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Acute-Phase Response Factor, a Nuclear Factor Binding to Acute-Phase Response Elements, Is Rapidly Activated by Interleukin-6 at the Posttranslational Level

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Interleukin-6 (IL-6) is known to be a major mediator of the acute-phase response in liver. We show here that IL-6 triggers the rapid activation of a nuclear factor, termed acute-phase response factor (APRF), both in rat liver in vivo and in human hepatoma (HepG2) cells in vitro. APRF bound to IL-6 response elements in the 5'-flanking regions of various acute-phase protein genes (e.g., the α_2-macroglobulin, fibrinogen, and α_1-acid glycoprotein genes). These elements contain a characteristic hexanucleotide motif, CTGGGA, known to be required for the IL-6 responsiveness of these genes. Analysis of the binding specificity of APRF revealed that it is different from NF-IL6 and NF-κB, transcription factors known to be regulated by cytokines and involved in the transcriptional regulation of acute-phase protein genes. In HepG2 cells, activation of APRF was observed within minutes after stimulation with IL-6 or leukemia-inhibitory factor and did not require ongoing protein synthesis. Therefore, a preexisting inactive form of APRF is activated by a posttranslational mechanism. We present evidence that this activation occurs in the cytoplasm and that a phosphorylation is involved. These results lead to the conclusions that APRF is an immediate target of the IL-6 signalling cascade and is likely to play a central role in the transcriptional regulation of many IL-6-induced genes.

During an acute inflammation, cytokines released by different cell types, including monocytes, fibroblasts, and endothelial cells, stimulate the synthesis and secretion of a set of plasma proteins, the so-called acute-phase proteins, by the liver. These proteins play a protective role during the acute-phase reaction, e.g., by inactivating proteases, supporting the wound-healing process, or scavenging free oxygen radicals (for a review, see reference 26). According to their regulation by different cytokines, acute-phase proteins have been divided into two subclasses (8). The synthesis of class 1 acute-phase proteins (e.g., α_1-acid glycoprotein, C-reactive protein, haptoglobin, and serum amyloid A) is induced by interleukin-1 (IL-1) or combinations of IL-1 and IL-6, whereas the genes for class 2 acute-phase proteins (e.g., α_2-macroglobulin, α_1-antichymotrypsin, and fibrinogen) are regulated mainly by IL-6 and glucocorticoids. The 5'-flanking regions of many acute-phase protein genes have been studied in detail, with the goal of identifying regulatory elements required for the cytokine induction of these genes. One type of cytokine response elements found in the promoters of several class 1 acute-phase protein genes represents binding sites for members of the C/EBP family of transcription factors (15, 21, 49, 51, 62). Of this family, human NF-IL6 and its rat (IL-6DBP or LAP) and mouse (AGP/EBP) homologs were shown to be implicated in the regulation of acute-phase protein genes by inflammatory cytokines (1, 15, 16, 47, 52). Both posttranslational activation of NF-IL6 and transcriptional induction of its gene in response to cytokines have been demonstrated (1, 52). More recently, cloning of an additional member of the C/EBP gene family, termed NF-IL6β, was reported (36). Expression of NF-IL6β, like expression of NF-IL6, is induced by cytokines, and the protein has been shown to heterodimerize and thereby cooperate with NF-IL6 in the transactivation of acute-phase protein genes (36). In addition, NF-κB or NF-κB-like factors were found to be involved in the regulation of the angiotensinogen, serum amyloid A, and complement factor B genes by IL-1 (17, 48, 54).

Less is known about transcription factors regulating the promoters of class 2 acute-phase protein genes. On the basis of a sequence comparison of the 5'-flanking regions of the rat α-, β-, and γ-fibrinogen genes, a hexanucleotide motif, CTGGGA, present in all three promoters has been proposed to be implicated in their regulation (19). In fact, in the human and rat β-fibrinogen genes, a promoter region containing this motif has been shown to be required for the responsiveness to IL-6 (6, 33). The IL-6 response elements of the rat T-kininogen gene were found to contain motifs highly homologous to this hexanucleotide (42). Similarly, the acute-phase response element (APRE) of the rat α_2-macroglobulin promoter, localized by us and others, is composed of two such hexanucleotide motifs which functionally cooperate to confer the full IL-6 response of the promoter (28, 30, 34, 38). Regulatory elements containing the CTGGGA hexanucleotide were thought to be implicated mainly in the regulation of class 2 acute-phase protein genes (30, 31). However, homologous elements were demonstrated to be required for the induction of the class 1 acute-phase haptoglobin and α_1-acid glycoprotein genes by IL-6 (49, 63).

The transcription factors binding to the CTGGGA motif have not been purified or cloned, and the mechanism of their activation by IL-6 is unknown. Nuclear factors from rat liver were shown to specifically bind to the APRE of the rat α_2-macroglobulin gene (28, 29, 34). In gel retardation experiments, the mobility of the DNA-protein complexes formed by these factors changed slightly during an acute-phase response, indicating that either mutual exclusive binding of different factors or modification of the same factors occurred (28, 34). Recent reports suggested that IL-6 induces a

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TABLE 1. Sequences of the synthetic double-stranded oligonucleotides used in the gel retardation assays

Oligonucleotide	Sequence[a]	Reference
Palindrome	5′-gatcttccgggaa aaggcccttctag-5′	
Rat α_2-macroglobulin gene promoter		60
α_2M CTGGGA	5′-gATCCTTCTGGGAATTCcta GAAGACCCTTAAGgatctag-5′	
CTGGGA-mutant	5′-gATCCTTCTctagATTCcta GAAGAgatcTAAGgatctag-5′	
2× CTGGGA	5′-ATCCTTCTGGGAATTCTGATCCTTCTGGGAATTCTG acgtTAGGAAGACCCTTAAGACTAGGAAGACCCTTAAGACgatc-5′	
α_2M CTGGAA	5′-GAGAAAAAGTGAGCAGTAACTGGAAAGTCCTTAgat acgtCTCTTTTTCACTCGTCATTGACCTTTCAGGAATctagatc-5′	
CTGGAA-mutant	5′-gatccAACTGGAAAGTCCTa gTTGACCTTTCAGGAtctag-5′	
Rat APRE	5′-agcttCAGTAACTGGAAAGTCCTTAATCCTTCTGGGAATTCTGt aGTCATTGACCTTTCAGGAATTAGGAAGACCCTTAAGACagatc-5′	
Human α_2-macroglobulin gene promoter		60
Human APRE	5′-GCTGTACGGTAAAAGTGAGCTCTTACGGGAATGGGAAT acgTCGACATGCCATTTTCACTCGAGAATGCCCTTACCCTTAGatc-5′	
Rat α_1-acid glycoprotein gene, distal enhancer		63
α_1AGP C	5′-gatCTGGGCTTCTGGGAAAAACTCAAG ACCCGAAGACCCTTTTTGAGTTCctag-5′	
Human C-reactive protein gene promoter		21
CRPα	5′-agcttCATAGTGGCGCAAACTCCCTTACTGa aGTATCACCGCGTTTGAGGGAATGACtctag-5′	
Mouse *H-2K^b* class I gene promoter		4
H-2Kb	5′-gatccgGCTGGGGATTCCCCATCta gcCGACCCCTAAGGGGTAGatctag-5′	

[a] Nucleotides corresponding to genomic sequences are given in capitals; all others are in lowercase. Binding sites for APRF and binding sites destroyed by mutation are underlined by double and single lines, respectively.

nuclear factor in human hepatoma cells which binds to the APRE and is likely to be the human homolog of the APRE-binding factor observed in rat liver (30). This factor, termed IL-6 RE-BP, was induced maximally 4 to 18 h after IL-6 stimulation, and its appearance was shown to depend on ongoing protein synthesis. The apparent molecular size of IL-6 RE-BP was estimated to be 46 kDa in Southwestern (DNA-protein) blotting and UV cross-linking experiments (30). More recently, the same authors reported a molecular size of 102 ± 10 kDa on the basis of further UV cross-linking experiments (31).

We now report that IL-6 rapidly induces the activation of a DNA-binding factor, termed acute-phase response factor (APRF), in rat liver in vivo as well as in human hepatoma cells in culture. Activation of APRF occurs within minutes after the application of IL-6 and is not dependent on ongoing protein synthesis. Therefore, a preexisting, inactive form of APRF is activated posttranslationally. We present evidence that a phosphorylation is involved and that the activation occurs in the cytoplasm. APRF binds specifically to CTGGGA hexanucleotide motifs in the IL-6 response elements of various acute-phase protein genes. These results imply that the activation of APRF is part of a general, rapid pathway of IL-6 signal transduction to the nucleus.

