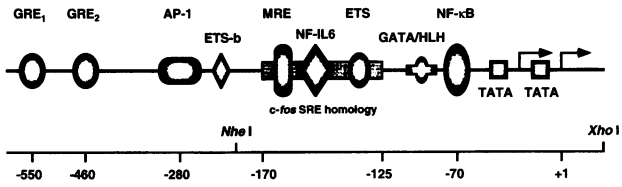

1. GRE_1	wt	AGCCAGAACACAGCA	5. NF-IL6	wt	TCATTGCACAATCTT
	mut	AGCCAGAATTCAGCA		mut	TCAGATATCAATCTT
2. GRE_2	wt	GTTCAGAACATCTTT	6. ETS	wt	TAAGGTTTCCAATCA
	mut	GTTCAGCTGATCTTT		mut	TAAGTCGACCAATCA
3. AP-1	wt	GTGCTGAGTCACTAA	7. GATA/HLH	wt	ATTTATCAAATGTGG
	mut	GTGCTGCAGCACTAA		mut	ATTCTAGAAATGTGG
4. MRE	wt	AAAGGACGTCATTGC	8. NF-κB	wt	GTGGGATTTTCCCAT
	mut A	AAAGGGATCCATTGC		mut	GTGGGATTTTAGACT
	mut B	AAAGCTTGTCATTGC			

FIG. 1. Putative *cis*-regulatory elements of the human IL-6 promoter with their approximate locations relative to the major transcription start site (+1). Abbreviations: GRE, glucocorticoid response element; SRE, serum response element. Below, sequences of the individual transcription factor binding sites are indicated together with the mutations used in this study (altered nucleotides underlined).

binding to AP-1 (5, 6, 10, 19, 24), NF-IL6 (51), and NF-κB (78) sites are also considered candidates for the transmission of cAMP-mediated signals to the transcriptional machinery, thereby suggesting the existence of several potential cAMP-inducible regulatory elements in the IL-6 promoter. The precise mechanism of cAMP- and prostaglandin-mediated transcriptional activation of the IL-6 gene has not been previously explored. In order to define prostaglandin- and cAMP-responsive elements (CREs) in the IL-6 promoter, we therefore studied the effect of prostaglandins and cAMP on IL-6 promoter-reporter gene constructs in transiently transfected monocytic cells. We show here that multiple *cis*-acting elements, namely, AP-1, MRE, NF-IL6, and NF-κB, are participating in full stimulation of IL-6 gene expression by cAMP and prostaglandin, although none of these elements appears to be absolutely required for cAMP or prostaglandin induction. In contrast, a single regulatory element appears to be critical for LPS response of the IL-6 promoter. Constitutive and/or cAMP-inducible binding of nuclear factors to the AP-1, MRE, NF-IL-6, and NF-κB sites can be demonstrated. We furthermore confirm that cAMP induces the endogenous IL-6 gene in the monocytic cell line PU5-1.8. Our results suggest that at least four regulatory elements cooperate to activate IL-6 gene transcription in response to cAMP and prostaglandins.

MATERIALS AND METHODS

Cell culture. The murine monocyte-macrophage cell line PU5-1.8 (63) (American Type Culture Collection, Rockville, Md.) was grown in RPMI-1640 (BioWhittaker, Walkersville, Md.) containing 10% fetal calf serum (HyClone, Logan, Utah), 2 mM L-glutamine, 50 U of penicillin per ml, and 50 μg of streptomycin per ml (all from BioWhittaker).

IL-6 ELISA. PU5-1.8 cells at 10^6/ml in complete media with 5% fetal calf serum were stimulated for 24 h with dibutyryl cAMP (Bt_2cAMP) (Sigma Chemical Co., St. Louis, Mo.), LPS from *Salmonella typhosa* (Difco Laboratories, Detroit, Mich.), or both agents at concentrations varying from 3.8 μM to 125 mM and from 0.31 ng/ml to 10 μg/ml, respectively. IL-6 concentrations in the supernatants were assayed by an enzyme-linked immunosorbent assay (ELISA) using a pair of monoclonal antibodies (Pharmingen, San Diego, Calif.) according to the protocol supplied by the manufacturer. Recombinant murine IL-6 (Genzyme, Cambridge, Mass.) served as the standard for quantitative evaluation.

Plasmid constructs and site-directed mutagenesis. A *Bam*HI-*Xho*I fragment of the human IL-6 gene extending from −1179 to +9 relative to the transcription start site (66) was cloned into the *Sma*I site of pUC-CAT (26) as previously described (45). Site-directed mutagenesis was performed according to the gapped heteroduplex method (82) and with the following oligonucleotides (5′-end position is given before sequence, and altered bases are underlined) (see also Fig. 1): GRE_1, −567, 5′-GGGAGAGCCAGAATTCAGCAAGAAC TC-3′; GRE_2, −475, 5′-AACTCAGTTCAGCTGATCTTTG GTTTT-3′; AP-1, −291, 5′-ATGCCAAGTGCTGCAGCAC TAATAAAGAA-3′; MRE(A), −176, 5′-GCGATGCTAA AGGGATCCACATTGCACAAT-3′; MRE(B), −177, 5′-TGCGATGCTAAAGCTTGTCACATTGCACA-3′; NF-IL6, −168, 5′-AAAGGACGTCACAGATATCAATCTTAATA AG-3′; Ets, −152, 5′-CAATCTTAATAAGTCGACCAAT CAGCCCCA-3′; GATA-HLH, −99, 5′-CTCCAACAAA GATTCTAGAAATGTGGGATTT-3′; NF-κB, −80, 5′-AA TGTGGGATTTTAGACTGAGTCTCAATATT-3′. Mutant clones were screened for the presence of newly created restriction sites and retransformed into bacteria to exclude contamination with wild-type plasmid. The mutations were introduced into pIL6-CAT by replacing a *Bsu*36I-*Nhe*I fragment (for GREs and AP-1; the unique *Bsu*36I site is located at −1154 in the IL-6 promoter) or an *Nhe*I-*Sph*I fragment (for the remaining mutations; the unique *Sph*I site is derived from the pUC19 polylinker and lies downstream from the *Xho*I site) with its mutated counterpart.

Four double mutant plasmids were prepared by replacing the *Bsu*36I-*Nhe*I (Fig. 1) fragment from the MRE, NF-IL6, and NF-κB mutant plasmids with the same region from the AP-1 mutant plasmid. The accuracy of the mutated constructs was confirmed by sequence analysis.