MATERIALS AND METHODS

Synthetic oligonucleotides and plasmid construction. Oligonucleotides were synthesized by a Gene Assembler (Pharmacia). The sequences of the double-stranded oligonucleotides used in gel retardation assays are given in Table 1. The rat α_2-macroglobulin promoter fragments used for labeling originated from plasmid pIC-215α_2M, obtained by insertion of the *Rsa*I (−215)-to-*Bgl*II (+8) fragment of this promoter into the *Sma*I and *Bam*HI sites of the vector pIC20R (43).

Cell culture and animals. Human hepatoma (HepG2) cells were cultured in Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1, vol/vol), supplemented with 10% fetal calf serum and 100 U each of penicillin and streptomycin per ml. For stimulation with cytokines, cells were grown to 70 to 90% confluency, and cytokines were added to the medium. The final concentrations of the recombinant human cytokines in the medium were as follows: IL-6 (a gift from T. Kishimoto and T. Hirano, Osaka, Japan), 100 BSF-2 (B-cell-stimulatory factor 2) units/ml; leukemia-inhibitory factor (a gift from N. A. Nicola, Melbourne, Australia), 10 U/ml; IL-1β (a gift from A. R. Shaw, Glaxo, Geneva, Switzerland), 100 U/ml; and tumor necrosis factor alpha (TNFα; BASF/Knoll, Mannheim, Germany), 100 U/ml.

Male Sprague-Dawley rats (200 to 300 g) were injected intraperitoneally with 10 mg of *Escherichia coli* lipopolysaccharide (LPS; Sigma) per kg of body weight or with 60,000 BSF-2 units of recombinant human IL-6 per kg of body weight. After the times indicated, the animals were killed by asphyxiation, and the livers were removed immediately.

Cytosolic and nuclear extracts. Nuclear extracts from HepG2 cells were prepared according to Shapiro et al. (58), with the following modifications. Cells were homogenized in homogenization buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES, pH 7.8], 0.75 mM spermidine, 0.15 mM spermine, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) by 15 strokes in a glass Dounce homogenizer. When cytosolic extracts were prepared from the cells, phosphatase inhibitors (1 mM sodium fluoride, 1 mM sodium diphosphate, and 0.1 mM sodium orthovanadate) and further

protease inhibitors (pepstatin [2 μg/ml], leupeptin [2 μg/ml], and aprotinin [10 μg/ml]) were included in the homogenization buffer. After homogenization, 0.1 volume of 75% sucrose was added to stabilize the nuclei. Nuclei were pelleted by centrifugation at 12,000 × *g* for 10 min at 4°C. From the supernatant, cytosolic extracts were obtained by centrifugation at 100,000 × *g* for 1 h at 4°C. For nuclear extracts, the isolated nuclei were resuspended in nuclear extraction buffer (50 mM Tris [pH 7.5], 10% sucrose, 420 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol). The suspension was rocked gently for 30 min at 4°C, after which the chromatin was sedimented at 100,000 × *g* for 1 h at 4°C. The supernatant was collected and dialyzed against dialysis buffer (20 mM HEPES [pH 7.8], 50 mM KCl, 12.5 mM $MgCl_2$, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40, 20% glycerol).

Nuclei from rat livers were isolated according to Gorski et al. (27). Briefly, livers were minced and homogenized in liver homogenization buffer (10 mM HEPES [pH 7.6], 0.5 mM spermidine, 0.15 mM spermine, 25 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol) with 0.3 M sucrose and 0.05% Triton X-100 (3 ml/g of tissue) by 15 strokes in a motor-driven Teflon-glass homogenizer at 0°C. Nuclei were isolated by centrifugation through a sucrose cushion (liver homogenization buffer with 2 M sucrose) at 24,000 rpm in an SW28 rotor for 30 min at 4°C. The isolated nuclei were extracted as described above for the HepG2 cell nuclei. Protein concentrations were measured by the Bio-Rad colorimetric assay (13).

Gel retardation assays. Oligonucleotides or DNA fragments were labeled by filling in 5′ protruding ends with the Klenow enzyme, using [α-^{32}P]dATP (3,000 Ci/mmol). Gel retardation assays were carried out according to published procedures (20, 56). Nuclear extract (1 to 5 μg of protein) was incubated with about 10 fmol (5,000 cpm) of probe in gel shift incubation buffer [10 mM HEPES (pH 7.8), 50 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 5 mM dithiothreitol, 0.7 mM phenylmethylsulfonyl fluoride, 1 mg of bovine serum albumin per ml, 0.1 to 0.2 mg of poly(dI-dC) · poly(dI-dC) (Pharmacia)] for 10 min at room temperature. Then the DNA-protein complexes formed were separated by electrophoresis on a 4% polyacrylamide gel containing 15% glycerol in 0.25× TEA (1× TEA is 40 mM Tris [pH 7.8], 1.1 mM EDTA, and 37 mM sodium acetate). Electrophoresis was performed in 0.25× TEA at 20 V/cm for 1.5 h. The gels were then dried and autoradiographed.

Expression of NF-IL6 in *E. coli*. The NF-IL6 gene was amplified by polymerase chain reaction from HepG2 DNA, using oligonucleotide primers corresponding to the published sequence (1). For binding studies using recombinant NF-IL6, a truncated form of NF-IL6 (ΔNF-IL6), which consists of the 112 carboxy-terminal amino acids of full-length NF-IL6 comprising its DNA-binding and leucine zipper domains, was used. A gene for ΔNF-IL6 was constructed by polymerase chain reaction, using a primer which introduced a start codon and an *Nco*I site instead of codon 233 of the wild-type gene. The gene was subcloned into the vector pRSET5d (57) and expressed in *E. coli* BL21(DE3)pLysS (59). Recombinant ΔNF-IL6 was extracted by 6 M guanidinium-HCl from the bacterial inclusion bodies.

Methylation interference analysis. Methylation interference analysis was performed essentially as described previously (4). The probes used in the experiments were prepared as follows. For labeling of the noncoding strand, plasmid pIC-215α_2M was linearized by *Asp*-718, labeled with Klenow enzyme by using [α-^{32}P]dATP (3,000 Ci/mmol), and digested with *Sty*I. The coding strand was labeled by linearizing with *Sty*I, filling in with Klenow enzyme by using [α-^{32}P]dTTP, and digesting with *Asp*-718. The resulting probes (−215 to −134 of the rat α_2-macroglobulin promoter) were gel purified and either partially methylated with dimethyl sulfate or subjected to a G+A sequencing reaction, both according to Maxam and Gilbert (44). A gel retardation assay using HepG2 nuclear extract from cells treated with IL-6 for 15 min was scaled up to 30,000 cpm of probe and carried out as described above. The free and bound probes were located by autoradiography and purified by using DEAE-paper. After cleavage with piperidine (44), 8,000 cpm of the product was loaded onto each lane of an 8% denaturing polyacrylamide gel.

Southwestern blot experiments. Southwestern blotting was carried out by using a procedure modified from that of Miskimins et al. (45). As a probe for the Southwestern blot experiment, double-stranded oligonucleotide α_2M CTGGGA was labeled by phosphorylation with [γ-^{32}P]ATP (3,000 Ci/mmol) and multimerized by ligation. Nuclear proteins (15 μg) extracted from rat liver nuclei were subjected to electrophoresis on a sodium dodecyl sulfate (SDS)-polyacrylamide gel (39) and electroblotted to nitrocellulose. The nitrocellulose was immersed in binding buffer (20 mM HEPES [pH 7.9], 60 mM KCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, 10% glycerol), blocked with 5% (wt/vol) low-fat dry milk in binding buffer for 10 min at 4°C, and hybridized with 10^5 cpm of probe per ml of binding buffer (containing 1 μg of herring sperm DNA per ml) overnight at 4°C. After three washes with binding buffer, the filter was autoradiographed.