DNA transfection assays. The DEAE-dextran method was used to transiently transfect 10^7 PU5-1.8 cells with 10 μg of plasmid DNA (29). Cotransfection of a second plasmid for determination of transfection efficiency was omitted because potential artifacts with this technique have been reported (22). At 24 h after transfection, cells were stimulated with LPS, Bt_2cAMP, prostaglandins E_1 (PGE_1) and PGE_2, theophylline (all from Sigma), misoprostol, or enisoprost (kindly provided by B. Struthers, Searle Pharmaceuticals, Skokie, Ill.). The cells were harvested another 24 h later, and extracts were prepared by the freeze-thawing procedure (28). The protein concentration was measured with a kit from Bio-Rad (Richmond, Calif.) and normalized for all samples in each individual experiment. Following heating to 60°C for 7 min, chloramphenicol acetyltransferase (CAT) activity was determined by a phase-extraction protocol (71) which directly yields quantitative results. The test background was always lower than 0.05% of the total counts of [^{14}C]chloramphenicol added into the reaction mixture. All assays were performed in duplicate, and transfections were repeated three times with similar results.

Nuclear extracts and EMSAs. A total of 2×10^8 cells were stimulated with 1 mM Bt_2cAMP or 10 μg of LPS per ml for 5 h, and nuclear extracts were prepared according to the method of Dignam et al. (21). Recombinant human CREB and NF-κB p50 proteins were generously provided by A. Wechsler and K. P. LeClair. The double-stranded oligonucleotides used as probes in electrophoretic mobility shift assays (EMSAs) are shown in Table 1.

TABLE 1. Double-stranded oligonucleotides used as probes

Gene	Oligonucleotide[a]
IL-6	
AP-1 wild type	tcgacGTGCTGAGTCACTAAc
	gCACGACTCAGTGATTgagct
AP-1 mutant	tcgacGTGCTGCAGCACTAAc
	gCACGACGTCGTGATTgagct
MRE wild type	tcgacATGCTAAAGGACGTCACATTc
	gTACGATTTCCTGCAGTGTAAgagct
MRE mutant	tcgacATGCTAAAGGGATCCACATTc
	gTACGATTTCCCTAGGTGTAAgagct
NF-IL6 wild type	tcgacACATTGCACAATCTTAAc
	gTGTAACGTGTTAGAATTgagct
NF-IL6 mutant	tcgacACAGATATCAATCTTAAc
	gTGTCTATAGTTAGAATTgagct
NF-κB wild type	tcgacATGTGGGATTTTCCCATGAc
	gTACACCCTAAAAGGGTACTgagct
Other	
Rat somatostatin	
CRE (53)[b]	gatccTTCCTTGGCTGACGTCAGAGAGAg
	gAAGGAACCGACTGCAGTCTCTCTcctag
Human MT-II$_A$	
AP-1 (44)[b]	tcgacCAAGTGACTCAGCGCc
	gGTTCACTGAGTCGCGgagct

[a] Uppercase nucleotides are derived from the promoter sequences. Lowercase nucleotides indicate flanking sequences creating a restriction site.
[b] Numbers in parentheses are references.

DNA binding reactions and EMSAs were performed as described previously (45). The 5′ ends of the oligonucleotides were ^{32}P-labeled with T4 DNA polynucleotide kinase to a specific activity of approximately 5×10^4 cpm/ng and purified on a 12% polyacrylamide gel. Samples of 20 μl containing either 5 μg of nuclear extract, 1 μg of crude bacterial control lysate, 1 μg of recombinant CREB, or 40 ng of NF-κB p50 protein were incubated with 10,000 cpm of labeled oligonucleotides; 1 μg of poly(dI-dC) (Pharmacia, Piscataway, N.J.) for MRE, AP-1, and NF-κB or 3 μg of poly(dI-dC) for NF-IL6; 2 μl of buffer D (21); and 2 μg of bovine serum albumin (New England Biolabs, Beverly, Mass.), in 10 mM Tris (pH 7.5)–50 mM NaCl–1 mM dithiothreitol–1 mM EDTA–5% glycerol for NF-IL6 and NF-κB probes, or in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9)–50 mM KCl–1 mM $MgCl_2$–1 mM dithiothreitol–1 mM EDTA–5 mM spermidine–5% glycerol (65) for AP-1 and MRE probes. Unlabeled competitor oligonucleotides were used at 1, 10, and 100 ng per reaction. The samples were incubated in the presence or absence of competitor oligonucleotides for 15 min at room temperature and run on 4% polyacrylamide gels in either 0.5× TBE (1× TBE = 90 mM Tris-borate, 2 mM EDTA) for AP-1, MRE, and NF-IL6, or 1× TAE (6.7 mM Tris-HCl [pH 7.5], 3.3 mM sodium acetate, 1 mM EDTA) for NF-κB, at 150 V.

RESULTS

cAMP and LPS induce IL-6 secretion in the murine monocyte/macrophage cell line PU5-1.8. We first tested the ability of cAMP and LPS to induce expression of the endogenous IL-6 gene in murine monocytic PU5-1.8 cells by measuring the IL-6 concentration in culture supernatants with an IL-6-specific ELISA 24 h after stimulation. Unstimulated PU5-1.8 cells secrete virtually no IL-6 (<10 pg/ml/10^6 cells per 24 h). Upon stimulation by the membrane-permeable cAMP derivative Bt_2cAMP, however, increasing amounts of IL-6 are released into the medium with increasing concentrations of cAMP (Fig. 2A). A peak of ~300 pg of secreted IL-6 per ml/10^6 cells per 24 h is reached at ~30 mM cAMP (Fig. 2A). The minimum Bt_2cAMP dose required to elicit an effect in PU5-1.8 cells lies in the range of 2 mM; the rapid decline in IL-6 levels at Bt_2cAMP concentrations beyond 30 mM is most likely due to unspecific toxicity of the agent. Bacterial endotoxin (LPS), one of the strongest inducers of IL-6 in monocytes (45) as well as in many other cell types, causes secretion of much larger quantities of IL-6 than Bt_2cAMP, even at doses as low as 10 ng/ml (Fig. 2B). The maximum IL-6 generation stimulated by 10 μg of LPS per ml is almost 2 orders of magnitude higher than for cAMP induction (Fig. 2B), suggesting either more transient kinetics of cAMP action or a differential effect between cAMP and LPS on transcriptional or posttranscriptional events involved in IL-6 production. Addition of Bt_2cAMP at 80 μM together with LPS causes an approximately twofold synergistic enhancement of IL-6 secretion, even though Bt_2cAMP alone has only marginal effects at the same concentration. These results demonstrate that Bt_2cAMP alone induces IL-6 secretion in PU5-1.8 cells, although at a highly reduced level compared with LPS, but strongly synergizes with LPS in IL-6 secretion.

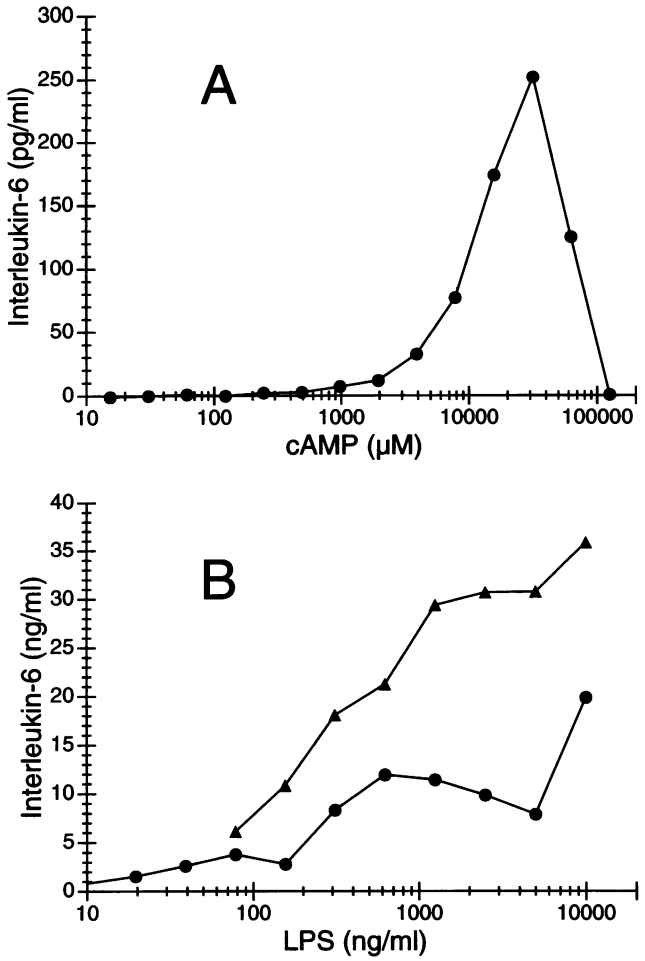

FIG. 2. cAMP and LPS stimulate IL-6 secretion in the murine monocyte/macrophage cell line PU5-1.8. (A) Cells were plated at a density of 10^6/ml and cultured with varying concentrations of Bt_2cAMP for 24 h. IL-6 immunoreactivity in the supernatant was determined by ELISA. (B) Incubation of cells with varying concentrations of LPS without (circles) or with (triangles) 80 μM Bt_2cAMP.

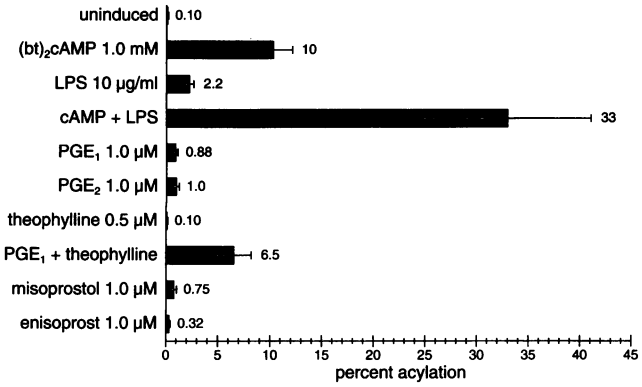

FIG. 3. Transient transfection assay of the wild-type IL-6 promoter–CAT construct in the PU5-1.8 cell line. Cells were stimulated 24 h after transfection with the indicated inducers, and CAT activity in the lysates was determined another 24 h later as described in Materials and Methods. Data shown are the averages of duplicate measurements with the standard deviation. The experiment was repeated three times with comparable results.

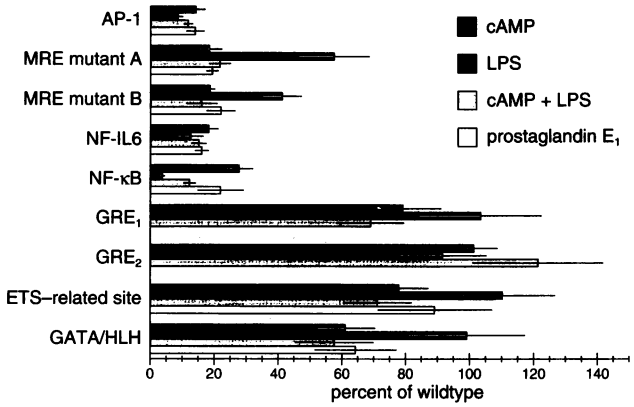

FIG. 4. Site-directed mutations within the AP-1, MRE, NF-IL6, and NF-κB elements diminish IL-6 promoter activity in response to cAMP (1.0 mM), PGE_1 (1.0 μM), and LPS (10 μg/ml), or a combination of cAMP and LPS, whereas mutations of four additional putative transcription factor binding sites have little or no effect. Full-length promoter-CAT constructs in which the specified *cis*-regulatory elements had been eliminated were transiently transfected into PU5-1.8 cells, and CAT activity was determined after a 24-h stimulation as described above. The CAT activity of the mutant IL-6 promoters is shown as the percentage of induction of the wild-type IL-6 promoter as indicated on the left. The effect of PGE_1 on GRE_1 and GRE_2 mutations was not measured. Data shown are the averages of duplicate measurements in two independent experiments with the standard deviations.

The IL-6 promoter is strongly inducible in PU5-1.8 cells by cAMP, prostaglandins, or LPS. In order to determine the effect of cAMP, prostaglandins, and LPS on transcriptional regulation of IL-6 gene expression, we used a transient transfection assay and reporter constructs with the CAT gene placed under the transcriptional control of the human IL-6 promoter (45). The recombinant IL6-CAT plasmid was introduced into PU5-1.8 cells for transient expression. After 24 h, the cells were incubated for an additional 24 h either in medium alone or in the presence of Bt_2cAMP, LPS, PGE_1, PGE_2, theophylline, misoprostol, enisoprost, or combinations thereof. In unstimulated cells, the IL-6 promoter is apparently silent, since activity of the IL-6 promoter is indistinguishable from the extremely low test background (Fig. 3) and from the parental plasmid, pUC-CAT (data not shown), correlating also with the virtual absence of endogenous IL-6 gene expression prior to stimulation (see above). Treatment with 1.0 mM Bt_2cAMP, however, causes a more than 100-fold stimulation of IL-6 promoter activity over unstimulated cells. Similarly, LPS at 10 μg/ml drastically increases CAT activity more than 20-fold in PU5-1.8 cells, although this effect is much weaker than the response to cAMP stimulation. LPS is a more powerful stimulator of IL-6 secretion than cAMP, yet transcriptional activation via the IL-6 promoter appears to be stronger in response to cAMP than to LPS. These results indicate that either cAMP has a destabilizing effect or LPS has a stabilizing effect on mRNA, or that other posttranscriptional events such as translation, posttranslational modification, or secretion are affected. Combination of cAMP and LPS results in a dramatic, more than 300-fold induction of CAT activity (Fig. 3), clearly indicating a synergistic cooperation between the two signals, which is also observed for IL-6 secretion. The two E series prostaglandins, PGE_1 and PGE_2, which elevate intracellular cAMP levels, lead to an approximately 10-fold increase in CAT activity (Fig. 3). We hypothesized that the reduced stimulation by prostaglandins compared with their second messenger cAMP might be related to a diminished amplitude or duration of intracellular cAMP accumulation with prostaglandin treatment. To examine this possibility further, we incubated cells in the presence of the phosphodiesterase inhibitor theophylline, which by itself has no effect on IL-6 promoter activity. Theophylline in the presence of PGE_1, however, enhances induction of the IL-6 promoter provided by PGE_1 almost to the same level as that observed for cAMP. The two orally bioavailable PGE_1 analogs, misoprostol and enisoprost, elicit relatively moderate effects similar to those seen with PGE_1. These data demonstrate the ability of cAMP-dependent pathways to stimulate IL-6 gene transcription.