RESULTS

A nuclear factor, APRF, binding to the CTGGGA motif is induced by LPS and IL-6 in rat liver. Bacterial LPS induces a strong acute-phase reaction which in rats is characterized by dramatically increased levels of plasma α_2-macroglobulin (18, 55). To identify the nuclear factor(s) interacting with the CTGGGA hexanucleotide motif in cytokine response elements of acute-phase protein genes, we intraperitoneally injected rats with LPS, prepared nuclear extracts from the livers, and, by gel retardation assays, analyzed proteins binding to the APRE of the rat α_2-macroglobulin promoter. As a probe, we used a ^{32}P-labeled double-stranded synthetic oligonucleotide spanning positions −176 to −161 of this promoter (Table 1). This sequence contains the proximal hexanucleotide motif, CTGGGA (38). In nuclear extracts isolated from untreated rats, only a low level of protein(s) forming a complex with this probe was detected (Fig. 1). Proteins extracted from liver nuclei of rats 1 h after LPS administration, however, showed dramatically increased CTGGGA-binding activity. With longer treatments, this signal decreased gradually and returned to low levels 10 h after injection. To reflect the LPS-induced binding to the APRE, we propose to call the protein forming this complex the acute-phase response factor (APRF).

LPS is known to release many cytokines, including IL-1, IL-6, and TNF from various cell types. Since IL-6 is known to be the major inducer of α_2-macroglobulin synthesis in rat liver (3, 22), we next examined whether this cytokine is able to directly induce APRF activity in liver. Liver nuclear

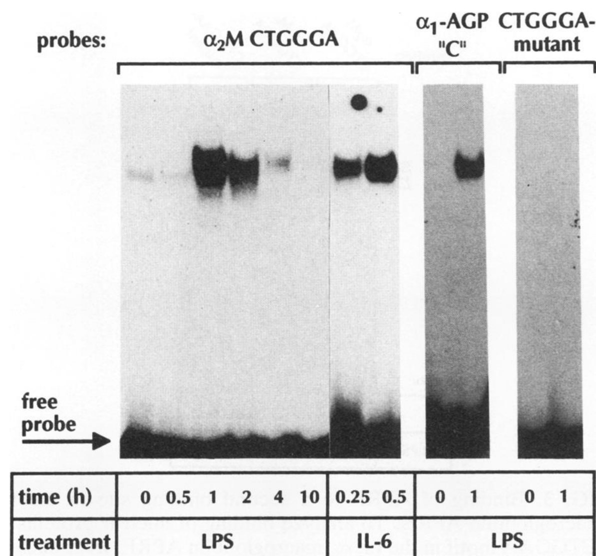

FIG. 1. Gel retardation assay of liver nuclear extracts from rats treated with LPS or IL-6. Rats were injected intraperitoneally with LPS (10 mg/kg of body weight) or IL-6 (60,000 BSF-2 units/kg). At the times indicated, the animals were killed, the liver was excised, and nuclear extracts were prepared. For the gel retardation assay, 1 μg of nuclear protein was incubated with the double-stranded ^{32}P-labeled probes. Subsequently, the DNA-protein complexes formed were separated on a native 4% polyacrylamide gel. After drying, the gel was exposed overnight to X-ray film. Sequences of the probes used are shown in Table 1.

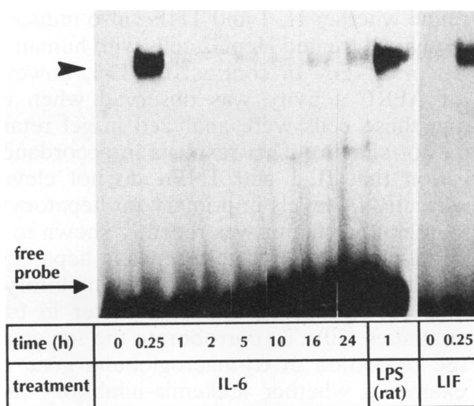

FIG. 2. Activation of APRF in HepG2 cells by IL-6 and leukemia-inhibitory factor. HepG2 cells were treated with IL-6 (100 BSF-2 units/ml of medium) or leukemia-inhibitory factor (LIF; 10 U/ml) and harvested at the times indicated. Protein was extracted from nuclei isolated from these cells and subjected to a gel retardation assay as described for Fig. 1, using 5 μg of nuclear protein per lane. A ^{32}P-labeled α_2M CTGGGA probe was used. The position of the APRF-DNA complex is indicated by an arrowhead. For comparison, 1 μg of liver nuclear extract from a rat treated with LPS for 1 h was used in one lane.

extracts from rats injected intraperitoneally with human recombinant IL-6 were analyzed in a gel retardation assay. In fact, IL-6 rapidly induced a nuclear factor forming a complex with the CTGGGA probe (Fig. 1). The mobility of this complex was indistinguishable from that observed for the LPS-induced complex. We conclude that IL-6, like LPS, activates APRF in the liver. However, IL-6 induced the appearance of APRF activity more rapidly than did LPS. After LPS treatment, active APRF was first observed after 60 min, whereas injection of IL-6 caused the appearance of detectable APRF activity within 15 min. Levels comparable to those found in LPS-treated rats were reached 30 min after addition of IL-6 (Fig. 1). This time course is in accordance with the assumption that LPS acts indirectly via the release of IL-6 to activate APRF in the liver.

To prove that binding of APRF is specific for the CTGGGA motif in the α_2-macroglobulin APRE, we used a mutated oligonucleotide probe in which four bases of the hexanucleotide were exchanged (CTGGGA→CTCTAG). APRF failed to bind to this probe in gel retardation assays (Fig. 1, CTGGGA-mutant probe). In addition to being involved in regulation of the rat α_2-macroglobulin gene and other class 2 acute-phase protein genes, IL-6 response elements containing the CTGGGA motif have been demonstrated to play an important role in the regulation of the class 1 genes coding for rat α_1-acid glycoprotein and human haptoglobin (49, 63). Hence, we next addressed the question whether APRF generally binds to this type of regulatory element in both classes of acute-phase protein genes. For this purpose, we used a radiolabeled oligonucleotide containing the C element in the distal enhancer of the rat α_1-acid glycoprotein gene as a probe in gel retardation assays (α_1AGP C probe). As shown in Fig. 1, an LPS-induced complex of the same mobility as that observed for the α_2M CTGGGA probe was formed. In additional gel retardation experiments, we were able to demonstrate specific binding of APRF to CTGGGA elements of the human γ-fibrinogen and α_1-antichymotrypsin genes and to the B element of the human haptoglobin promoter (data not shown). These observations lead to the conclusion that APRF is a general CTGGGA-binding factor and may be involved in the IL-6 response of many acute-phase protein genes.

IL-6 and leukemia-inhibitory factor, but not IL-1 and TNF, rapidly induce APRF activity in human hepatoma cells. The results of the in vivo experiments described above, although suggesting that IL-6 triggers APRF activation directly, do not formally eliminate the possibility that IL-6 acts indirectly via the release of other cytokines. Furthermore, because IL-1 and TNF both release IL-6 from monocytes and other cell types (66), in vivo experiments are not suited to examine whether these cytokines induce APRF activity directly. Therefore, we next addressed the question of whether the activation of APRF can be observed in cultured cells. Since human hepatoma (HepG2) cells have been successfully used as a model system for the functional analysis of the rat α_2-macroglobulin promoter and its regulation by IL-6 (28, 38), we chose this cell line for the further studies. HepG2 cells were treated with human recombinant IL-6 for various periods, and the proteins extracted from the nuclei were analyzed in gel retardation assays. A strong retarded band with a mobility identical to that of the complex formed with rat liver APRF appeared when extracts from cells treated for 15 min with IL-6 were used (Fig. 2). One hour after IL-6 stimulation, the signal returned to levels only slightly higher than that in control cells and did not significantly change thereafter. We conclude that IL-6 causes a rapid activation of APRF in both rat liver and human hepatoma cell nuclei. The kinetics of this activation is significantly different from that observed by Hocke et al. for the nuclear factor IL-6 RE-BP, which was found to bind to the rat α_2-macroglobulin APRE after long-term treatment of HepG2 cells with IL-6 (30).

To determine whether IL-1 and TNFα also induce APRF activity directly, we treated HepG2 cells with human recombinant IL-1β or TNFα. In contrast to IL-6, however, no induction of APRF activity was observed when nuclear extracts from these cells were analyzed in gel retardation assays (data not shown). This result is in accordance with the observation that IL-1 and TNFα do not elevate α_2-macroglobulin mRNA levels in primary rat hepatocytes (3).

Leukemia-inhibitory factor was recently shown to induce the same set of acute-phase protein genes in hepatocytes as does IL-6 (9, 10). In HepG2 cells, leukemia-inhibitory factor stimulates the rat α_2-macroglobulin promoter in transient transfection studies (30). To corroborate the importance of APRF in the regulation of α_2-macroglobulin gene expression, we examined whether leukemia-inhibitory factor is similarly able to induce APRF in HepG2 cells. In fact, nuclear extracts from HepG2 cells treated with leukemia-inhibitory factor for 15 min exhibited strong APRF activity (Fig. 2). Taken together, the results show that the cytokines (IL-6 and leukemia-inhibitory factor) that induce α_2-macroglobulin expression also activated APRF, whereas IL-1 and TNFα were inactive in both respects. Thus, APRF activation correlates well with the stimulation of α_2-macroglobulin synthesis, indicating a pivotal role of APRF in the cytokine-induced expression of rat α_2-macroglobulin.

APRF binds to two sites in the α_2-macroglobulin promoter and can be distinguished from a constitutive CTGGAA-binding factor by its binding specificity. In addition to containing the proximal CTGGGA motif at −170, the APRE of the rat α_2-macroglobulin promoter contains another hexanucleotide motif, CTGGAA, at −190 which has been demonstrated to functionally cooperate with the CTGGGA motif and to bind similar or identical nuclear factors (28, 34). We next examined whether this CTGGAA motif also represents a binding site for APRF. When an oligonucleotide probe spanning positions −208 to −178 of the rat α_2-macroglobulin promoter was used in gel retardation assays, the LPS-induced appearance of a specific DNA-protein complex with rat liver nuclear extracts was observed (Fig. 3, α_2M CTGGAA probe). This band exhibited the same mobility as did the complex formed by APRF with the CTGGGA probe. The interaction of APRF with this motif was further confirmed by the observation that an excess of this oligonucleotide completely and specifically inhibited binding of APRF to an APRE probe (see Fig. 5A). It should be pointed out, however, that APRF bound less efficiently (i.e., with lower affinity) to the CTGGAA probe than to the CTGGGA probe.

In addition to the LPS-induced binding of APRF to the CTGGAA motif, a complex of slightly higher mobility was observed with nuclear extracts from both control and LPS-treated rats (Fig. 3). The intensity of this complex significantly decreased after LPS treatment. A similar complex was also observed when nuclear extracts from untreated HepG2 cells were analyzed (data not shown). We used a mutated CTGGAA probe to examine whether this constitutive factor recognizes the same binding sequence as does APRF. In this probe, the CTGGAA hexanucleotide itself remained unchanged but nucleotides 5′ of it were changed, generating a *Bam*HI site (G<u>CAGT</u>AACTGGAA→G<u>ATCC</u> AACTGGAA). The constitutive complex was formed with this probe when liver nuclear extracts from control rats were tested (Fig. 3, CTGGAA-mutant probe). As observed with the wild-type CTGGAA probe, the signal was reduced after LPS treatment. APRF, however, was not able to bind to this probe (Fig. 3), nor did an excess of unlabeled CTGGAA-mutant oligonucleotide compete for APRF binding to the

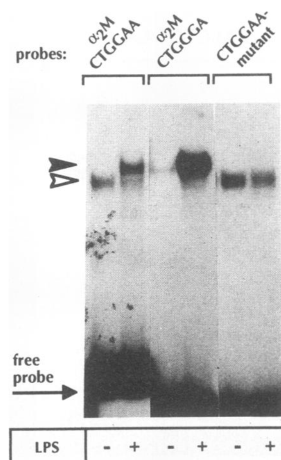

FIG. 3. Binding of APRF to a second binding site in the rat α_2-macroglobulin APRE. To analyze binding of nuclear proteins to the CTGGAA motif in the rat α_2-macroglobulin APRE, liver nuclear extracts (1 μg) from either untreated rats or rats treated with LPS for 1 h were subjected to a gel retardation assay as described in the legend to Fig. 1. The indicated ^{32}P-labeled double-stranded oligonucleotide probes were used. The positions of the complexes formed by APRF and a constitutive factor binding to the CTGGAA element are indicated by closed and open arrowheads, respectively.

CTGGGA probe (see Fig. 5A). Therefore, APRF can be distinguished from the constitutive factor on the basis of binding specificity, indicating that the two proteins are different. Furthermore, the findings demonstrate that sequences 5′ of the hexanucleotide core were important for APRF binding.

We next used probes containing the entire APRE region of the rat α_2-macroglobulin promoter in gel retardation assays. With a ^{32}P-labeled DNA fragment (−215 to −131) of the promoter or a synthetic oligonucleotide covering positions −196 to −159 (rat APRE probe), two retarded bands were observed with nuclear extracts from livers of LPS-treated rats as well as from IL-6-treated HepG2 cells (Fig. 4). This finding could indicate binding of an additional factor to the APRE region. However, the same two bands were also observed when a probe containing two CTGGGA motifs in tandem (2× CTGGGA probe) was used in the experiment (Fig. 4). Therefore, no additional sequence was required for the appearance of the second band, since the presence of two identical APRF binding sites in a single probe was sufficient to generate the slower-migrating complex. Similarly, the formation of both complexes could be competed for by adding an excess of unlabeled oligonucleotides containing only one APRF binding site (i.e., the CTGGGA and CTGGAA oligonucleotides) but not by the mutant CTGGAA oligonucleotide, which lacked the ability to bind APRF (Fig. 5A). These findings clearly demonstrate that the appearance of the second complex was not due to the presence of an additional binding site for a different nuclear factor. Rather, this complex very likely represents probe molecules in which both binding sites were occupied by APRF.

Binding of two protein molecules to a single probe even in the presence of an excess of free probe cannot occur unless binding of the second protein molecule is facilitated by the occupation of the first binding site. To examine this possibility, we carried out gel retardation assays using various concentrations of nuclear extract. The ratio of formation of

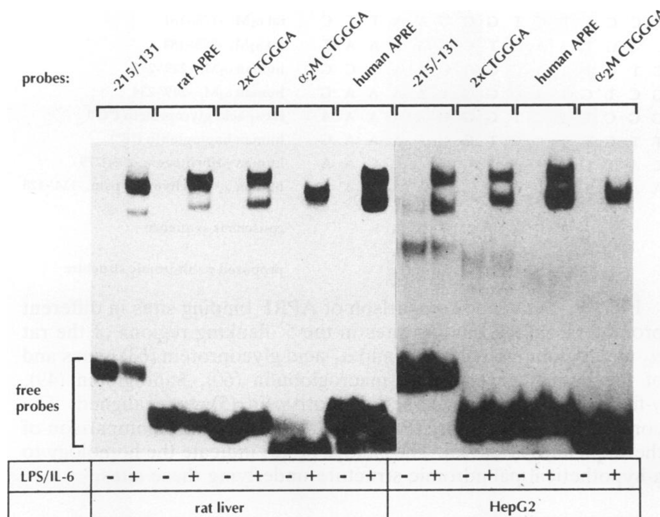

FIG. 4. Formation of two complexes with probes containing two APRF binding sites. Liver nuclear proteins (2 μg) from either untreated rats or rats treated with LPS for 1 h or 5 μg of nuclear extract from HepG2 cells treated without or with IL-6 for 15 min was analyzed by gel retardation assays as described for Fig. 1. As probes, a radiolabeled fragment (−215/−131) of the rat α_2-macroglobulin promoter and double-stranded oligonucleotides comprising the entire rat α_2-macroglobulin APRE (rat APRE), the CTGGGA motif of this element (α_2M CTGGGA), two CTGGGA motifs in tandem (2× CTGGGA), or the APRE of the human α_2-macroglobulin gene (human APRE) were used.