Multiple regulatory elements are required for induction of the IL-6 gene by cAMP, LPS, and prostaglandins. To examine which regulatory elements in the IL-6 promoter are responsive to cAMP, LPS, and prostaglandins, we introduced 2- to 5-bp mutations into the core regions of eight potential transcription factor binding sites within the context of the 1.2-kb IL-6 promoter as outlined in Fig. 1, with the intention to completely abolish the interaction between individual *trans*-acting factors and their cognate recognition sequences. In contrast to deletional studies, this approach should enable us to identify the contribution of single regulatory elements to gene activation in the context of the full-length promoter. Upon transient transfection of the mutant IL-6 promoter constructs into PU5-1.8 cells, a number of regulatory elements were found to be functionally required for inducibility of the IL-6 promoter by cAMP, PGE_1, and LPS (Fig. 4). Surprisingly, the strongest effect is obtained when the AP-1 site around position −280 is eliminated, reducing the responses to cAMP, PGE_1, and LPS stimulation to 14, 11, and 9% of wild-type promoter activity, respectively. The two mutations within the MRE at position −150 diminish inducibility down to 18 and 19% for cAMP and 20 and 16% for PGE_1, but reduce inducibility by LPS only to 58 and 41%. Disruption of the NF-IL6 binding site attenuates expression of the reporter gene equally for all agents, namely, to 18% for cAMP, 15% for PGE_1, and 13% for LPS stimulation. In contrast, mutation of the NF-κB site virtually abolishes induction by LPS (4%), but retains a reasonable level of cAMP (28%) and PGE_1 (20%) inducibility. The remaining four mutations in the two putative GREs, the ETS-related and the

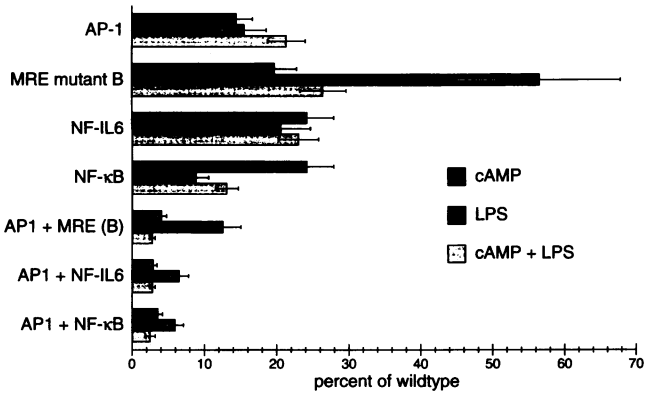

FIG. 5. Elimination of two transcription factor binding sites abolishes inducibility of the IL-6 promoter. *y*-axis labels denote the *cis*-regulatory elements that have been eliminated by site-directed mutagenesis in the respective IL-6 promoter–CAT constructs. Stimulations were performed with either cAMP (1.0 mM), LPS (10 μg/ml), or a combination of both agents. The CAT activity of the mutant IL-6 promoters is shown as the percentage of induction of the wild-type IL-6 promoter as indicated on the left. Data shown are the averages of duplicate measurements with the standard deviations.

GATA-HLH sites, do not significantly affect promoter inducibility by LPS. Responsiveness to cAMP and PGE_1 is only altered to some extent for the GATA-HLH mutation, which reduces it to 61 and 64% of that of the wild type, respectively. These data suggest that at least four of the eight putative *cis*-acting elements tested are involved in activation of the IL-6 promoter through cAMP or LPS signalling mechanisms, even though there are quantitative differences among individual regulatory elements. Elimination of each of these four elements markedly decreases promoter inducibility by cAMP, PGE_1, or LPS. Regarding the specificity for cAMP, PGE_1, or LPS signal transduction pathways, none of the four transcriptional regulatory elements can be exclusively implicated in one of the signalling cascades. However, the effect of MRE mutations on LPS inducibility is relatively minor (~50% inhibition), whereas alteration of the NF-κB site abrogates LPS responsiveness almost completely. The same degree of inhibition is not observed for the cAMP- or PGE_1-dependent pathway, where 14 or 11% inducibility, respectively, is retained even for the AP-1 mutation. These data demonstrate that whereas a single regulatory element, NF-κB, appears to be essential for LPS inducibility, no individual regulatory element is essential for cAMP inducibility.

The effect of the point mutations on the synergistic activation of the IL-6 gene by LPS and cAMP was examined as well (Fig. 4). Reduced CAT expression corresponding to the effect of the individual inducer on the particular regulatory element was observed in all cases. Since cAMP appears to be a stronger inducer of IL-6 transcription than LPS, the effect of cAMP on the particular mutation is relatively stronger than the effect of LPS.

Double mutations in two transcription factor binding sites virtually abrogate inducibility of the IL-6 promoter. In an attempt to delineate potential cooperative versus additive effects of different regulatory elements in response to cAMP or LPS, we created double mutants combining the mutation within the AP-1 site at −280 with mutations of any one of three downstream elements, MRE, NF-IL6, and NF-κB. CAT activity of the three double mutants in response to cAMP or LPS treatment is drastically reduced to 2 to 4% of wild-type activity for induction with cAMP and 6 to 12% for induction by LPS (Fig. 5). Interestingly, mutation of the MRE in context with the AP-1 site has only a small additional effect on inducibility by LPS, indicating again the marginal involvement of the MRE in LPS responses. Elimination of the AP-1 site in combination with a mutation in a second regulatory element thus prevents IL-6 gene activation almost completely even in response to synergistic stimulation and suggests additive rather than cooperative interaction between the AP-1 site and any one of the other three elements.