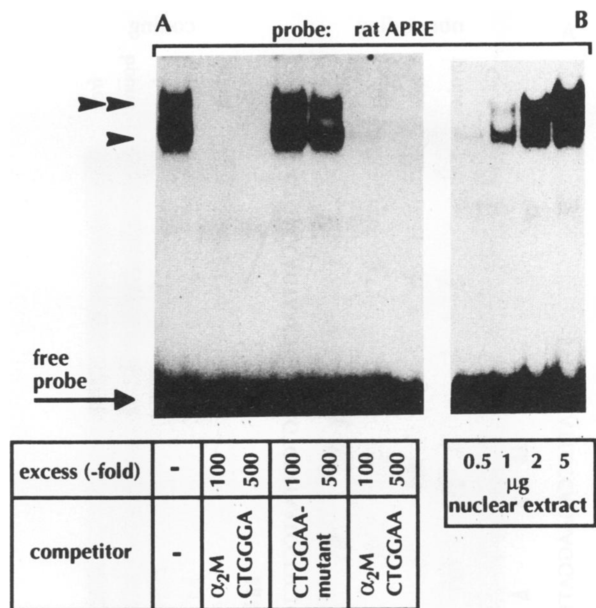

FIG. 5. Evidence for facilitated binding of APRF to adjacent binding sites. Gel retardation assays using liver nuclear extract from rats treated with LPS for 1 h and the ^{32}P-labeled rat APRE oligonucleotide containing both binding sites for APRF were performed as described for Fig. 1. (A) Competition gel retardation assay. Each lane contained 2 μg of nuclear extract. An excess of unlabeled oligonucleotides was included in the incubation mixture as competitor where indicated. (B) Gel retardation assay at various protein concentrations. The amounts of nuclear extract used in the assay are shown. The complexes representing probes in which one and two binding sites are occupied by APRF are marked by one and two arrowheads, respectively.

the two complexes was found to depend on the amount of protein added to the incubation mixture (Fig. 5B). At dilute protein concentrations, only the faster-migrating complex was formed, whereas upon increasing the amounts of nuclear extract, the low-mobility complex also appeared. At the highest protein concentrations used, this latter complex was the predominant band. This finding is in agreement with facilitated binding of APRF to the second adjacent binding site but does not enable distinction between different possible mechanisms underlying the observed behavior. An additional protein interacting with two bound APRF molecules or direct interaction between adjacently bound APRF molecules could stabilize the complex. Alternatively, APRF could induce DNA bending and thereby change the affinity of the adjacent binding site. For the nuclear factor IL-6 RE-BP, cooperative binding to the CTGGGA and CTGGAA motifs has been reported (30). From our results, it seems possible that binding of APRF to the α_2-macroglobulin APRE region exhibits the same characteristic.

We recently have shown that the 5′-flanking region of the human α_2-macroglobulin gene contains an APRE which responds to IL-6 to an extent comparable to that found for the rat α_2-macroglobulin APRE (37). Using an oligonucleotide probe spanning the APRE (−248 to −211) of the human α_2-macroglobulin promoter, we examined whether APRF is able to bind to this region as well. As shown in Fig. 4, two retarded bands of mobilities identical to those observed for the rat APRE probe appeared in gel retardation assays with nuclear extracts from IL-6-treated HepG2 cells or livers of LPS-treated rats. This finding indicates the presence of two binding sites for APRF in the human α_2-macroglobulin promoter in an arrangement similar to that demonstrated above for the rat α_2-macroglobulin APRE. Binding of APRF to this element was further confirmed by the ability of unlabeled human APRE oligonucleotide to compete for binding of APRF to the rat APRE (data not shown).

To locate the contact points between APRF and its binding sites in the rat α_2-macroglobulin APRE, we performed methylation interference assays using end-labeled fragments (−215 to −131) of the rat α_2-macroglobulin promoter. When nuclear extracts from IL-6-treated HepG2 cells were analyzed, an interference pattern that fit exactly with patterns of the two hexanucleotide motifs present in the APRE was obtained (Fig. 6).

The consensus sequence for APRF binding sites suggests a palindromic structure. Comparison of the different APRF binding sites defined above revealed that the homologies between them extend, by two nucleotides, into the 5′-flanking sequence of the originally proposed hexanucleotide (Fig. 7). This finding corresponds well with the observation that a mutation 5′ of the rat α_2-macroglobulin CTGGAA motif abolished APRF binding (Fig. 3). The consensus sequence derived from these data reads (T/G)T(C/A)(C/T)(G/T)G(G/T)AA (Fig. 7). Furthermore, as indicated in Fig. 7 by shaded boxes, we propose that the APRF binding motif is of palindromic nature, being composed of two GGAA or GTAA half sites separated by one base pair. In accordance with this view is the observation that an oligonucleotide containing the perfect palindromic sequence TTCCGGGAA bound APRF more efficiently than did the α_2-macroglobulin CTGGGA motif (Fig. 8).

APRF is different from known cytokine-induced transcription factors and has an apparent molecular size of 110 kDa.

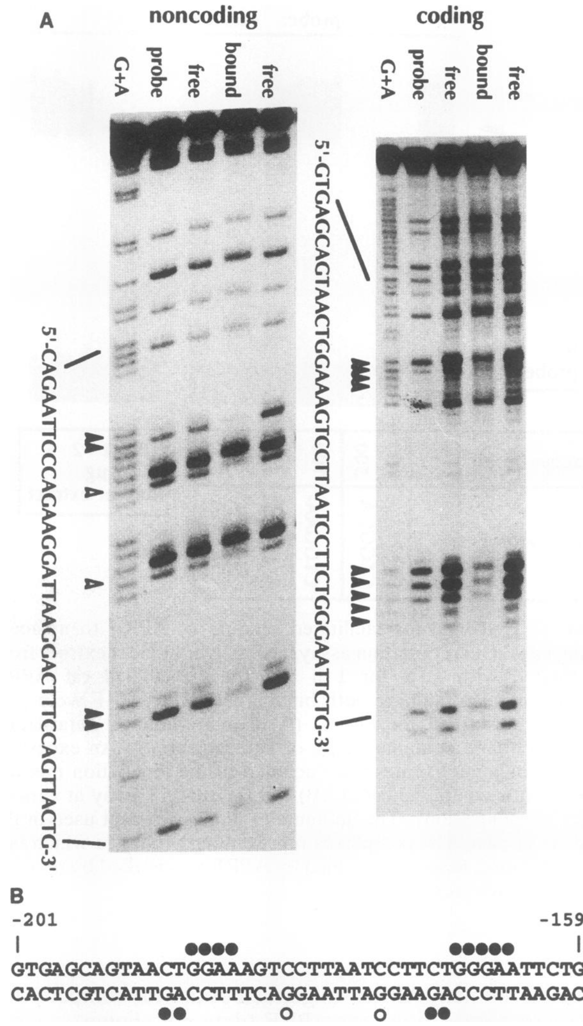

FIG. 6. (A) Methylation interference analysis of APRF binding sites in the rat α_2-macroglobulin promoter. Fragments (−215 to −134) of the rat α_2-macroglobulin promoter were end labeled at either the noncoding or coding strand, partially methylated by dimethyl sulfate, and used as probes in preparative gel retardation assays with liver nuclear extract from IL-6-treated rats. The free and bound probes were isolated from the gel, cleaved by treatment with piperidine, and analyzed on an 8% sequencing gel. As a control, partially methylated probe which had not been incubated with nuclear extract was cleaved and analyzed (probe). Unmethylated probe was subjected to a G+A sequencing reaction (G+A). Closed and open arrowheads indicate strong and weak interference, respectively. (B) Summary interference pattern of both strands. Closed and open circles represent strong and weak interference, respectively.

IL-6 is known to posttranslationally activate and transcriptionally induce transcription factors of the C/EBP family, NF-IL6 (IL-6DBP/LAP) and NF-IL6β (1, 16, 36, 52). These factors bind to IL-6 response elements of many if not all class 1 acute-phase protein genes containing the consensus sequence T(T/G)NNGNAA(T/G) (1). We next wanted to prove that APRF is different from NF-IL6. As shown in Fig. 9A, binding of APRF to the rat α_2-macroglobulin APRE was not competed for by an oligonucleotide containing the NF-IL6 binding site in the α element of the human C-reactive protein gene. Therefore, APRF does not bind to NF-IL6

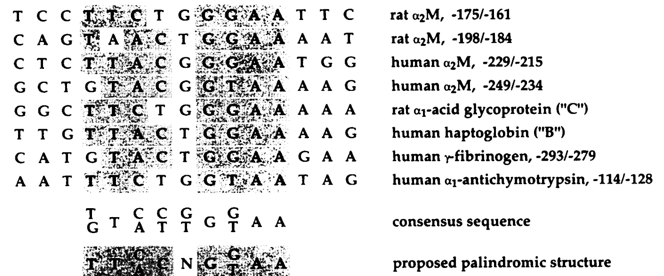

FIG. 7. Sequence comparison of APRF binding sites in different promoters. APRF binding sites in the 5′-flanking regions of the rat α_2-macroglobulin (α_2M) (38) and α_1-acid glycoprotein (63) genes and of the human genes for α_2-macroglobulin (60), haptoglobin (49), γ-fibrinogen (53), and α_1-antichymotrypsin (5) were aligned. The consensus sequence for APRF binding derived from comparison of the sequences is shown. The shaded boxes indicate the homology to a hypothetical palindromic structure underlying these sites.

binding sites. In addition, bacterially expressed NF-IL6 bound to the C-reactive protein probe in gel retardation assays but did not interact with the rat α_2-macroglobulin APRE (Fig. 9B). These findings clearly show that APRF is distinct from members of the C/EBP family of transcription factors.