An inducible nuclear factor binds specifically to the IL-6 promoter AP-1 element. To confirm that nuclear proteins bind specifically to the regulatory elements functionally implicated in activation of the IL-6 promoter by cAMP or LPS but no longer interact with the mutant sites, and to determine whether binding of these factors would be affected by stimulation, we performed EMSAs. We compared the abilities of wild-type and mutant synthetic double-stranded oligonucleotides encompassing the relevant regulatory elements to form complexes with proteins present in nuclear extracts from PU5-1.8 cells grown for 5 h in the presence or absence of cAMP or LPS. When a double-stranded oligonucleotide encoding the IL-6 promoter AP-1 site is allowed to interact with nuclear proteins in EMSAs, three DNA-protein complexes of different mobilities which are present under all stimulation conditions can be distinguished (Fig. 6). The most slowly migrating complex (indicated by an arrow in Fig. 6) is approximately threefold enhanced upon induction with both cAMP and LPS. This protein-DNA complex, but not the two other complexes, is completely abolished when an oligonucleotide encoding the functionally disabled, mutant AP-1 site is used as a probe. Furthermore, this largest complex with similar properties in terms of mobility and inducibility also appears when an oligonucleotide from the human metallothionein II_A (MT-II_A) gene harboring a canonical AP-1 site is used, indicating that identical or similar nuclear proteins bind to both sequences. These results suggest that the inducible protein binding to the IL-6 AP-1 site might be the functionally relevant factor and might be identical with the factors interacting with the MT-II_A AP-1 site. This was further substantiated by competition experiments. Increasing amounts of either unlabeled IL-6 AP-1 competitor oligonucleotide or MT-II_A AP-1 oligonucleotide abrogate the induced highest complex while the mutant IL-6 AP-1 oligonucleotide or the unrelated NF-IL6 oligonucleotide has no effect (Fig. 6). Similarly, if the MT-II_A AP-1 site is used as the probe, both the IL-6 AP-1 and the MT-II_A AP-1 oligonucleotide compete specifically with the highest complex. There are no apparent differences in affinities of nuclear proteins binding to the IL-6 and MT-II_A AP-1 sites.

The same constitutively expressed nuclear proteins interact specifically with the IL-6 MRE and the somatostatin CRE. EMSA analysis of the IL-6 promoter MRE with nuclear extracts from unstimulated and stimulated PU5-1.8 cells reveals one specific protein-DNA complex in the uninduced extract that is not formed with the mutant IL-6 MRE oligonucleotide and might be slightly enhanced by stimulation with LPS (Fig. 7). cAMP treatment does not alter the intensity of this band but leads to the formation of two additional weaker bands with greater mobility. Since the functionally relevant region of the IL-6 MRE contains a sequence with strong similarity to the classical CRE, we examined whether a canonical CRE site from the rat somatostatin gene binds the same proteins which interact with the IL-6 MRE. If the somatostatin CRE is used as the probe, strong binding of nuclear proteins which appear to comigrate with complexes formed with the IL-6 MRE is observed with the three nuclear extracts. Com-

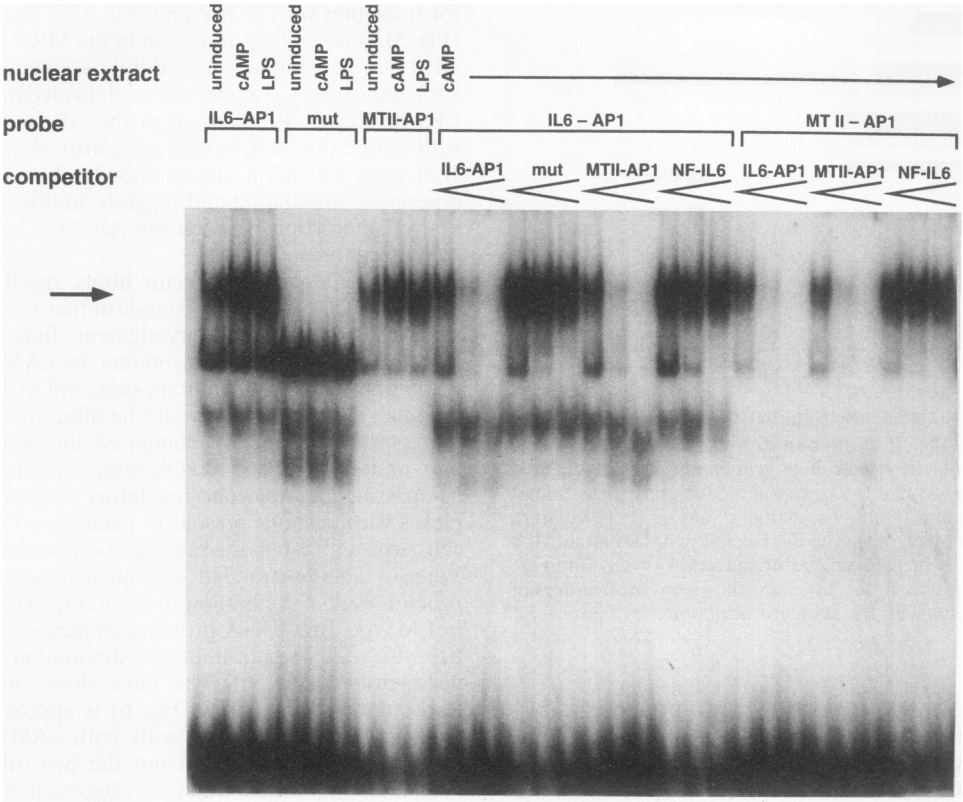

FIG. 6. EMSA for the AP-1 element. PU5-1.8 cells were either left untreated or stimulated with cAMP (1.0 mM) or LPS (10 μg/ml) for 5 h before preparation of nuclear extracts. Five micrograms of extracts was incubated with ^{32}P-labeled oligonucleotides, and the DNA-protein complexes were visualized on a nondenaturing polyacrylamide gel. Unlabeled competitor oligonucleotides were added at 1, 10, and 100 ng per reaction. The MTII-AP1 oligonucleotide contains the canonical AP-1 element from the human MT-II$_A$ promoter. The arrow indicates the position of the specific complex.

petition analysis demonstrates that the two highest protein-DNA complexes are specific for the IL-6 MRE sequence, because they are effectively inhibited by the wild-type MRE, but not by the mutant MRE. Furthermore, these proteins appear to be identical to the factors binding to the somatostatin CRE, since unlabeled somatostatin CRE oligonucleotide inhibits these proteins very efficiently. Comparing the competition efficiencies of wild-type MRE and somatostatin CRE oligonucleotides suggests that the affinity of nuclear factors binding to the CRE is much higher than the affinity to the IL-6 MRE (Fig. 7). This is also observed when the MRE is used as the probe. Differences can be most clearly appreciated at the 1- and 10-ng concentrations of competitors. When CRE is used as the probe, displacement by CRE itself is significant at 10 ng whereas MRE even at 100 ng has only a marginal effect. In conclusion, the factors interacting specifically with the IL-6 MRE appear to be identical to proteins binding to CREs, although the affinity is apparently weaker for the MRE.

Recombinant CREB protein binds to the IL-6 MRE in vitro. To further confirm that the IL-6 MRE represents a lower-affinity CRE, we tested whether the CREB protein, a classical CRE-binding transcription factor, can interact with the IL-6 MRE oligonucleotide. Human recombinant CREB was expressed in *Escherichia coli* (14), and the crude bacterial extract was used to perform in vitro DNA binding assays (Fig. 7). Incubation of the extract with the somatostatin CRE yields several strong complexes which are not formed with a control bacterial lysate. The IL-6 MRE forms the same complexes, albeit with somewhat lower intensity. Competition analysis again indicates that the affinity of CREB to the CRE is higher than to the IL-6 MRE, since the degree of competition achieved with 100 ng of unlabeled MRE oligonucleotide equals that for only 10 ng of CRE.

A highly inducible factor binds to the IL-6 promoter NF-IL6 site. Examination of the interactions of nuclear factors with the NF-IL6 element as a probe reveals that cAMP and LPS both induce the formation of a complex which is practically absent in unstimulated cells (Fig. 8). The mutation which functionally inactivates the NF-IL6 site also abrogates binding to this inducible factor. The specificity of the inducible protein-DNA complex for the NF-IL6 site is again verified by efficient competition with the wild-type NF-IL6 oligonucleotide and the failure of the mutant NF-IL6 oligonucleotide to displace this factor.

cAMP and LPS upregulate proteins interacting with the IL-6 NF-κB site. EMSAs using the IL-6 NF-κB site as a probe demonstrate that both cAMP and LPS induce proteins able to interact with the IL-6 NF-κB site (Fig. 9). LPS appears to induce the formation of an additional more slowly migrating complex not present or much weaker in cAMP-stimulated cells. Two constitutively expressed protein-DNA complexes are also visible. Unlabeled NF-κB wild-type oligonucleotide displaces the probe from both the inducible and the constitutive proteins, whereas an unrelated competitor NF-IL6 oligonucleotide has no effect. The failure of the mutant NF-κB site

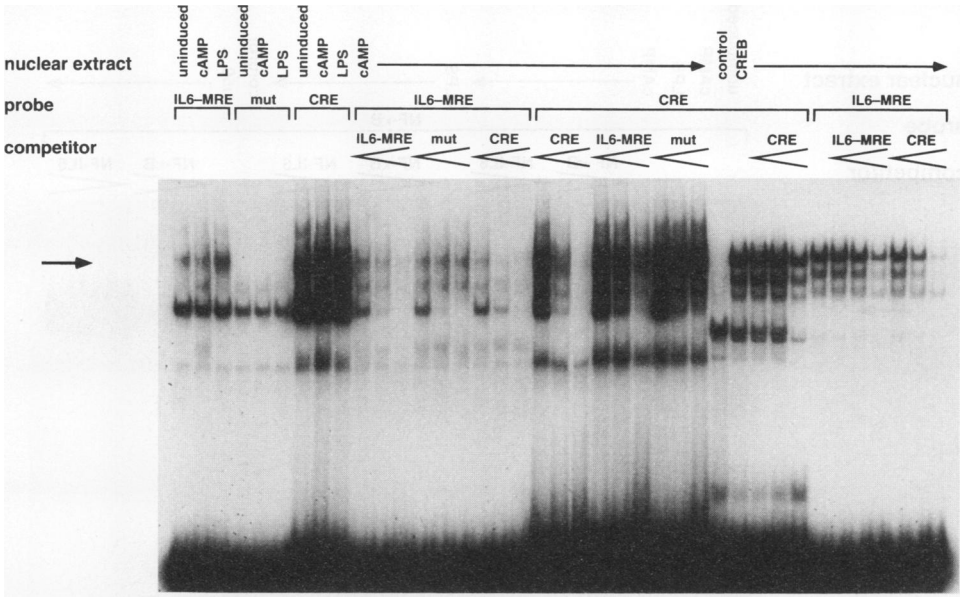

FIG. 7. EMSA for the IL-6 MRE. In addition to the nuclear extracts, 1 μg of crude bacterial lysate from *E. coli* BL21(DE3) expressing human CREB protein was incubated with the probes. A lysate from bacteria transformed with the expression vector without the cDNA insert served as control. CRE denotes an oligonucleotide derived from the canonical cAMP response element of the rat somatostatin gene.

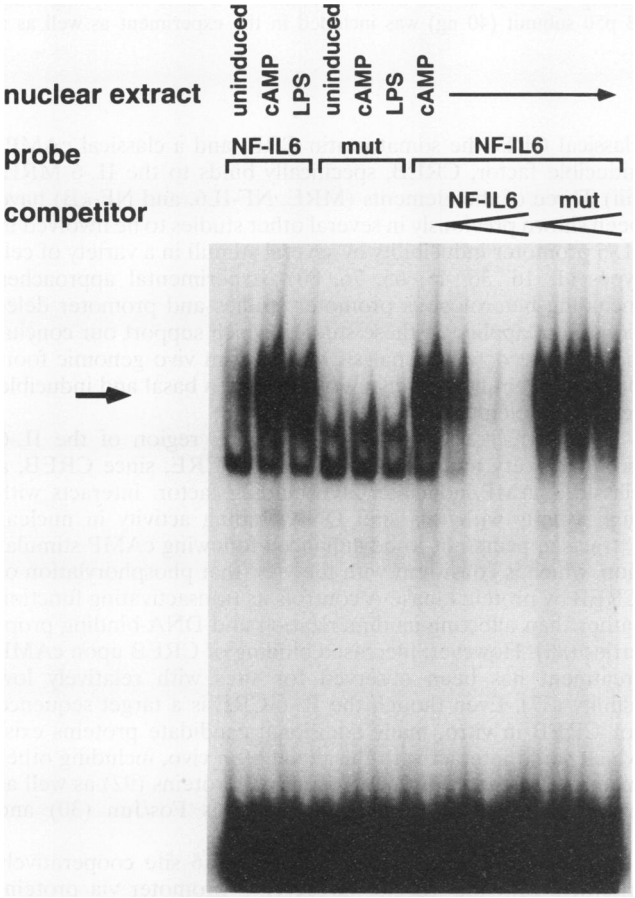

FIG. 8. EMSA for the NF-IL6 binding site in the IL-6 promoter. The specific DNA-protein complex (arrow) is strongly inducible by both cAMP and LPS stimulation.

to bind κB-specific proteins has been confirmed previously (45) and is therefore not shown here.

Recombinant p50 NF-κB binds specifically to the IL-6 κB site. To evaluate the ability of recombinant NF-κB p50 protein to interact with the IL-6 κB site, we used crude bacterial lysates expressing the human p50 subunit of NF-κB in EMSAs (Fig. 9). A single specific complex, most likely consisting of p50 homodimers, is formed with the IL6-κB probe, whereas the control bacterial extract does not form this complex. This p50-DNA complex comigrates with the complex formed by nuclear extracts from both cAMP- and LPS-stimulated cells and is specifically inhibited by the unlabeled IL-6 κB oligonucleotide, but not by the NF-IL6 oligonucleotide.