The cytokine response elements of the genes for angiotensinogen, serum amyloid A, and complement factor B have been shown to be bound and regulated by NF-κB or NF-κB-like factors (17, 48, 54). Since some APRF binding sites exhibit a limited homology to the κB site, we tested whether APRF binding can be competed for by an unlabeled oligonucleotide containing the NF-κB binding site of the murine *H-2K^b* class I gene (4). As shown in Fig. 10, this oligonucleotide did not interfere with binding of APRF to the CTGGGA probe, indicating that APRF does not interact with NF-κB binding sites. Furthermore, no IL-6-induced binding to the *H-2K^b* probe was observed in HepG2 nuclear extracts. IL-1 is known to activate NF-κB in HepG2 cells (48). Accordingly, extracts from IL-1β-treated cells con-

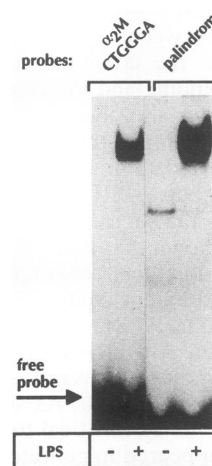

FIG. 8. Strong binding of APRF to a palindromic sequence. Double-stranded oligonucleotides containing the CTGGGA motif of the rat α_2-macroglobulin APRE (α_2M CTGGGA) or the palindromic sequence TTCCGGGAA were ^{32}P labeled to the same specific activity and used in a gel retardation assay with 2 μg of liver nuclear extract from LPS-treated rats as described in the legend to Fig. 1.

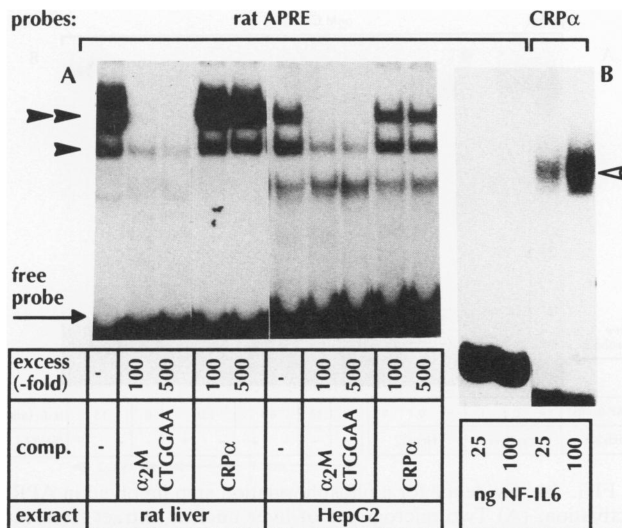

FIG. 9. Evidence that APRF is different from NF-IL6. (A) Competition gel retardation assay. Liver nuclear proteins (2 μg) from rats treated with LPS for 1 h or 5 μg of HepG2 nuclear extract from cells stimulated with IL-6 for 15 min was incubated with radiolabeled rat APRE oligonucleotide and the indicated excess of unlabeled oligonucleotides as competitors (comp.). The protein-DNA complexes formed were analyzed as described for Fig. 1. α_2M CTGGGA is an oligonucleotide containing the hexanucleotide CTGGGA motif of the rat α_2-macroglobulin APRE: CRPα is an oligonucleotide comprising the NF-IL6 binding site of the α element of the C-reactive protein gene (21). Complexes representing probes with one and two binding sites occupied by APRF are marked by one and two arrowheads, respectively. (B) Gel retardation assay with recombinant NF-IL6. NF-IL6 protein truncated at the amino terminus (ΔNF-IL6) was expressed in *E. coli*. Bacterial extract (25 and 100 ng) was incubated with radiolabeled rat APRE or CRPα oligonucleotide and analyzed by a gel retardation assay. The complex formed by ΔNF-IL6 with the CRPα probe is indicated by an open arrowhead.

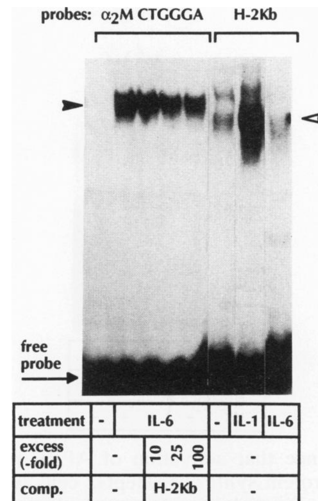

FIG. 10. Evidence that APRF does not bind to binding sites for NF-κB. Nuclear extracts (5 μg) from either untreated HepG2 cells or cells treated with IL-1β or IL-6 for 15 min were used in a gel retardation assay with the radiolabeled oligonucleotide α_2M CTGGGA (the hexanucleotide motif of the rat α_2-macroglobulin APRE) or H-2Kb (the NF-κB binding site of the mouse *H-2K^b* class I major histocompatibility gene [4]). Where indicated, an excess of unlabeled oligonucleotide H-2Kb was added as a competitor (comp.). Incubation and electrophoresis were performed as described in the legend to Fig. 1. The closed and open arrowheads mark the complexes formed by APRF and IL-1β-induced NF-κB-like factors, respectively.

tained factors which strongly bound to the *H-2K^b* probe (Fig. 10). Thus, APRF can be distinguished from factors binding to κB-like motifs.

By Southwestern blotting, we attempted to estimate the molecular size of APRF. Nuclear extracts from either untreated or IL-6-treated HepG2 cells were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with a ^{32}P-labeled, multimerized CTGGGA oligonucleotide. An IL-6-inducible band with an apparent molecular size of about 110 kDa was observed (Fig. 11). This band did not appear when the mutated CTGGGA probe shown above not to bind APRF was used (data not shown).

The rapid activation of APRF occurs at the posttranslational level. To investigate whether the induction of APRF activity by IL-6 is due to de novo synthesis of APRF protein or to the activation of a preexisting, inactive form of APRF, we used cycloheximide to inhibit protein synthesis in HepG2 cells. The cells were preincubated with cycloheximide for 1 h, and IL-6 was added to the medium. After 15 min of further incubation, nuclear extracts were prepared and analyzed by a gel retardation assay. As shown in Fig. 12, cycloheximide did not prevent the rapid appearance of active APRF in IL-6-treated HepG2 cells, indicating that ongoing protein synthesis was not required for the activation of APRF.

Various transcription factors are known to be activated in the cytosol in response to external stimuli and to subsequently be translocated to the nucleus. A well-known example is the phosphorylation-induced dissociation of NF-κB from its cytoplasmic inhibitor IκB (25). We therefore next addressed the question whether active APRF can be detected in cytosolic fractions of either untreated or IL-6-

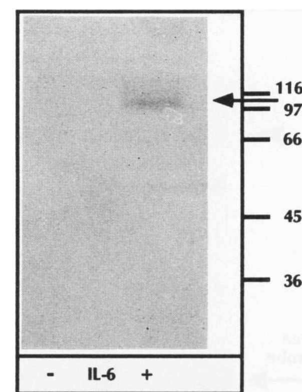

FIG. 11. Southwestern blot of rat APRF. The apparent molecular size of APRF was estimated in a Southwestern blot experiment. Liver nuclear proteins (15 μg) from either untreated or LPS-treated rats were subjected to denaturing SDS-polyacrylamide electrophoresis and subsequently transferred to nitrocellulose. The nitrocellulose membrane was blocked with milk, incubated with an oligomerized, radiolabeled oligonucleotide α_2M CTGGGA comprising the proximal APRF binding site of the rat α_2-macroglobulin gene, and analyzed by autoradiography. The positions of molecular weight marker proteins (in kilodaltons) are indicated. The arrow marks the specific LPS-induced APRF band.