DISCUSSION

The goals of our study were to identify regulatory elements which are involved in IL-6 gene activation by cAMP, prostaglandins, and LPS. We have previously demonstrated that the IL-6 κB site is a crucial element in the induction of IL-6 gene expression in monocytic cells in response to a variety of stimulants including LPS (45). In the present report, we show that multiple regulatory elements in the IL-6 promoter apparently mediate induction of the IL-6 gene by cAMP, prostaglandins, and LPS in the monocytic cell line PU5-1.8. Furthermore, we demonstrate that induction by cAMP or prostaglandins can be distinguished at least partially from induction by LPS. Whereas point mutations of single regulatory elements do not completely eliminate inducibility of the IL-6 promoter by cAMP or prostaglandins, mutation of the IL-6 κB site virtually abolishes responsiveness to LPS. Thus, at least four, partly redundant regulatory elements may be required for IL-6 activation by cAMP or prostaglandins. These include (i) the AP-1 site at position −280, (ii) a putative CRE in the MRE sequence at −160, (iii) the NF-IL6 element at −150, and (iv) the κB site at −70.

Although regulatory elements in the IL-6 promoter have been characterized in several previous studies (73), a system-

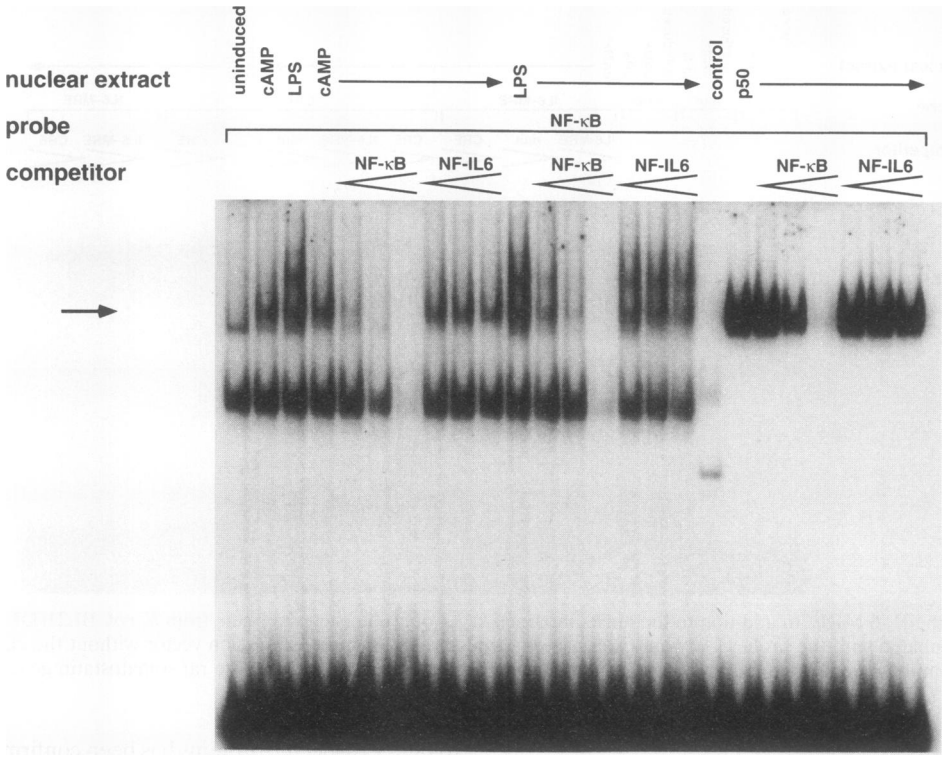

FIG. 9. EMSA for the NF-κB element showing inducibility of NF-κB binding by cAMP as well as the formation of a more slowly migrating complex after LPS stimulation. A crude bacterial lysate expressing NF-κB p50 subunit (40 ng) was included in the experiment as well as a nonexpressing lysate as the negative control.

atic analysis of cAMP- or prostaglandin-responsive elements has not been carried out. Ray et al. (65) first called attention to the 23-bp IL-6 MRE region which confers cAMP inducibility on a heterologous promoter. cAMP responsiveness in the IL-6 MRE is likely to be mediated through the sequence GGACGTCA, which corresponds to the consensus CRE TGACGTCA (15). Our results confirm and extend these observations by showing that two site-directed mutants, GG GATCCA and GCTTGTCA, in the context of the full-length IL-6 promoter greatly diminish IL-6 inducibility in response to cAMP, but to a much lesser extent to LPS, suggesting also that the signal transduction pathway for LPS is largely independent of cAMP induction in PU5-1.8 cells. Our studies evaluate the function of the various regulatory elements in the context of 1.2 kb of IL-6 5′ flanking region, a more physiological situation in comparison with heterologous promoter experiments or deletion mutant studies in which most of the naturally occurring protein-protein interactions are disrupted. Since the basal level of IL-6 promoter activity prior to induction is identical to the test background and to the activity of the parental promoterless vector, pUC-CAT, we cannot rule out the possibility that some of the regulatory elements implicated in inducibility of the IL-6 promoter might actually be involved in the basal transcription machinery. The endogenous IL-6 gene appears to be silent in unstimulated cells, which coincides with the lack of promoter activity. We have several reasons to believe that the implicated regulatory elements are indeed required for induction of the IL-6 promoter rather than basal activity. (i) Three of the four elements (AP-1, NF-IL6, and NF-κB) interact with nuclear factors whose binding is enhanced by cAMP or LPS as shown in Fig. 6 to 9. (ii) Proteins interacting with the IL-6 MRE appear to be identical with factors interacting with a classical CRE, the somatostatin CRE, and a classical cAMP-inducible factor, CREB, specifically binds to the IL-6 MRE. (iii) Three of the elements (MRE, NF-IL6, and NF-κB) have been shown previously in several other studies to be involved in IL-6 promoter inducibility by several stimuli in a variety of cell types (1, 16, 36, 45, 65, 76, 80). Experimental approaches including heterologous promoter studies and promoter deletions were applied in these studies, which support our conclusions. More detailed analysis including in vivo genomic footprinting might help to distinguish between basal and inducible regulatory elements.

The CRE-like sequence in the MRE region of the IL-6 promoter very much acts like a typical CRE, since CREB, a classical cAMP-inducible CRE-binding factor, interacts with high affinity with this site. DNA-binding activity in nuclear extracts appears not to be enhanced following cAMP stimulation, which is consistent with the view that phosphorylation of CREB by protein kinase A controls its transactivating function rather than affecting its dimerization and DNA-binding properties (88). However, increased binding of CREB upon cAMP treatment has been observed for sites with relatively low affinity (57). Even though the IL-6 CRE is a target sequence for CREB in vitro, many additional candidate proteins exist which could interact with this element in vivo, including other members of the ATF/CREB family of proteins (92) as well as other leucine zipper molecules such as Fos/Jun (30) and C/EBP (8).

It has been speculated that the NF-IL6 site cooperatively interacts with the κB site in the IL-8 promoter via protein-protein interactions between NF-IL6 and NF-κB (46, 49, 81), and the ability of the Rel homology domain of NF-κB p50 or p65 to bind to the leucine zipper motif of NF-IL6 has been

shown directly (42, 49, 81). Similar to the IL-8 promoter, the IL-6 promoter contains a κB site adjacent to the NF-IL6 site. Recently, it was shown that the p65 subunit of NF-κB and NF-IL6 cooperatively activate the IL-6 promoter when overexpressed in P19 cells (49). Indeed, mutation of either the NF-IL6 or the κB site reduces activity of the IL-6 promoter in response to cAMP or LPS, indicating potential cooperativity. Nevertheless, there are some distinct differences in the effects of mutations in either site. Whereas mutation of the κB site abrogates responsiveness to LPS, mutation of the NF-IL6 site only partially reduces the LPS effect, suggesting that an intact κB site, but not the NF-IL6 site, is essential for LPS signal transduction. On the other hand, the response to cAMP is partially diminished when either of the two sites is mutated. These results suggest that cooperativity of the NF-IL6 site with the κB site might depend on the particular stimulus and might diverge for different signal transduction pathways activated by the stimuli. It is very striking that the κB site appears to be far more responsive to the signals transduced by LPS, where the κB site is essential for IL-6 promoter activation, than to cAMP, where mutation of the κB site results in a more modest reduction of promoter activity. It is conceivable that LPS induces a different subset of homo- or heterodimers of proteins belonging to the Rel/NF-κB family than does cAMP, thus affecting both the activity of the κB site and potentially cooperative interactions with factors binding to the NF-IL6 site. Further experiments using specific antibodies should help to clarify these questions. Our data show clearly that the NF-IL6 and NF-κB sites are functionally relevant for IL-6 gene activation in response to both cAMP and LPS in a macrophage cell line and that both of these agents induce specific binding of nuclear proteins to the NF-IL6 and NF-κB sites.

Data regarding the functional properties of the IL-6 AP-1 site have been inconclusive. Several studies employing various cell types and stimulants showed either a small reduction (66, 80) or no alteration (16, 76, 90) of inducibility, if the 5′ part of the promoter containing the AP-1 site was deleted. In a recent study by Janaswami et al. (37), the AP-1 deletion construct had a somewhat higher basal activity than a 742-bp promoter fragment and responsiveness to cAMP but not to tumor necrosis factor alpha was lost. Mutation of the AP-1 element in our hands greatly reduced the activity of the full-length promoter in response to cAMP, prostaglandins, and LPS, clearly arguing in favor of a functional role for the IL-6 AP-1 element. Apparent discrepancies with deletional data mentioned above might again be explained by the inherent limitations of these types of studies. Thus, deletions eliminating the AP-1 site could have also removed a negative-regulatory element, thereby compensating for a loss of activity.

AP-1 activity might be controlled by the cAMP-dependent signal transduction pathway on several levels including transcriptional induction of members of the *fos* and *jun* gene families (10, 24, 50, 55) and posttranslational protein modifications like nuclear translocation (68), phosphorylation (reviewed in reference 35), dephosphorylation (4), and targeted degradation (60). An additional alternative involves indirect regulation via intermediary factors (5, 19, 68). Finally, members of the ATF/CREB family of leucine zipper transcription factors can directly interact with AP-1 sites (30, 48).

The ability of prostaglandins and other agonists of the cAMP-dependent second messenger pathway to stimulate IL-6 gene expression in fibroblasts was appreciated early (38) and confirmed at the level of promoter activity (66). Most of the work addressing transcriptional regulation of the IL-6 gene has been carried out in fibroblasts, and information on the properties of monocytes/macrophages with respect to IL-6 expression is comparatively limited, even though cells of the monocyte/macrophage lineage contribute in a major way to systemic IL-6 release (9, 77, 85). We have previously reported data concerning transcriptional activation of the IL-6 promoter in the human monocytic cell line U-937. We now extend our transcriptional regulation studies with a murine monocyte/macrophage cell line, PU5-1.8, with relatively mature phenotypic characteristics (56), which can be induced to secrete large quantities of several cytokines including IL-1, tumor necrosis factor alpha, and IL-6 in response to bacterial endotoxin (23). Several groups have shown that steady-state mRNA levels for IL-6 are markedly increased in purified human monocytes (87), leading to secretion of IL-6 when cultured with cAMP-elevating agents (32, 61, 70). In mouse macrophage cell lines, however, release of IL-6 following induction by cAMP agonists has not been detectable even though mRNA concentrations were slightly upregulated (47). Our data show that only high concentrations of cAMP induce significant IL-6 secretion in PU5-1.8 cells and the maximum amount of secreted IL-6 is almost 2 orders of magnitude lower compared with LPS stimulation. Nevertheless, IL-6 promoter activity in response to cAMP was approximately fivefold higher than in response to LPS, suggesting that posttranscriptional mechanisms must be operative to diminish actual release of cytokine protein from PU5-1.8 cells. One possible explanation for this phenomenon involves UA-rich sequence motifs in the 3′-untranslated region of the IL-6 mRNA. These regions have been found in a number of cytokines and proto-oncogenes including granulocyte-macrophage colony-stimulating factor, tumor necrosis factor alpha, and c-*fos* and have been shown to regulate mRNA stability as well as translation (12, 13, 31).

With respect to the combined actions of cAMP and LPS on monocytes/macrophages, evidence that cAMP synergistically enhances LPS-induced IL-6 production has previously been reported (7, 47). We have shown here that the synergistic effect is mediated at the transcriptional level. Prostaglandins and their second messenger cAMP have been traditionally viewed as inhibitory agents downregulating a variety of macrophage functions in an auto- and paracrine fashion and also suppressing T- and B-lymphocyte responses (18, 41, 67, 83). It has become increasingly apparent, however, that prostaglandins and cAMP can enhance certain aspects of immunological reactions and thus influence the character and dimension of an immune response rather than generally limiting it (62). Our finding that these immune modulators increase IL-6 expression in macrophages strongly supports their role in promoting a T_H2-type immune response and antibody secretion by B cells.

In conclusion, our results indicate that the activity of at least four transcription factors is simultaneously required to maximally induce IL-6 gene transcription upon stimulation with cAMP or LPS. Despite redundancy of regulatory sites, each of the sites appears to contribute a necessary signal to the transcriptional machinery, and different sites vary in their responsiveness to cAMP or LPS. Signal transmission most likely occurs through protein-protein interactions, and it is becoming increasingly apparent that the DNA-binding proteins of the Fos/Jun, ATF/CREB, C/EBP, and Rel/NF-κB families contain structural motifs involved in homo- and heterodimerization necessary for cooperative protein-protein interactions.

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