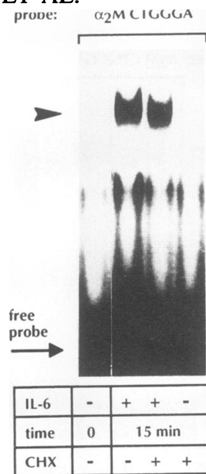

FIG. 12. Evidence that activation of APRF by IL-6 does not require ongoing protein synthesis. HepG2 cells were preincubated for 1 h with 10 μg of cycloheximide per ml where indicated (CHX). Then IL-6 (100 BSF-2 units/ml) was added to the medium, and incubation was continued for 15 min. Nuclear proteins (5 μg) extracted from these cells were analyzed in a gel retardation assay using ^{32}P-labeled oligonucleotide α_2M CTGGGA as a probe. The arrowhead indicates the APRF complex.

treated HepG2 cells. Cells were incubated with IL-6 for 1 to 15 min, quickly harvested, and homogenized at 0°C in the presence of protease and phosphatase inhibitors, and cytosolic and nuclear extracts were prepared. As shown in Fig. 13, cytosolic extracts from untreated cells did not contain any detectable APRF activity. Cytosolic fractions from cells incubated with IL-6 for only 1 min, however, caused a low level of complex formation with the α_2M CTGGGA probe. This complex had the same mobility as did the one formed with nuclear APRF, indicating that APRF was present in cytosolic extracts. The level of active APRF found in cytosolic fractions increased until 15 min after IL-6 stimulation. In nuclear extracts, the appearance of APRF followed a similar but slightly delayed time course. When cells were incubated with IL-6 for more than 15 min, APRF activity

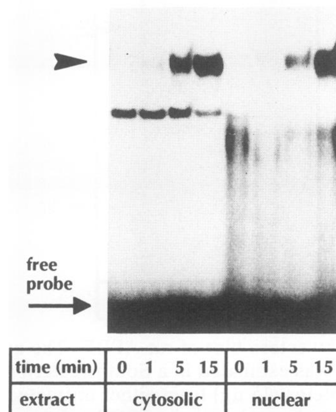

FIG. 13. APRF activation in cytosolic fractions. HepG2 cells were incubated with 100 BSF-2 units of IL-6 per ml for the periods indicated, the cells were quickly harvested, and cytosolic and nuclear extracts were prepared; 5 μg of each extract was analyzed by a gel retardation assay using radiolabeled oligonucleotide α_2M CTGGGA as described for Fig. 1. The arrowhead indicates the position of the complexes formed by APRF.

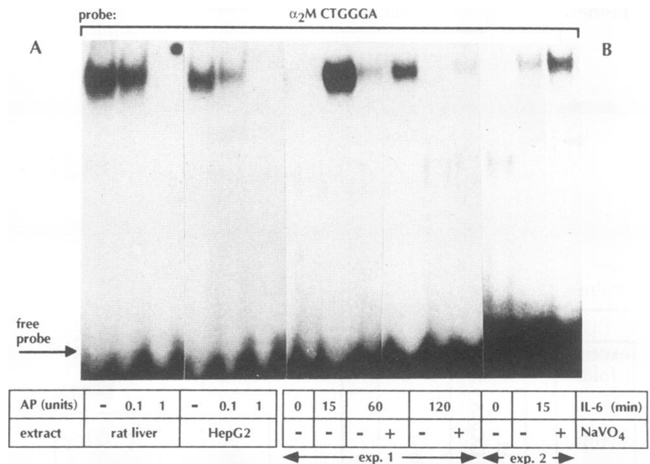

FIG. 14. Evidence for a phosphorylation step involved in APRF activation. (A) Two micrograms of liver nuclear extract from rats treated with LPS for 1 h or 5 μg of nuclear proteins from HepG2 cells stimulated with IL-6 for 15 min was incubated with the indicated amounts of calf intestine alkaline phosphatase (AP) for 15 min at 37°C. Then APRF activity was analyzed in a gel retardation assay using the α_2M CTGGGA probe as described for Fig. 1. (B) HepG2 cells were preincubated for 1 h with 50 μM sodium orthovanadate where indicated. Then IL-6 (100 BSF-2 units/ml) was added to the medium, and the incubations were continued for 15, 60, or 120 min. The cells were harvested, and proteins were extracted from the nuclei and analyzed in a gel retardation assay using the α_2M CTGGGA probe. exp. 1 and exp. 2 indicate two independent experiments. The gel in experiment 2 was exposed for less time to X-ray film in order to demonstrate the influence of vanadate on APRF levels in cells treated with IL-6 for 15 min.

decreased in both compartments and returned to near basal levels after about 1 h (data not shown). Theoretically, the APRF detected in the cytoplasmic fractions could originate from nuclei leaking during the homogenization procedure. However, this is very unlikely for two reasons. First, calculation of the absolute activity of APRF in both fractions revealed that cytosolic extracts contained 5- to 10-fold-larger amounts of total APRF than did nuclear extracts. Second, other DNA-binding factors, e.g., C/EBP-like factors binding to the α element of the C-reactive protein promoter (see above), were detected almost exclusively in the nuclear extracts (data not shown). Therefore, the detection of APRF in cytosolic fractions clearly is not due to an artifact.

Since many transcription factors are regulated by protein kinases (12), we examined whether the DNA-binding activity of APRF requires phosphorylation. Nuclear extracts from IL-6-treated HepG2 cells and from livers of LPS-treated rats were incubated with alkaline phosphatase and subsequently analyzed by a gel retardation assay. The DNA-binding activity of APRF was completely abolished by this treatment (Fig. 14A). This result indicates that APRF is phosphorylated and that this modification is important for its DNA-binding activity.

IL-6 has been demonstrated to induce tyrosine phosphorylation of proteins in target cells (41). To study whether tyrosine phosphorylation may be part of the signal transduction pathway leading to APRF activation, we used sodium orthovanadate to inhibit the cellular tyrosine-specific protein phosphatases. After preincubation with vanadate, stimulation of HepG2 cells with IL-6 for 15 min to 2 h resulted in higher levels of active APRF than in the absence of the

inhibitor (Fig. 14B). This finding provides evidence that APRF is activated either directly or indirectly via tyrosine phosphorylation.

DISCUSSION

In this report, we demonstrate that IL-6, the major mediator of the acute-phase response in liver, triggers the rapid activation of a nuclear factor, APRF, by a posttranslational mechanism. After activation, APRF binds to the IL-6 response elements of several acute-phase protein genes and is likely to play a pivotal role in their transcriptional regulation.

The interaction of nuclear factors with the APRE of the rat α_2-macroglobulin gene in response to IL-6 is biphasic. Among class 2 acute-phase protein genes, the rat α_2-macroglobulin gene has been studied most extensively with respect to its regulation by cytokines in rat liver in vivo and primary hepatocytes in vitro as well as the function of the APRE present in its promoter. Here we have shown that APRF, a nuclear factor binding to this APRE, is rapidly activated in rat liver after injection of LPS. The effect is likely to be mediated by IL-6, since injection of human recombinant IL-6 caused an even faster activation of APRF. In fact, treatment of human hepatoma (HepG2) cells with IL-6 led to the activation of a nuclear factor exhibiting the same binding specificity and forming complexes comparable in mobility to those formed by APRF. It therefore very likely represents the human homolog of rat APRF. In contrast, IL-1β and TNFα, cytokines known to be released after LPS treatment in vivo as well, failed to activate APRF in HepG2 cells.

Changes in APRE-binding factors observed after treatment of HepG2 cells with IL-6 occurred in two phases. The first phase is characterized by the rapid, strong, and transient activation of APRF. At 1 min after the addition of IL-6 to the medium, APRF activation could be detected. The response, which did not depend on ongoing protein synthesis, reached maximal levels after as early as 15 min. In the second phase, after inactivation of APRF, low levels of a factor forming a complex with the APRE persisted for at least 24 h when IL-6 treatment was continued. At present, it is unclear how the biphasic response described here can be related to the data presented by other authors (28, 30, 31, 34). Even though essentially the same probes for gel retardation analysis were used, the rapid activation of APRF has not previously been observed. Instead, Hocke et al. described a factor, IL-6 RE-BP, which is induced only several hours after IL-6 treatment (30, 31). This induction was reported to depend on de novo protein synthesis. Thus, APRF and IL-6 RE-BP bind to the same element, and the binding activity of both factors is stimulated by IL-6. Furthermore, the apparent molecular size of 110 kDa reported here for APRF is similar to that recently estimated for IL-6 RE-BP (102 ± 10 kDa) (31).

It seems very likely that IL-6 RE-BP corresponds to the low level of APRE-binding protein which we observed in HepG2 cells during the second phase of the response to IL-6. Hence, the question arises as to whether APRF and IL-6 RE-BP are different proteins or whether they are identical. The similar if not identical binding specificities, molecular sizes, and mobilities in native gel electrophoresis support the latter possibility. In this case, the strong and rapid activation of APRF after addition of IL-6 to HepG2 cells would be followed by a down-modulation of the IL-6-induced signalling pathway, causing the low levels of APRF/IL-6 RE-BP activation observed during the second phase. This down-modulation could be explained by a feedback inhibition of component(s) of the signal transduction pathway or by the internalization of IL-6 receptors known to occur after ligand binding (67).

On the other hand, the differences in both the activation kinetics and requirements for protein synthesis of APRF and IL-6 RE-BP provide a strong argument for two different nuclear factors binding to the same regulatory elements. The rapid posttranslational activation of APRF may be followed by the induction of a different factor (IL-6 RE-BP), a model similar to that discussed for activation and induction of NF-IL6 and NF-IL6β, respectively (36). Thus, at present the question of whether APRF and IL-6 RE-BP are the same or different factors cannot be answered. Further characterization of the factors and antibodies against the purified proteins are needed to resolve this issue.

The binding sites for APRF define a distinct class of IL-6 response elements. Two classes of transcription factors have been reported to be regulated by inflammatory cytokines. NF-IL6 (IL-6DBP/LAP) and NF-IL6β, members of the C/EBP family of leucine zipper factors, respond to IL-1 and IL-6 (1, 36, 47), whereas NF-κB and NF-κB-like factors are regulated by IL-1 and TNF (50, 66). By comparing their binding specificities with that of APRF, we could clearly demonstrate that APRF is different from both factor families. Therefore, APRF (and possibly IL-6 RE-BP) defines a new class of cytokine-regulated transcription factors.

A comparison of the identified binding sites for APRF has led us to propose a palindromic structure underlying the binding motif that is composed of two GGAA or GTAA half sites which are separated by one base pair. One of these half sites is completely conserved in all binding sites, whereas the other one shows a higher degree of degeneracy (Fig. 7). Many transcription factors, including the factors of the leucine zipper, helix-loop-helix, and steroid receptor families, are known to bind to DNA as dimers and recognize palindromic sequences (11, 14). Therefore, one might speculate that APRF also forms dimers. Intriguingly, this binding motif shares similarities with binding sites for members of the family of *ets*-related transcription factors which bind to GGAA motifs and also resembles the heat shock element (NTTCNNGAAN) binding the heat shock factor (2, 64). Whether APRF is related to either one of these factors remains to be elucidated.

In addition to the APRE of the rat α_2-macroglobulin gene, IL-6 response elements containing sequences highly homologous to the above-defined APRF binding motif are found in all other class 2 acute-phase protein genes so far studied (6, 33, 42). Furthermore, by analyzing the promoter regions of the human γ-fibrinogen and α_1-antichymotrypsin genes, we could demonstrate that APRF binds to IL-6 response elements in these promoters as well (65). Binding sites for APRF are, however, not restricted to promoters of class 2 acute-phase protein genes. As we have shown here, APRF also binds to IL-6 response elements in the rat α_1-acid glycoprotein and human haptoglobin genes, which are class 1 acute-phase protein genes.

Therefore, APRF seems to be a general factor involved in the IL-6 induction of the class 2 acute-phase protein genes. However, direct proof for the transactivating potential of APRF will require the use of purified APRF protein in in vitro transcription assays and eventually the cloning of its cDNA. Further circumstantial evidence for its role in transcriptional regulation originates from the observation that APRF binding sites confer IL-6 inducibility to heterologous promoters (29–31, 37). In addition, a single point mutation in the APRE of the rat α_2-macroglobulin gene abolishing APRF

binding to the proximal hexanucleotide motif rendered the promoter unresponsive to IL-6 in transient transfection studies, indicating a close connection between APRF binding and transcriptional regulation (32). It should be pointed out, however, that because of the similar or identical binding specificities of APRF and IL-6 RE-BP, such experiments do not elucidate the individual contribution of either factor to the transactivation of IL-6-responsive promoters. Therefore, additional criteria are necessary to determine the functional importance of both factors. Since the activation kinetics and the requirement for ongoing protein synthesis are different for APRF and IL-6 RE-BP, the time course of acute-phase protein gene induction is one such criterion. The transcriptional induction of most acute-phase protein genes by cytokines was shown to be immediate and independent of de novo protein synthesis and to persist for at least 24 h (7). Conflicting results have been reported for α_2-macroglobulin. In H-35 rat hepatoma cells, an onset of transcription delayed by 2 h was noted (7), whereas in rat liver in vivo, intraperitoneal injection of IL-6 caused liver α_2-macroglobulin mRNA levels to rise as early as after 1 h (24). From these data, it can be concluded that neither APRF nor IL-6 RE-BP activation alone can sufficiently explain the full time course of acute-phase protein gene induction by IL-6. APRF activation, being rapid but transient, does not account for the persistent induction of these genes. The activation of IL-6 RE-BP is delayed by at least 4 h and requires de novo protein synthesis and hence cannot be expected to be involved in the early responses of target genes. Rather, it seems attractive to speculate that the two factors act consecutively to transactivate acute-phase protein genes.

APRF is an early target of IL-6 and leukemia-inhibitory factor signalling pathways. We have shown that the rapid activation of APRF does not require ongoing protein synthesis and thus occurs by a posttranslational mechanism. The finding that APRF is present in cytosolic fractions indicates that this activation may take place in the cytoplasm followed by translocation of APRF to the nucleus. This view is supported by the observation that the time course of APRF activation was slightly delayed in the nuclear compared with the cytosolic extracts.

At present, we do not know how APRF is activated. Several mechanisms are possible, including the signal-induced release from an inhibitor protein analogous to the situation described for NF-κB/IκB (40), the assembly of a multimeric transcription factor complex as in the case of ISGF3 activation by alpha interferon (35), or a posttranslational modification, e.g., phosphorylation. We have presented evidence that phosphorylation is involved in APRF activation. In fact, protein kinases are likely to be involved in IL-6 signal transduction. A rapid phosphorylation of a 160-kDa protein at tyrosine residues after IL-6 action has been documented (41, 46). In addition, the induction of immediate-early genes by IL-6 could be blocked by specific inhibitors of protein kinases (46). Recently, the plasma membrane receptors for IL-6 and leukemia-inhibitory factor have been shown to interact with a common, signal-transducing subunit, the so-called gp130 protein (23). In this context, our finding that leukemia-inhibitory factor also triggers the rapid activation of APRF is not unexpected. Accordingly, leukemia-inhibitory factor is known to induce the same set of acute-phase proteins in the liver as does IL-6 (9, 10). It is likely, therefore, that APRF is an immediate or indirect target of a protein phosphorylation cascade initiated by the gp130 subunit of the IL-6 and leukemia-inhibitory factor receptors. Whether APRF is directly phosphorylated by a tyrosine protein kinase or whether its activation requires additional steps awaits further elucidation. Since the activation of APRF is among the most rapid IL-6-induced responses so far reported, it represents an excellent tool for studying the still unknown signal transduction pathway of IL-6.

Recently, the induction of immediate-early response genes by IL-6 has been studied in several cell lines. Various genes, including the *junB*, TIS11, IRF-1, and ICAM-1 genes, have been shown to be transcriptionally induced within 30 min to 1 h after stimulation of cells with IL-6 (41, 46). This induction did not depend on protein synthesis. Because of its quick posttranslational activation, it is tempting to speculate that APRF might be involved in the transactivation of immediate-early genes in response to IL-6. In fact, preliminary results from studies with other IL-6-responsive cell types indicated that APRF is not restricted to cells of hepatic origin. In primary human monocytes or murine myeloma cells, activation of APRF or an APRF-like factor occurred as rapid as in HepG2 cells (61). APRF therefore is likely to be involved in the transcriptional regulation of IL-6-induced genes in various cell types.

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